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# Purification of a heat-stable activator protein for ADP-ribosylation factor-dependent phospholipase D

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**Abstract A heat-stable activator for ADP-ribosylation factor (ARF)-dependent phospholipase D (PLD) was purified to near homogeneity from rat kidney cytosol by a sequential column chromatography. The purified activator has a molecular mass of 23 kDa on SDS-PAGE. Using a partially purified ARF-dependent PLD from rat kidney, the activator synergistically stimulates PLD with ARF in time- and dose-dependent manner. In the absence of ARF, the activator has little or no effect. The purified activator also stimulates PLD under several conditions including permeabilized cell system, suggesting that the activator is a physiologically relevant regulator of PLD.**



*Key words:* Phospholipase D; ADP-ribosylation factor; Phospholipase D activator; Phosphatidylcholine; Phosphatidylethanolamine

## 1. Introduction

The hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D (PLD) is elicited by a wide variety of external signals (for reviews, see [1,2]). The mechanism of PLD activation has attracted considerable attention because the enzyme has been implicated in the regulation of many cellular responses including mitogenesis in fibroblasts, intracellular protein trafficking, and apoptosis [3–5]. Recently, it has become evident that several factors are involved in the activation of PLD. As lipid factors, phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-$P_2$) [6–8] and phosphatidylethanolamine (PtdEtn) [9] are shown to be essential for exhibiting PLD activity. Small GTP-binding regulatory proteins (G-proteins) such as ADP-ribosylation factor (ARF) [6,10] and RhoA [11,12] are shown to activate PLD. PLD obtained from hematopoietic cell lines is activated by coexistence of a 50-kDa soluble protein and either ARF [13,14] or RhoA [15]. Brain PLD is activated by a cytosolic factor in a manner synergistic to ARF [16], and this cytosolic factor is indistinguishable from protein kinase C (PKC) [17,18].

A preceding report from this laboratory has described that under resting conditions membrane-bound PLD is in a latent form, but exhibits a high activity in the presence of ammonium sulfate, guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), and cytosol [19]. The active component in the cytosol consists of three protein factors: ARF, RhoA, and a soluble heat-stable protein [20]. The heat-stable protein is now purified to near homogeneity from rat kidney cytosol. Some properties of this activator are described here.

## 2. Materials and methods

### 2.1. Materials

1,2-Di-[1-$^{14}$C]palmitoyl-*sn*-glycero-3-phosphocholine ([$^{14}$C]PtdCho, 115 mCi/mmol) and 1-[1-$^{14}$C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine ([$^{14}$C]lysoPtdCho, 57.0 mCi/mmol) were purchased from DuPont-New England Nuclear. [γ-$^{32}$P]ATP was from Amersham. 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. Streptolysin-*O* was obtained from Murex Diagnostics. Phorbol 12-myristate 13-acetate (PMA) was a product of LC services (Woburn, MA). Other chemicals were of analytical grade.

### 2.2. Cells

HL-60 cells were maintained at a cell density between 0.1 and $1.0\times10^6$ cells per ml as a suspension in RPMI 1640 medium supplemented with 5% fetal bovine serum (Flow Laboratories) at 37°C in a humidified 5% $CO_2$/95% $O_2$ atmosphere.

### 2.3. Preparation of PLD

PLD was partially purified from kidney particulate fraction as described previously [20] except that rat kidney was used instead of bovine kidney, and that hydroxyapatite column chromatography was omitted from the purification procedure. The PLD preparation after TSK heparin 5PW column chromatography was purified about 200-fold in its specific activity from the particulate fraction. This preparation was free of ARF and the heat-stable PLD activator. The PLD activity was stable at least for two weeks at −80°C.

### 2.4. Preparation of ARF

ARF was purified from bovine brain as described [21]. Final preparation of ARF after gel filtration (HiLoad 16/60 Superdex 75 (Pharmacia; $1.6\times60$ cm)) was about 87% pure by silver stain. This preparation was free of the heat-stable PLD activator as judged by SDS-PAGE [22].

