J. Biochem. 2011;150(4):385–394 doi:10.1093/jb/mvr064

JB THE JOURNAL OF BIOCHEMISTRY

Identification of a 42-kDa Group IV cPLA$_2$-activating protein, cPLAPγ, as a GTP-binding protein in the bovine brain

Received November 5, 2010; accepted April 11, 2011; published online May 25, 2011

Jae Hyung Bach, Kwang Mook Jung, Jae Sin Choi, Sung Yun Jung, Mi Reyoung Chin, Kyong Hoon Ahn, Seok Kyun Kim and Dae Kyong Kim*

Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, 221 Huksuk-dong, Dongjak-ku, Seoul 156-756, South Korea

*Dae Kyong Kim, Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, 221 Huksuk-Dong, Dongjak-Ku, Seoul 156-756, South Korea. Tel: +82-2-820-5610, Fax: +82-2-825-7920, email: proteinlab@hanmail.net

Brain tissue contains multiple forms of Phospholipase A$_2$ (PLA$_2$) whose activities are involved in intracellular and intercellular signalling related to normal functions such as long-term potentiation, neurotransmitter release, cell growth and differentiation. Among them, we focused on regulatory mechanism of cPLA$_2$α (Group IVA cytosolic PLA$_2$) in brain tissue. In the present study, we report the identification of a cPLA$_2$-activating protein (cPLAP) in the bovine brain. cPLAP activity appeared as two major peaks with molecular masses of 200 and 42 kDa in a Superose 12 gel filtration FPLC column. The 42-kDa form of cPLAP, designated cPLAPγ, was further purified using a Mono S FPLC column to near homogeneity and characterized to as a GTP-binding protein (G protein). Metabolic labelling and immunoprecipitation studies revealed that cPLAPγ associates with cPLA$_2$ *in vitro* and co-immunoprecipitates with [^{35}S]-cPLA$_2$. Notably, cPLAPγ rendered cPLA$_2$ fully activated at submicromolar concentrations of Ca^{2+}. These results suggest that cPLAPγ may act as a G protein, activating cPLA$_2$α prior to reaching full intracellular Ca^{2+} concentrations.

Keywords: calcium/cPLA$_2$-activating protein/Group IV cytosolic phospholipase A$_2$/GTP-binding protein/purification.

Abbreviations: 2-[1-^{14}C]AA-GPC, 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine; 2-[1-^{14}C]AA-GPE, 1-acyyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine; AA, arachidonic acid; AppNHp, adenylylimidotriphosphate; CaLB domain, Ca^{2+}-dependent lipid-binding domain; cPLA$_2$, cytosolic phospholipase A$_2$; cPLAP, cPLA$_2$-activating protein; GDP, guanosine diphosphate; G protein, GTP-binding regulatory protein; GTP, guanosine triphosphate; MAP kinase, mitogen-activated protein kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI-PLC, phosphatidylinositol-specific phospholipase C; PLA$_2$, phospholipase A$_2$ (EC 3.1.1.4); PLAP, PLA$_2$-activating protein.

Phospholipase A$_2$ (PLA$_2$: EC 3.1.1.4) are ubiquitous enzymes that specifically hydrolyze the acyl ester bond at the *sn*-2 position of membrane phospholipids to produce a free acid and lysophospholipid (*1*).

Brain tissue contains multiple forms of PLA$_2$, whose activities are involved in intracellular and intercellular signalling related to normal functions such as long-term potentiation, neurotransmitter release, cell growth, and differentiation (*2*). Increased levels or activity of PLA$_2$ and excessive production of arachidonic acid (AA) and lysophospholipids may lead to disease states and neuronal injury or neurodegenerative diseases (*3*), including Alzheimer's disease (*4*), multiple sclerosis (*5*), olivopontocerebellar atrophy (*6*), as well as psychiatric disorders such as schizophrenia (*7*).

Recent advances in molecular biology of PLA$_2$ have led to the identification of more than 20 isoforms with PLA$_2$ activity. The PLA$_2$ can be divided into several groups depending upon enzymatic properties, subcellular localization, their structure and cellular function (*2*). These are secretory PLA$_2$ (sPLA$_2$), cytosolic PLA$_2$ (cPLA$_2$) and calcium-independent PLA$_2$ (iPLA$_2$). Among them, the most thoroughly investigated group of PLA$_2$ is cPLA$_2$ (Group IV PLA$_2$) in brain tissue. Our laboratory has focused on the study of the group IVA cPLA$_2$ (Group IVA PLA$_2$ or cPLA$_2$α). Although brain tissue contains cPLA$_2$ activity, it has never been purified to homogeneity and characterized from brain (*2*). Thus, not only its regulatory mechanism of AA release in the brain but also the existence of cPLA$_2$ as an active enzyme in the brain remains largely uncertain.

Although to date extensive studies have focused on the regulatory mechanism of cPLA$_2$, the precise mechanism remains not fully understood. At present, three major mechanisms have been elucidated. First, Ca^{2+} is an important regulator of cPLA$_2$ activity. This enzyme has a Ca^{2+}-dependent lipid-binding (CaLB) domain, which is responsible for translocation of cPLA$_2$ from the cytosol to the membrane compartment in the presence of 10^{-6} M Ca^{2+} (*8*, *9*). That is, cPLA$_2$ requires Ca^{2+} only for translocation and association with its substrate membrane via the CaLB domain. Second, phosphorylation of cPLA$_2$ is also postulated as a

possible mechanism of activation. Diverse agonists that provoke the release of AA also activate a variety of kinases (*10–12*). Third, ceramide can interact directly with $cPLA_2$ via the CaLB domain and thereby serves as a membrane-docking device that facilitates $cPLA_2$ action in inflammatory diseases (*13*). Moreover, ceramide-1-phosphate not only increases the membrane affinity of $cPLA_2$ but also may act as an allosteric activator of the membrane-associated enzyme (*14*). However, detailed mechanisms of its regulation remain to be elucidated.