### 2.5. PLD assay in cell-free systems

PLD activity was determined by measuring the formation of [$^{14}$C]PtdEtOH from [$^{14}$C]PtdCho in the presence of ethanol. The reaction mixture contained PLD partially purified from rat kidney, ARF, GTP-γ-S, ammonium sulfate, [$^{14}$C]PtdCho, PtdEtn, and each fraction to be assayed. The detailed conditions are described elsewhere

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*Abbreviations:* PtdCho, phosphatidylcholine; PLD, phospholipase D; ARF, ADP-ribosylation factor; PtdIns-4,5-$P_2$, phosphatidylinositol 4,5-bisphosphate; PtdEtn, phosphatidylethanolamine; G-protein, GTP-binding regulatory protein; PKC, protein kinase C; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); [$^{14}$C]PtdCho, 1,2-di-[1-$^{14}$C]palmitoyl-*sn*-glycero-3-phosphocholine; [$^{14}$C]lysoPtdCho, 1-[1-$^{14}$C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine; PtdEtOH, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; Octylglucoside, 1-octyl β-glucopyranoside

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


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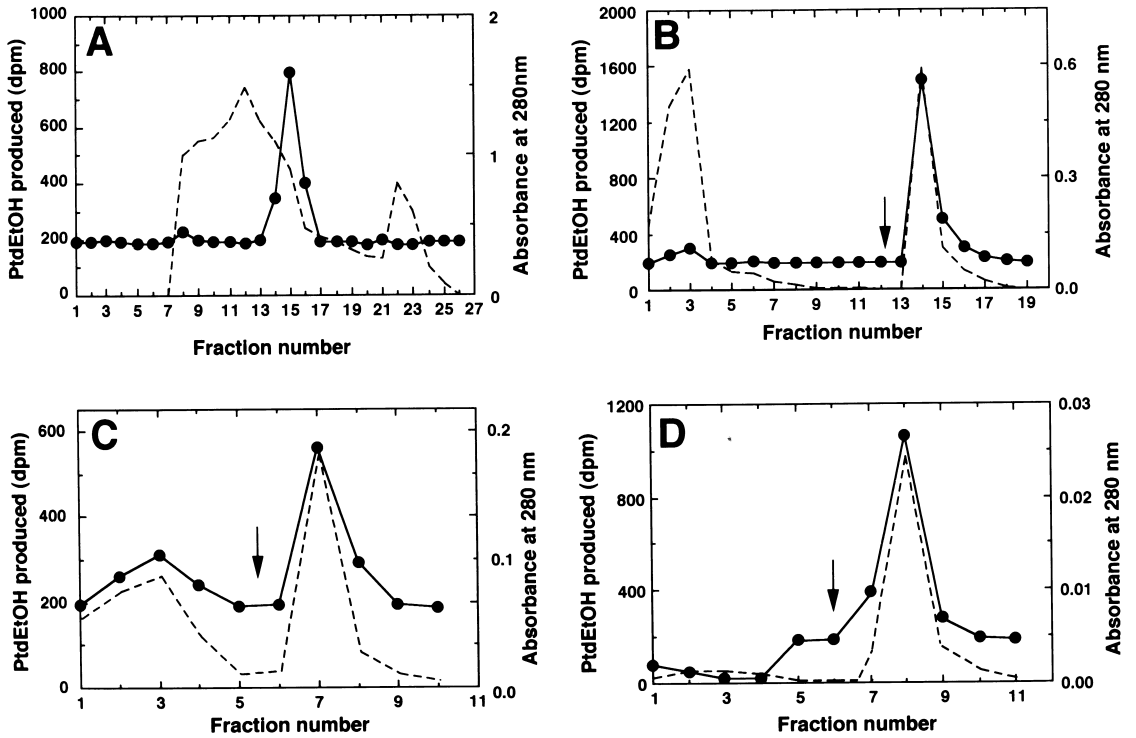


Fig. 1. Elution profiles of a heat-stable activator protein for PLD. Each fraction (20 μl) was assayed for PLD activation in the presence of 200 nM ARF under standard conditions. ●, PLD activity; dotted line, absorbance at 280 nm. A: Superdex 75 column chromatography. B: DEAE 5PW column chromatography. The arrow indicates the start of elution with buffer A containing 0.1 M NaCl. C: Phenyl 5PW column chromatography. The arrow indicates the start of elution with buffer A. D: Resource Q column chromatography. The arrow indicates the elution with buffer A containing 0.1 M NaCl.

[20]. [$^{14}$C]PtdCho and PtdEtn were separately dried and dispersed by sonication in distilled water and added to the reaction mixture. In some studies PLD activity was determined by measuring the formation of [$^{14}$C]PtdEtOH from [$^{14}$C]PtdCho with PtdIns-4,5-$P_2$ and PtdEtn as lipid activators as described [6].

*2.6. PLD assay in permeabilized cells*

HL-60 cells were preincubated with [$^{14}$C]lysoPtdCho (0.5 μCi/$1\times10^7$ cells) for 1 h at 37°C. Labeled cells ($5\times10^5$ cells) were incubated in a total volume of 100 μl with 0.4 i.u./ml streptolysin-*O*, 100 μM GTP-γ-S, 3 mM $MgCl_2$, 0.5 mM ATP, 0.1 μM free $Ca^{2+}$ in $Ca^{2+}$-EGTA buffer, 2% ethanol, and various concentrations of heat-stable PLD activator as indicated in the presence or absence of 100 nM PMA for 20 min at 37°C. Radioactive PtdEtOH produced was extracted and quantitated as described [23].

*2.7. Other procedures*

Conventional PKC was purified from rat brain as described [24]. One unit of PKC was expressed as 1 pmol of [γ-$^{32}$P]ATP incorporated into myelin basic protein per 5 min. Protein was determined by the method of Bradford [25].

## 3. Results

*3.1. Purification of heat-stable PLD activator*

Cytosolic fraction from rat kidney was prepared as described earlier [19]. Protein of the cytosolic fraction (1250 mg protein) was precipitated with ammonium sulfate (30–70% saturation), dissolved and dialyzed as described [19]. The dialysate was heated for 10 min at 60°C. After cooling on ice, heat-denatured materials were removed by centrifugation for 10 min at $20\,000\times g$. The supernatant was concentrated to about 5 ml with an Amicon ultrafiltration cell equipped with PM-10 membrane, and applied to a gel filtration column, HiLoad 16/60 Superdex 75 (Pharmacia; $1.6\times60$ cm) which had been equilibrated with buffer A (50 mM HEPES-NaOH at pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, and 1 μg/ml leupeptin). The activator was eluted from the column with the same buffer at a flow rate 1 ml/min using an FPLC system (Pharmacia). Fractions (5 ml each) were collected. An aliquot (20 μl) of each fraction was assayed for PLD activation (Fig. 1A). PLD activator (fractions 14 through 16) was applied to a DEAE 5PW column (Toyo Soda (Tokyo); $2.15\times15$ cm) which had been equilibrated with buffer A. After washing the column with buffer A, PLD activator was eluted stepwise with buffer A containing 0.1 M NaCl at a flow rate of 1 ml/min (Fig. 1B). Fractions (2 ml each) were collected. Fractions 14 through 15 containing PLD activator were pooled, brought to 3 M NaCl by adding solid NaCl, and applied to a Phenyl 5PW column (Toyo Soda (Tokyo); $2.15\times15$ cm) which had been equilibrated with buffer A containing 2 M NaCl. After washing the column with buffer A containing 2 M NaCl, PLD activator was eluted stepwise with buffer A at a flow rate of 1 ml/min (Fig. 1C). Fractions (2 ml each) were collected. Fraction 7 which contained PLD activator was applied to a Hi-Trap Octyl Sepharose 4 FF column (Pharmacia; $0.7\times2.5$ cm; 1 ml) which had been equilibrated with buffer A. After washing the column with buffer A, PLD activator was eluted stepwise with buffer A containing 1% 1-octyl β-glucopyranoside (octylglucoside) at a flow rate of 1 ml/min. Fractions (2 ml each) were collected. Fractions 12 through 14 containing PLD activator were pooled and applied to a Resource Q column (Pharmacia; $0.64\times3$ cm; 1 ml) which had been equilibrated with buffer A. After washing the column with buffer A, PLD activator was eluted stepwise