In a signal transduction concept first proposed by Rodbell *et al*. (*15*), GTP-binding regulatory protein (G protein) was proposed as a transducer or a molecular switch. Many phospholipases have been found to interact with and be directly regulated by specific G proteins, *e.g*. phospholipase C (PLC) by heterotrimeric G proteins (*16*, *17*) and phospholipase D by ADP-ribosylation factor (*18*). In the brain, PLA_2 activity and AA release are linked to the receptors for dopamine, glutamate, serotonin, P2-purinergics, cytokines and growth factors through various coupling mechanisms (*19–23*). In the case of PLA_2, there have been many trials to identify the corresponding G protein that regulates PLA_2s, especially high-molecular weight $cPLA_2\alpha$, which is hormonally regulated (*24*). Using the non-hydrolysable guanine nucleotide analogues GTPγS/GDPβS and G protein-sensitive bacterial toxins such as cholera toxin and pertussis toxin, there are an increasing number of reports suggesting that AA release in various cells and tissues is regulated by G proteins (*25*). $cPLA_2$ is regulated by Ras-related C3 botulinum toxin substrate 1 (Rac1), a member of the Rho family of G proteins, which acts as a molecular switch by cycling between active GTP bound and inactive GDP-bound forms (*26*). Rac1 binds to effector molecules in its GTP bound form, and influences $cPLA_2$ activation and subsequent release of AA (*27–29*). However, at present, there is no evidence for direct interaction of $cPLA_2$ with a G protein either *in vivo* or *in vitro*.

On the other hand, 28-kDa PLA_2-activating protein (PLAP) has been identified and characterized (*30*). The PLAP stimulated $sPLA_2$ but not $cPLA_2$. The purified protein selectively stimulated $sPLA_2$ when phosphatidylcholine (PC) was used as a substrate but had no effect on activity with phosphatidylethanolamine (PE) as a substrate. Interestingly, a homology search identified that PLAP has a domain exhibiting significant homology with β-transducin, a member of the G_β superfamily. However, the importance of this domain for PLAP activity remains unknown, as PLAP activity has not been shown to be affected by GTPγS or GDPβS.

In the present study, we identified a $cPLA_2$-activating protein (cPLAP) in the bovine brain that specifically enhanced the *in vitro* activity of porcine spleen $cPLA_2$. The cPLAP activity appeared as two major peaks with molecular masses of 200 and 42 kDa on a Superose 12-gel filtration FPLC column. We also demonstrate here that a polyclonal antibody generated against the $cPLA_2\alpha$ protein immunoprecipitated 42 kDa cPLAP (cPLAPγ) from bovine brain as well as human promyelocytic leukaemia HL60 and human leukaemic monocyte lymphoma U937 cell lines in metabolic labelling study. These results strongly suggest that cPLAPγ may be a G protein which fully activates $cPLA_2$ at sub-micromolar concentrations of Ca^{2+}.

Materials and Methods

Materials

1-Stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (2-[1-^{14}C]AA-GPC) (55 mCi/mol), 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (2-[1-^{14}C]AA-GPE) (55.6 mCi/mol) and [α-^{32}P]GTP (1 mCi/ml, 3000 Ci/mmol) were purchased from Amersham Life Science, Ltd (Buckinghamshire, England). DEAE-cellulose gel (DE52) was purchased from Whatman Co (Maidstone, England) and Phenyl-Sepharose CL-4B gel, Resource S column and PD-10 were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). A Phenyl-5PW column was purchased from Tosoh (Tokyo, Japan). A Centricon YM10 centrifuge was purchased from Millipore (Bedford, MA, USA). Porcine pancreatic PLA_2 was purchased from Sigma Chemical Co. (St Louis, MO, USA). $cPLA_2\alpha$ was purified from porcine spleen as described previously (*31*). Rat platelet group II PLA_2 was partially purified from rat platelets as described previously (*32*).

Anti-$cPLA_2$ antibody was prepared as described previously (*31*). GTPγS and GDPβS were purchased from BIOMOL Research Laboratories, Inc (Plymouth, PA, USA) and guanosine 5′-triphosphate (GTP), ATP and adenylylimidotriphosphate (AppNHp) from Sigma Chemical Co. All other chemicals were of the highest purity or molecular biology grade available from commercial sources.

Assay for $cPLA_2$, $iPLA_2$ and cPLAP activities

To assay $cPLA_2$ activity, the substrate, 2-[1-^{14}C]AA-GPC or 2-[1-^{14}C]AA-GPE, was dried under nitrogen and resuspended in ethanol. The standard incubation system (100 µl) for assay of $cPLA_2$ activity contained the $cPLA_2$ preparation, 75 mM Tris–HCl at pH 7.5, 5 mM $CaCl_2$ and 4.5 nmol of the substrate (~55,000 cpm). $iPLA_2$ activity assay was performed in the standard reaction buffer (100 µl) contained $iPLA_2$ preparations, 200 mM Tris–HCl, pH 7.5, 5 mM EDTA and 4.5 nmol of the substrate. The reactions were carried out at 37°C for 5 min and stopped by adding 560 µl of modified Dole's reagent (*n*-heptane/isopropyl alcohol/1 N-H_2SO_4; 400/390/10, by volume) (*33*) and 110 µl of water, vortex mixed and centrifuged. Then, 150 µl of the upper phase was transferred to a new microtube, to which 800 µl of *n*-heptane and silica gel (~5 mg) was added. The sample was vortex mixed and centrifuged again and an aliquot (800 µl) of the supernatant was removed and added to 2.5 ml of β-scintillation solution (INSTA GEL-XF) and counted for radioactivity with a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co, Meriden, CT, USA). In order to characterize GTP effect, $cPLA_2$ activity was assayed in the presence of 2.5 mM of $MgCl_2$. The $cPLA_2$ activity itself was not affected by Mg^{2+}. To measure cPLAP activity, an aliquot of the sample preparation was added to the assay system for $cPLA_2$ activity and expressed as an *n*-fold increase in activity.

Detection and purification of cPLAPγ and $iPLA_2$

Bovine brain was freshly obtained from a local slaughterhouse and frozen at −70°C. A 600*g* portion of brain was thawed in five volumes of homogenizing buffer V (50 mM Tris–HCl at pH 7.5, 50 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol) with a Polytron PT-MR6000 (Kinematica AG, Rittau, Switzerland). The homogenates were centrifuged at 10,000*g* for 60 min. The resulting supernatants were adjusted to pH 5.0 with acetic acid and centrifuged again at 10,000*g* for 15 min. After centrifugation, precipitates were resuspended in buffer V using Polytron at 4°C. The resuspension of pH 5.0-precipitate was loaded onto a DEAE-cellulose anion exchange column (DE52, 2.5 mm × 12.5 cm, bed volume 200 ml; Whatman Ltd) designed for use with a P-3 peristaltic pump (Pharmacia LKB Biotechnology AB) with buffer A (50 mM Tris–HCl at pH 7.5, 1 mM EDTA and 10 mM 2-mercaptoethanol) at a flow rate of ~25 ml/min, and the column

was washed with buffer A. The washed column was eluted at a flow rate of 25 ml/min with a stepwise gradient of 0–1.0 M NaCl. The protein fractions eluted with 1.0 M NaCl were fractionated at 30 ml per fraction. A 20-μl aliquot of each fraction was assayed for $iPLA_2$ and $cPLA_2$-activating activities. The active fractions with $iPLA_2$ activity were pooled.

The active fractions from the DEAE-cellulose column were pooled and adjusted to 0.5 M $(NH_4)_2SO_4$ by adding 4.0 M $(NH_4)_2SO_4$ solution and applied to a Phenyl-Sepharose CL-4B column (30 mm × 25 cm, bed volume 30 ml; Pharmacia LKB Biotechnology) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. The column was eluted at a flow rate of 10 ml/min with sequential stepwise elution of buffer A containing 0.25 M $(NH_4)_2SO_4$ and buffer B (25 mM glycine–NaOH at pH 9.0, 1 mM EDTA, and 10 mM 2-mercaptoethanol). A 20-μl aliquot of each 10 ml fraction was assayed for $iPLA_2$ and $cPLA_2$-activating activities at 37°C for 5 min. The active pool of the Phenyl-Sepharose CL-4B column was adjusted to 0.75 M $(NH_4)_2SO_4$ and applied to Phenyl-5PW HPLC column, which was pre-equilibrated with buffer A containing 0.75 M $(NH_4)_2SO_4$. The column was eluted at a flow rate of 5 ml/min with a linear gradient with water. The active fractions with $cPLA_2$-activating activity were pooled, dialyzed with buffer S (25 mM imidazole–HCl at pH 6.6, 3 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol) for 18 h. The dialyzed sample was applied to a Resource S FPLC column (1 ml; Pharmacia LKB Biotechnology AB) pre-equilibrated with buffer S. The column was eluted at a flow rate of 1 ml/min with a linear gradient of 0.0–1.0 M NaCl. A 20-μl aliquot of each 1 ml fraction was assayed for $cPLA_2$-activating activity. The active fractions (~3.0 ml) were concentrated to 400 μl using a Centricon YM10 (Millipore). The active concentrate was applied to a Superose 12 gel filtration FPLC column (Pharmacia LKB Biotechnology AB) pre-equilibrated with buffer G (50 mM Tris–HCl at pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1 M NaCl). The column was eluted at a flow rate of 0.5 ml/min. A 50-μl aliquot of each 0.5 ml fraction was assayed for $cPLA_2$-activating activity at 37°C for 5 min. The 42-kDa peak fractions were pooled and dialyzed with buffer S (25 mM imidazole/HCl at pH 6.6, 3 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol) for 18 h. The dialyzed sample was applied to a Mono S FPLC column (1 ml/bed volume; Pharmacia LKB Biotechnology AB) pre-equilibrated with buffer S. The column was eluted at a flow rate of 1 ml/min with a linear gradient of 0.0–1.0 M NaCl. A 20-μl aliquot of each 1.0 ml fraction was assayed for $cPLA_2$-activating activity.