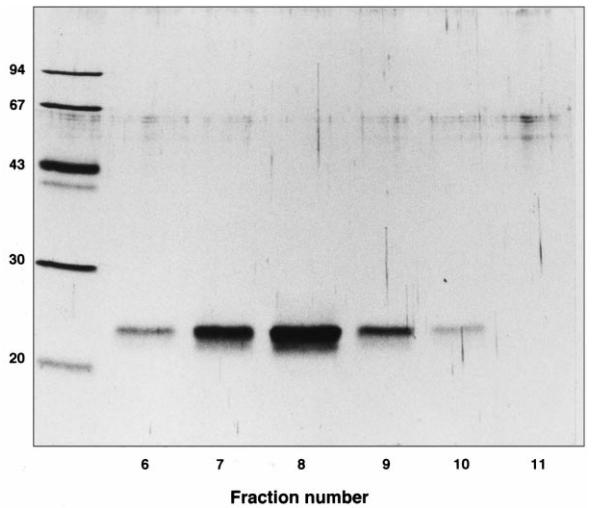


Fig. 2. Analysis of purified heat-stable activator on SDS-PAGE. Aliquots of the samples from Resource Q column chromatography (Fig. 1D) were subjected to SDS-PAGE on a 12.5% gel followed by silver stain. The positions of molecular weight markers are indicated in kDa.

with buffer A containing 0.1 M NaCl at a flow rate of 1 ml/min (Fig. 1D). Fractions (2 ml each) were collected. The active fractions contained 23-kDa protein estimated by SDS-PAGE (Fig. 2). The purity of the 23-kDa protein was estimated to be more than 95% by SDS-PAGE analysis; the distribution of the 23-kDa protein coincided exactly with that of PLD-stimulating activity. The final preparation showed a 11 700-fold increase in the specific activity with an overall yield of 28% (Table 1). PLD activator at this stage of purification was used for subsequent studies. PLD activator was stable at least several months at −80°C.

### 3.2. Characterization of heat-stable PLD activator

The heat-stable PLD activator caused a marked stimulation of ARF-dependent PLD in a dose-dependent manner (Fig. 3A). Half-maximal stimulation was observed at 45 ng/assay and a maximal stimulation was obtained at 130 ng/assay. Higher concentrations of the activator caused a slight inhibition. In the absence of ARF, the heat-stable activator showed a very weak stimulatory effect if any. The reaction in the presence of both ARF and the heat-stable activator linearly proceeded for 40 min and then became plateau (Fig. 3B). About 55% of total PtdCho was converted to PtdEtOH after 80 min incubation.