Photoaffinity labelling of cPLAPγ with [α-^{32}P]GTP

To determine whether GTP binds to cPLAPγ by photoreaction, photoaffinity labelling of cPLAPγ with [α-^{32}P]GTP was performed as previously described (*34*, *35*) with a minor modification. Briefly, the samples were mixed with 5–10 mCi of [α-^{32}P]GTP, 0.1 mM AppNHp and 2 mM $MgCl_2$ and pre-incubated for 5 min at 4°C. The samples were then placed in an ice bath and irradiated with UV light (254 nm) for 5–10 min using a UV cross-linker (SPECTROLINKER XL-1000, Spectronics Co, Manukau City, New Zealand). After irradiation, the samples were mixed with Laemmli's stopping solution (*36*) and allowed to stand at room temperature for 1 h. The samples were subjected to SDS–PAGE using 8–12% gels, and the gels were dried and exposed overnight on Kodak X-OMAT XAR-5 film using DuPont image intensifying screens.

Co-immunoprecipitation of cPLAPγ with $cPLA_2$ in HL60 and U937 cells metabolically labelled with L-[^{35}S]methionine

The cells cultured in DMEM (10% FCS) were collected by centrifugation at 400*g* for 5 min. The resulting pellets were resuspended in DMEM (10% FCS) without L-methionine supplemented with L-glutamine (2%) as a wash buffer and centrifuged at 400*g* at room temperature for 5 min. After removing the wash buffer as completely as possible, the cells were resuspended in methionine-free medium (1×10^7 cells/ml). After transferring to a small tissue culture plate (16 mm, 24 wells/plate), the cells were incubated for 30 min at 37°C to exhaust the remaining methionine.

Then 100 μCi [^{35}S]methionine was added to the plates and the cells were allowed to incorporate the radiolabel into total protein for 3 h. The cells were treated with 1 μM A23187 for 30 min, transferred to a test tube and washed twice with PBS. The resulting pellets were resuspended in 1 ml of a RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl at pH 7.5 and 1 mM EDTA) per 1×10^7 cells for lysis. The cells were kept on ice for 30 min with occasional mixing and spun down for 10 min at 10,000*g* at 4°C. The resulting supernatants were centrifuged at 100,000*g* for 30 min and the supernatants used as the soluble cell-free fractions.

To pre-clear proteins in the soluble fractions that bind non-specifically to immune complexes or the solid phase, 1 ml fractions were incubated with 50 μl of normal serum on ice for 1 h. Then 500 μl of 10% washed suspension of fixed *Staphylococcus aureus* Cowan I (SAC) was added to the lysates and further incubated for 30 min on ice. The resulting supernatants were incubated with 20 μl of anti-$cPLA_2$ antibody bound to Protein A-Sepharose beads for 12 h. Immune complexes bound to the beads were pelleted at 1,000*g* at 4°C for 3 min and washed five times with 1 ml of the lysis buffer. The $cPLA_2$ and associated proteins were eluted by incubating for 5 min at 100°C with 70 μl of 2 × Laemmli's buffer and subjected to 10% SDS–PAGE. After washing twice for 30 min with DMSO, the gel was reacted with 22% PPO in DMSO for 1 h and extensively washed for 30 min with water in a circulating chamber. Then, the gel was dried at 80°C for 40 min and exposed to film overnight at −70°C for fluorography.

Results

Detection and partial purification of cPLAPγ

Recently, we identified and purified a novel form of $iPLA_2$ in the pH 5.0-precipitate of the brain homogenate (Eui Man Jeong *et al.*, manuscript in preparation). The brain homogenates were resuspended with buffer V and applied to a DEAE-cellulose anion exchange column, where the $iPLA_2$ activity was eluted with a stepwise gradient of 1.0 M NaCl with an 82% yield and ~10.5-fold purification (Fig. 1A). The active fractions of $iPLA_2$ were adjusted to 0.5 M $(NH_4)_2SO_4$ and then applied to a Phenyl-Sepharose CL-4B hydrophobic column. The bound proteins were eluted with stepwise gradients of buffer A containing 0.25 M $(NH_4)_2SO_4$ and buffer B containing no salt, respectively. The $iPLA_2$ activity was eluted in the glycine-eluted fractions with a yield of <20%, but not in 0.25 M $(NH_4)_2SO_4$ elution (Fig. 1B). This low yield prompted us to examine an existence of an activator in other fractions. First, we observed a small increase by ~1.2–1.5-fold in $iPLA_2$ activity in the 0.25 M $(NH_4)_2SO_4$-elution fractions, but this result could not explain the low yield in this step (data not shown). Since we cannot exclude the possible existence of $cPLA_2$ in the glycine-eluted fraction, we added a highly purified $cPLA_2$ from porcine spleen to the 0.25 M $(NH_4)_2SO_4$-elution fractions. Interestingly, as shown in Figure 1B, a significant $cPLA_2$-activating activity was observed in the 0.25 M $(NH_4)_2SO_4$-elution fractions. One unit of $cPLA_2$-activating activity was defined as the ability that doubles 52 nmol/min of the total activity of the purified spleen $cPLA_2$ for 5 min/mg of each protein in the samples. For purification, the fractions showing $cPLA_2$-activating activity were readjusted to 0.75 M $(NH_4)_2SO_4$ and then applied to a Phenyl-5PW HPLC column (Fig. 1C). The active fractions were pooled and dialyzed for 18 h. Then, the dialyzed sample was applied to a Resource S FPLC column (Fig. 1D). The active fractions had 55% less protein and a total activity recovery of ~60% compared with the loaded sample. Finally, a 3.0-ml pool of the active fractions from the Resource S FPLC