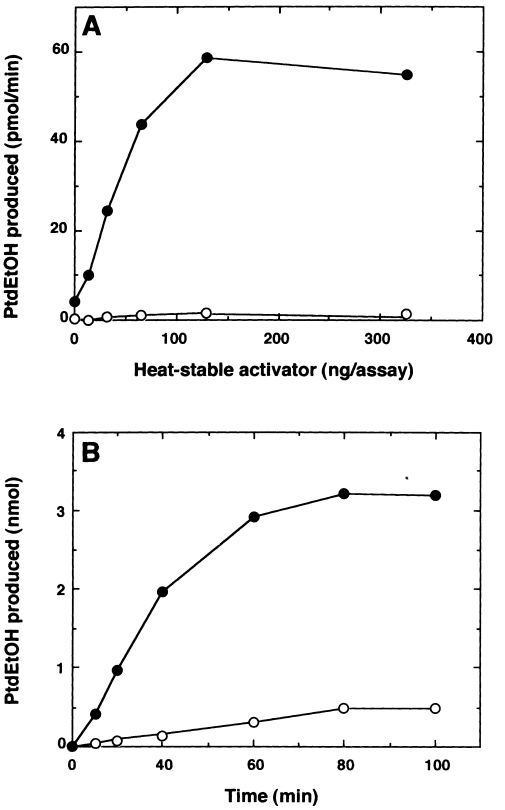


Fig. 3. Characteristics of enhancement of ARF-dependent PLD activity by purified heat-stable activator. A: Requirement of heat-stable activator for ARF-dependent PLD activation. Various concentrations of the purified heat-stable activator as indicated were assayed for PLD activation in the presence (●) or absence (○) of 200 nM ARF with the reaction mixture (100 μl) containing 100 ng of PLD, 60 μM [$^{14}$C]PtdCho (13 750 dpm/nmol), 70 μM PtdEtn, 2% ethanol, 1.6 M ammonium sulfate, 100 μM GTP-γ-S, 1 mM $MgCl_2$, and 20 mM HEPES-NaOH at pH 7.4 as described [20]. B: Time course of PLD activation. ●, with 200 nM ARF and heat-stable activator (130 ng/assay); ○, with 200 nM ARF.

### 3.3. Stimulation by heat-stable activator of PLD under different assay systems

The ability of the heat-stable activator was assessed by different PLD assay systems. Fig. 4A and B show the activation of ARF-dependent PLD by the heat-stable activator or by PKC under the method reported by Brown et al. [6]. The heat-stable activator caused PLD stimulation in a dose-dependent manner to the similar extent as caused by PKC plus PMA. In this condition the heat-stable activator (130

Table 1
Purification of a heat-stable activator protein for PLD

| Purification | Protein (mg) | Specific activity (pmol/min/mg) | Total activity (pmol/min) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Supernatant | 1250 | 1.24 | 1550 | 1 | 100 |
| AS precipitation | 178 | 17.1 | 3040 | 14 | 196 |
| Superdex 75 | 53 | 93.9 | 4980 | 76 | 321 |
| DEAE 5PW | 6.9 | 620 | 4280 | 500 | 276 |
| Phenyl 5PW | 2.9 | 562 | 1630 | 453 | 105 |
| Octyl Sepharose | 0.04 | ND | ND | ND | ND |
| Resource Q | 0.03 | 14 500 | 435 | 11 700 | 28 |

PLD activity was measured with 100 ng of enzyme, 200 nM ARF, and the heat-stable activator at various stages of purification in Section 2. The activator activity is defined as the increased amount of [$^{14}$C]PtdEtOH per min by subtracting the value without the activator. Octylglucoside was highly inhibitory to PLD reaction [9], the activity after Octyl Sepharose column was not detected. ND, not detected.

Table 2
Stimulation of PLD by a purified heat-stable activator in streptolysin-*O*-permeabilized HL-60 cells

| Addition | PtdEtOH formation (% of total counts) | |
|---|---|---|
| | −PMA | +PMA |
| None | 0.08 ± 0.02 | 0.36 ± 0.02 |
| Heat-stable activator (0.13 μg/assay) | 0.10 ± 0.01 | 0.55 ± 0.04 |
| Heat-stable activator (1.3 μg/assay) | 0.14 ± 0.02 | 1.0 ± 0.12 |

PLD activity was measured using metabolically labeled HL-60 cells permeabilized with streptolysin-*O* as described in Section 2. Results are the means ± S.E.M. of duplicate determinations from three separate experiments.

ng) and PKC (10 units) plus PMA caused almost additive stimulation of PLD (not shown). The ability of the heat-stable activator was also assessed by streptolysin-*O*-permeabilized HL-60 cell system in which membrane PtdCho was metabolically labeled with [$^{14}$C]lysoPtdCho. In streptolysin-*O*-permeabilized HL-60 cells the heat-stable activator enhanced PLD activity in a synergistic manner with PMA (Table 2).

## 4. Discussion

The activator described here was found earlier as a heat-stable protein which effectively enhanced PLD activity in the simultaneous presence of ARF and RhoA [20]. In the present studies RhoA was excluded from the PLD assay because this protein is not obligatory for PLD activation [20]. The purified heat-stable activator has a molecular mass of 23 kDa estimated by SDS-PAGE (Fig. 2). In the previous study the molecular mass of the heat-stable PLD activator was estimated to be 36 kDa by gel filtration [20]. Possible reasons for the discrepancy of the molecular size may be ascribed to different methodology used for the estimation or complex formation with other proteins in the crude preparations.

There are at least two types of mammalian PLD that have recently been cloned [26–28]. PLD1 is an ARF-dependent PLD which requires PtdIns-4,5-$P_2$ for its activity [26]. There are two splice variants of PLD1 (PLD1a and PLD1b) with similar biochemical properties and regulation [27]. PLD2 is an ARF-independent enzyme whose activity is also stimulated by PtdIns-4,5-$P_2$ [28]. Kidney PLD used in the present study is similar to PLD1a or PLD1b in that kidney PLD is an ARF-dependent enzyme and is also stimulated by RhoA [20,27]. Relationship between the kidney PLD and PLD1 remains to be studied.

There are several reports concerning the cytosolic protein activators for PLD [13–15,17,18]. The heat-stable activator may not be a proteolytic product of PKC. Polyclonal antibody against a synthetic peptide around the N-terminal portion of α-subspecies of PKC did not recognize the activator [20]. Furthermore, PMA has no effect on the activation of PLD by the activator. PLD obtained from hematopoietic cells is shown to be activated by a 50-kDa soluble protein and either ARF [13,14] or RhoA [15]. The heat-stable activator also activated PLD associated with HL-60 cell membrane in an ARF-dependent manner (not shown). The relationship between the heat-stable activator and the 50-kDa soluble protein remains to be clarified.

It is unlikely that the heat-stable activator stimulates PLD by interacting with and activating ARF because the activator has no effect on the [$^{35}$S]GTP-γ-S binding to ARF [20]. The precise mechanism of activation of ARF-dependent PLD by the heat-stable activator and the structural analysis of the protein remain to be elucidated.

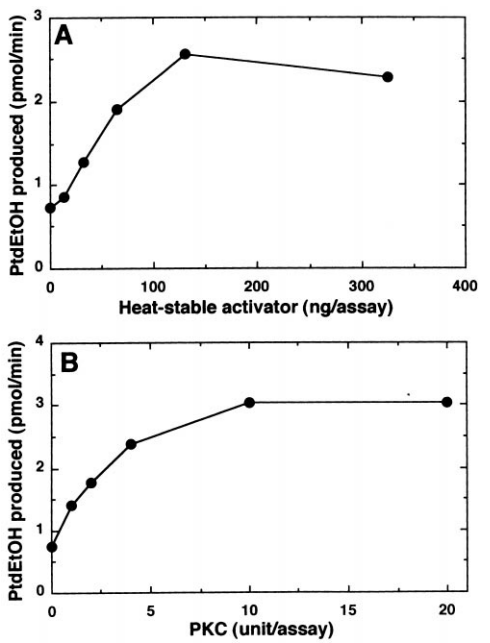


Fig. 4. Activation of ARF-dependent PLD by heat-stable activator or by PKC with PtdIns-4,5-$P_2$ and PtdEtn as lipid activators. PLD activity was measured in the presence of various concentrations of heat-stable activator (A) or PKC plus 100 nM PMA (B) with the reaction mixture (100 μl) containing 100 ng of PLD, 200 nM ARF, 8.6 μM [$^{14}$C]PtdCho (13 750 dpm/nmol), 12 μM PtdIns-4,5-$P_2$, 138 μM PtdEtn, 100 μM GTP-γ-S, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM EGTA, 80 mM KCl, 1 mM dithiothreitol, and 50 mM HEPES-NaOH at pH 7.4 as described [6].