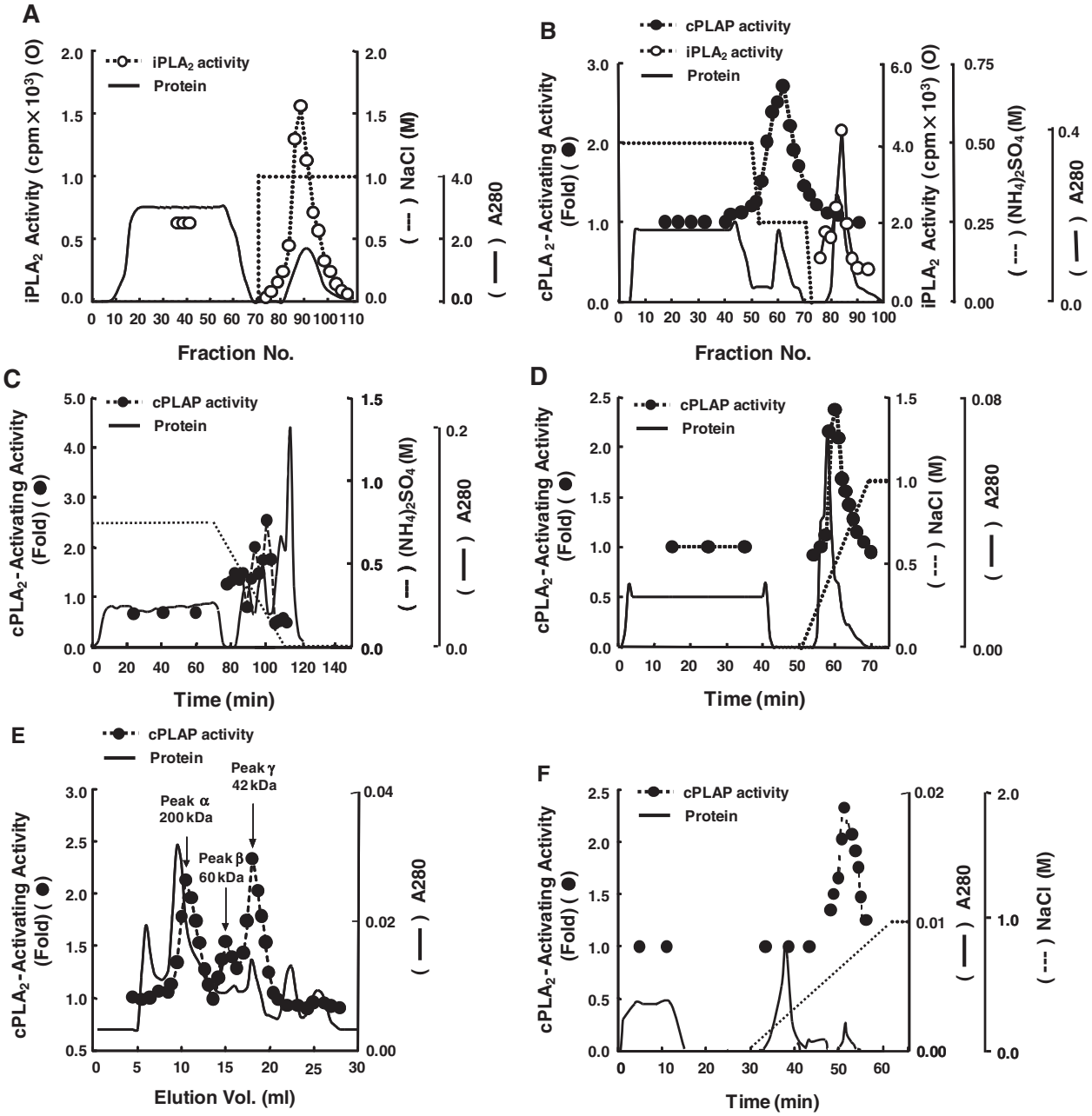

Fig. 1 Detection and purification of cPLAP. (A) Detection of $iPLA_2$ in bovine brain samples. The pH 5.0-precipitate of bovine brain homogenate was applied to a DE52 column pre-equilibrated with buffer A. The column was eluted and each fraction was assayed for $iPLA_2$ activity as described in 'Materials and Methods' section. The active fractions (numbers 83–96) were pooled for the next step. (B) Detection of cPLAP by Phenyl-Sepharose CL-4B hydrophobic column chromatography. The pool of DE52 was applied to a Phenyl-Sepharose CL-4B column pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. The column was eluted with a sequential stepwise elution and each fraction was assayed for cPLAP activity as described in 'Materials and Methods' section. The $iPLA_2$ activity was eluted in the elution step without salt. (C) Phenyl-5PW HPLC column profile of cPLAP. The pool of the active cPLAP fractions of the Phenyl-Sepharose CL-4B column was applied to a Phenyl-5PW HPLC column pre-equilibrated with buffer A. The column was eluted and each fraction was assayed for cPLAP activity as described in 'Materials and Methods' section. (D) Resource S FPLC profile of the active pool from the Phenyl-5PW column. The active pool was dialyzed and applied to a Resource S FPLC column pre-equilibrated with buffer S. The column was eluted and assayed for cPLAP activity as described in 'Materials and Methods' section. Fraction numbers 59–61 were pooled for the next step. (E) Superose 12-gel filtration FPLC profile of cPLAP. The active pool of Resource S was applied to a Superose 12 FPLC column pre-equilibrated with buffer T. The column was eluted and assayed for cPLAP activity as described in 'Materials and Methods' section. (F) Mono S cation exchange FPLC profile of cPLAP. The active pool of Superose 12 FPLC column was applied to a Mono S FPLC column, which was eluted and assayed for cPLAP activity as described in 'Materials and Methods' section.