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## References

[1] Billah, M.M. and Anthes, J.C. (1990) Biochem. J. 269, 281–291.
[2] Exton, J.H. (1990) J. Biol. Chem. 265, 1–4.
[3] Liscovitch, M. and Cantley, L.C. (1994) Cell 77, 329–334.
[4] Nishizuka, Y. (1995) FASEB J. 9, 484–496.
[5] Yoshimura, S., Sasaki, H., Ohguchi, K., Nakashima, S., Banno, Y., Nishimura, Y., Sakai, N. and Nozawa, Y. (1997) J. Neurochem. 69, 713–720.
[6] Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C. and Sternweis, P.C. (1993) Cell 75, 1137–1144.

[7] Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S. and Cantley, L.C. (1994) J. Biol. Chem. 269, 21403–21406.
[8] Pertile, P., Liscovitch, M., Chalifa, V. and Cantley, L.C. (1995) J. Biol. Chem. 270, 5130–5135.
[9] Nakamura, S., Kiyohara, Y., Jinnai, H., Hitomi, T., Ogino, C., Yoshida, K. and Nishizuka, Y. (1996) Proc. Natl. Acad. Sci. USA 93, 4300–4304.
[10] Cockcroft, S., Thomas, G.M.H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N.F., Truong, O. and Hsuan, J.J. (1994) Science 263, 523–526.
[11] Bowman, E.P., Uhlinger, D.J. and Lambeth, J.D. (1993) J. Biol. Chem. 268, 21509–21512.
[12] Malcolm, K.C., Ross, A.H., Qiu, R.-G., Symons, M. and Exton, J.H. (1994) J. Biol. Chem. 269, 25951–25954.
[13] Lambeth, J.D., Kwak, J.-Y., Bowman, E.P., Perry, D., Uhlinger, D.J. and Lopez, I. (1995) J. Biol. Chem. 270, 2431–2434.
[14] Bourgoin, S., Harbour, D., Desmarais, Y., Takai, Y. and Beaulieu, A. (1995) J. Biol. Chem. 270, 3172–3178.
[15] Kwak, J.-Y., Lopez, I., Uhlinger, D.J., Ryu, S.H. and Lambeth, J.D. (1995) J. Biol. Chem. 270, 27093–27098.
[16] Singer, W.D., Brown, H.A., Bokoch, G.M. and Sternweis, P.C. (1995) J. Biol. Chem. 270, 14944–14950.
[17] Singer, W.D., Brown, H.A., Jiang, X. and Sternweis, P.C. (1996) J. Biol. Chem. 271, 4504–4510.
[18] Ohguchi, K., Banno, Y., Nakashima, S. and Nozawa, Y. (1996) J. Biol. Chem. 271, 4366–4372.
[19] Nakamura, S., Shimooku, K., Akisue, T., Jinnai, H., Hitomi, T., Kiyohara, Y., Ogino, C., Yoshida, K. and Nishizuka, Y. (1995) Proc. Natl. Acad. Sci. USA 92, 12319–12322.
[20] Shimooku, K., Akisue, T., Jinnai, H., Hitomi, T., Ogino, C., Yoshida, K., Nakamura, S. and Nishizuka, Y. (1996) FEBS Lett. 387, 141–144.
[21] Taylor, T.C., Kahn, R.A. and Melançon, P. (1992) Cell 70, 69–79.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Inoue, H., Shimooku, K., Akisue, T. and Nakamura, S. (1995) Biochem. Biophys. Res. Commun. 210, 542–548.
[24] Kikkawa, U., Go, M., Koumoto, J. and Nishizuka, Y. (1986) Biochem. Biophys. Res. Commun. 135, 636–643.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Hammond, S.M., Altshuller, Y.M., Sung, T.-C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J. and Frohman, M.A. (1995) J. Biol. Chem. 270, 29640–29643.
[27] Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A. and Morris, A.J. (1997) J. Biol. Chem. 272, 3860–3868.
[28] Kodaki, T. and Yamashita, S. (1997) J. Biol. Chem. 272, 11408–11413.