column was concentrated to ~400 µl and applied to a Superose 12 gel filtration FPLC column. The $cPLA_2$-activating activity was separated into two major peaks of apparent molecular masses of 200 and 42 kDa, termed peak α and γ, respectively (Fig. 1E). The 42-kDa protein of peak γ was further purified using a Mono S cation exchange FPLC column (Fig. 1F).

Effects of cPLAPγ on various types of PLA$_2$

A 20-μl aliquot of the active pool obtained from the Mono S FPLC column was assayed for cPLAP activity. To examine the substrate specificity of the acyl chains at the *sn*-2 position, PLA$_2$-activating activity was assayed for 2-PA-PC, 2-LA-PC and 2-AA-PC. There was no preferential activity of cPLA$_2$ toward AA at the *sn*-2 position (Figure 2A). The effects of cPLAPγ on various types of PLA$_2$ were then examined. cPLAPγ enhanced group IVA PLA$_2$ activity by ~2-fold, but did not affect pancreatic group I, non-pancreatic group II or partially purified iPLA$_2$ from the bovine brain. The results indicate that cPLAPγ activated significantly cPLA$_2$ by up to ~2.5-fold (Figure 2B). Next, we also examined the effects of GTPγS on cPLAPγ activity. The addition of GTPγS further increased the activating effect of cPLAPγ on cPLA$_2$α (Figure 2A and B). The data suggest that cPLAPγ may be a GTP-binding protein.

To determine the nature of the cPLA$_2$-activating activity, the effects of boiling and digestion with trypsin on cPLA$_2$-activating activity were assessed (Figure 2C). A 20-μl aliquot of the active fractions from Mono S was pre-incubated for 15 and 30 min at 75°C, followed by 0.5 μg/μl of trypsin and then the trypsin inhibitor 0.25 μg/μl aprotinin, or vice versa, before addition of the cPLA$_2$ enzyme source. The cPLA$_2$-activating activity was sensitive to boiling and digestion with trypsin, thus the cPLA$_2$-activating activity is attributable to a protein in the preparation, and is named cPLA$_2$-activating protein accordingly.

cPLAPγ has characteristics of G protein

To characterize the cPLAPγ, the effects of guanine nucleotides on the activity of cPLAP were investigated. Because most of the GTP proteins require an appropriate concentration of Mg^{2+} for nucleotide binding

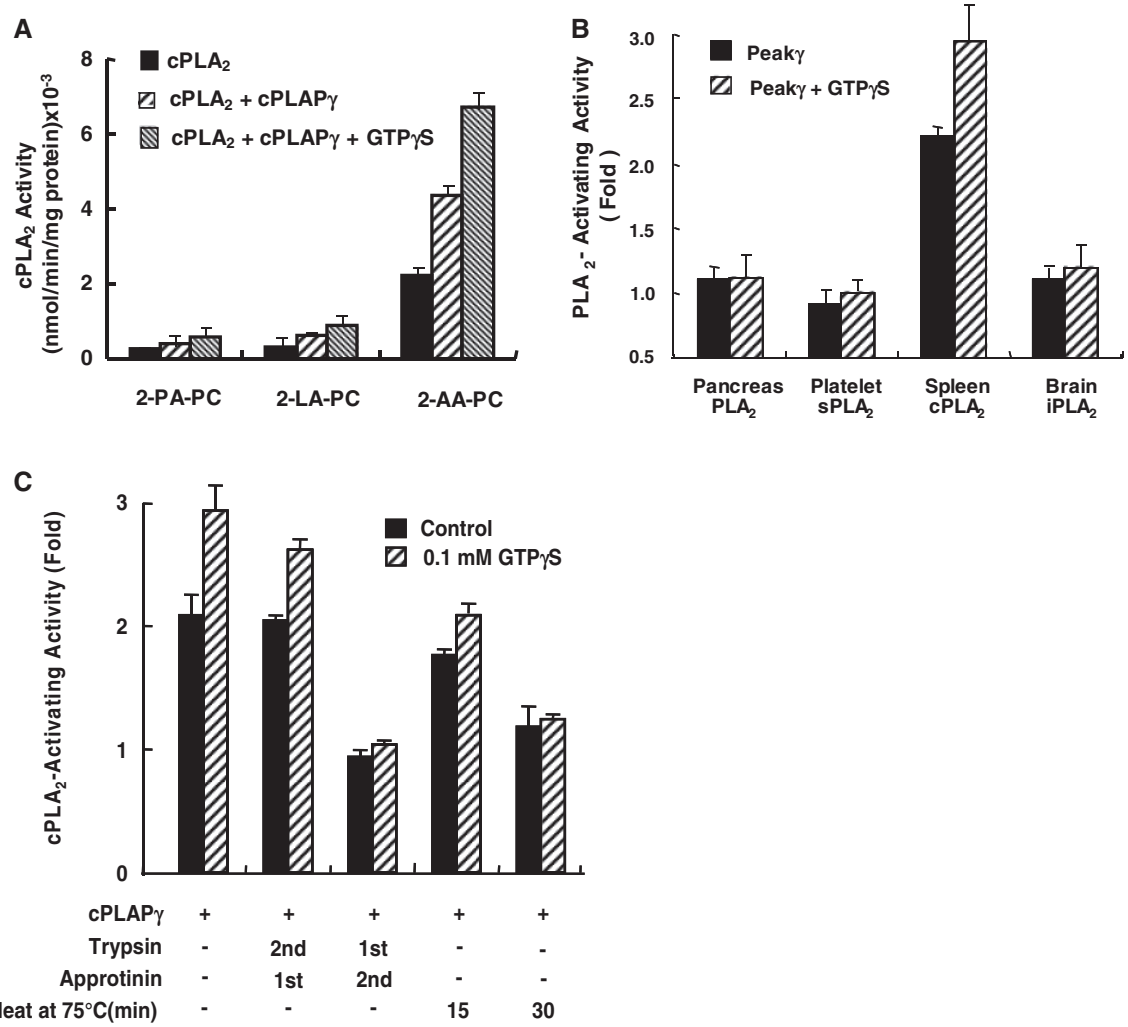

Fig. 2 Selectivity of cPLAPγ for subtypes of PLA$_2$ and fatty acids at the sn-2 position. A 20-μl aliquot of the active pool obtained from the Mono S FPLC column was assayed for cPLAP activity. (A) cPLAPγ activity was assayed using PCs containing various fatty acids at the *sn*-2 position as described in 'Materials and Methods' section. (B) The cPLAPγ activity was assayed using various types of PLA$_2$ as described in 'Materials and Methods' section. Pancreas PLA$_2$ (Group I PLA$_2$), platelet sPLA$_2$ (Group II), spleen cPLA$_2$ (Group IV), and brain iPLA$_2$ (Ca^{2+}-independent PLA$_2$) had 3,500, 3,200, 4,280 and 4,100 cpm under the assay conditions, respectively. (C) Effects of trypsin and heat on cPLAPγ activity. cPLAPγ activity was measured by incubating with the combination of trypsin and its inhibitor approtinin. Activity was also assayed by boiling at 75°C for 15 and 30 min. Values are the average of duplicate assays with a variance of <10%. The result is representative of three independent experiments for each condition.

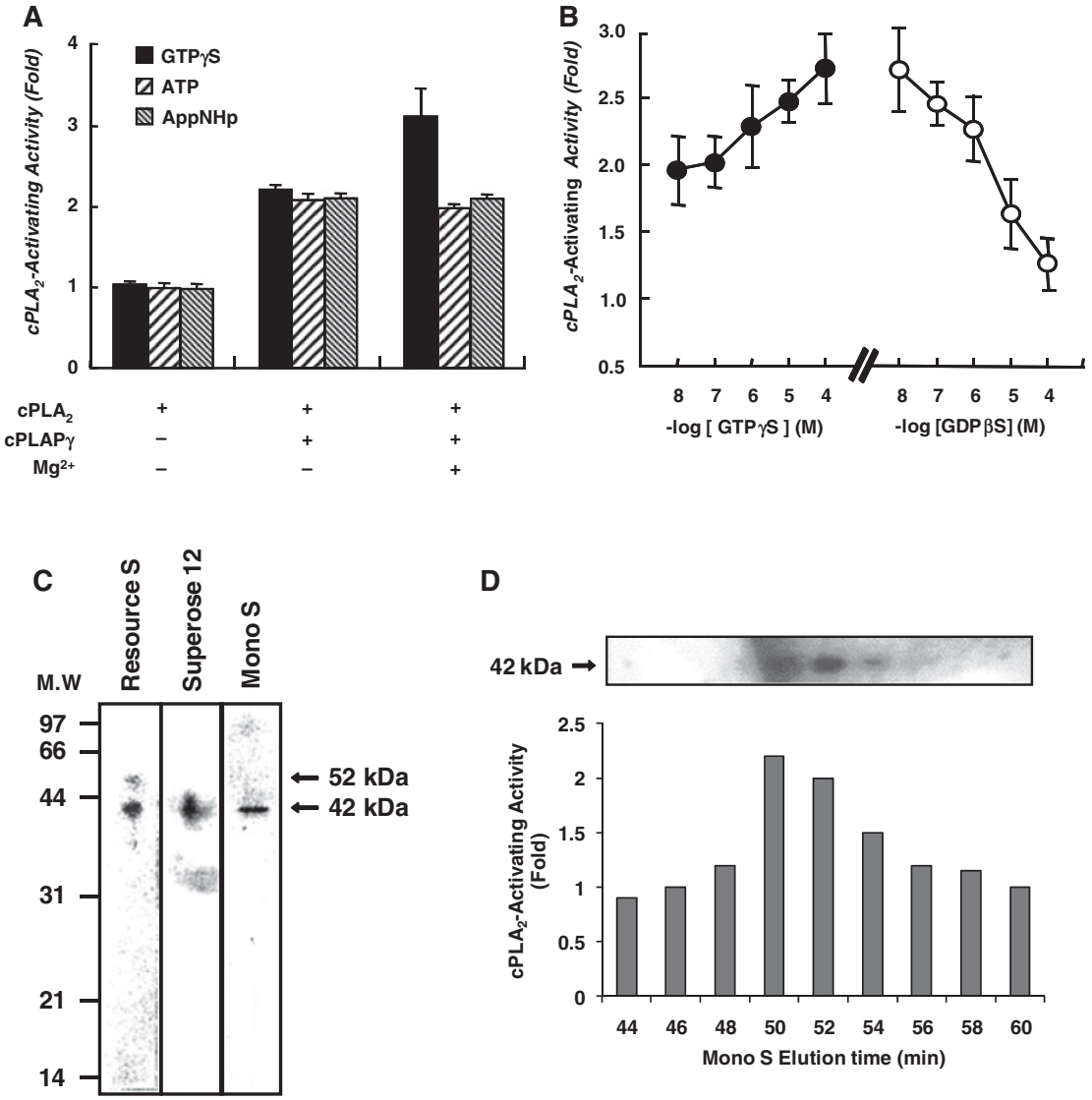

Fig. 3 Identification of cPLAPγ as a G protein. (A) Effects of Mg^{2+} ion and nucleotide analogues on cPLAPγ activity. cPLAPγ activity was assayed with 0.1 mM GTPγS, ATP and AppNHp under standard assay conditions with or without 2.5 mM $MgCl_2$. After incubation for 5 min, cPLAPγ activity was measured as described in 'Materials and Methods' section. (B) cPLAPγ activity was increased by GTPγS and decreased by GDPβS. A 50-μl aliquot of the active fractions from the Superose 12 FPLC column was assayed with increasing concentrations of GTPγS (dark circle) followed by additional treatment with increasing concentrations of GDPβS (open circle) as described in 'Materials and Methods' section. (C) Photoaffinity labelling of cPLAPγ with [α-^{32}P]GTP. A 30-μl aliquot of the active fractions from each column was incubated with 5 mCi of [α-^{32}P]GTP and 0.5 mM AppNHp in the presence of 2 mM $MgCl_2$ and labelled with UV light (254 nm). The proteins were analyzed with SDS/PAGE (12% gel) followed by autoradiography as described in 'Materials and Methods' section. (D) Photoaffinity labelling of cPLAPγ with [α-^{32}P]GTP. A 30-μl aliquot of the fractions from Mono S column was incubated with 5 mCi of [α-^{32}P]GTP and 0.5 mM AppNHp in the presence of 2 mM $MgCl_2$ and labelled with UV light (254 nm). The proteins were analyzed with SDS/PAGE (12% gel) followed by autoradiography as described in 'Materials and Methods' section.

and configuration, 2.5 mM of $MgCl_2$ was added to the standard assay system. The $cPLA_2$ activity itself was not affected by Mg^{2+} (data not shown). As shown in Figure 3A, cPLAP activity enhanced by GTPγS required the presence of Mg^{2+}. ATP and AppNHp, other purine nucleotides whose structures are similar to the guanine nucleotide, had no effect (Figure 3A). GTPγS dose-dependently enhanced cPLAPγ activity up to ~1.5- to 2.0-fold, whereas GDPβS, a non-hydrolyzable GDP analogue, dose-dependently decreased the enhanced cPLAPγ activity (Figure 3B). To further confirm the possibility that cPLAPγ may be a G protein, a photoaffinity labelling experiment with [α-^{32}P]GTP was performed (Figure 3C). When the active fractions from Resource S and Superose 12 columns were labelled with [α-^{32}P]GTP, proteins with molecular masses of 42 and 52 kDa were cross-linked with UV and the protein band at 42 kDa was seen at the same position as the major band from the Mono S column of the final step (Figure 3C). Furthermore, cPLAPγ was observed as a 42 kDa band, paralleled with the activity from the Mono S column (Figure 3D). These results strongly suggest that cPLAPγ may be a GTP-binding protein. The cPLAP activity appearing with ~200 kDa molecular mass in the Superose 12 gel filtration FPLC was not affected by guanine nucleotides nor was labelled by UV cross-linking (data not shown).

Identification of the 42 kDa protein which is co-immunoprecipitated with $cPLA_2$

$cPLA_2$ is found normally in a various cells including HL60 and U937 cells. Proteins from HL60 and U937 cells were radiolabelled by incubating the cells in [^{35}S]methionine-containing medium. The supernatants obtained from the [^{35}S]methionine-labelled cells were used as the soluble cell-free fractions for immunoprecipitation. As shown in Figure 4, Protein A-Sepharose beads bound to anti-$cPLA_2$ antibody precipitated a 42-kDa protein from both HL60 and U937 cells, but those bound to normal serum did not.

On the other hand, it is important whether interaction of $cPLA_2$ and cPLAPγ require an increase in intracellular Ca^{2+} concentration. Our results showed that proteins from A23187-untreated cell had no different effect on the interaction of cPLAPγ toward $cPLA_2$ (data not shown). Although intracellular Ca^{2+} is not required to interaction between cPLAPγ and $cPLA_2$, there is a possibility that cPLAPγ could make $cPLA_2$ more active through increase of Ca^{2+}sensitivity. Next, we investigated the effects of Ca^{2+} on the increase of $cPLA_2$ activity by cPLAPγ.

Ca^{2+}-dependency of the $cPLA_2$-activating activity of cPLAPγ

$cPLA_2$ is known to have full activity at a micromolar concentration of Ca^{2+}. Interestingly, the present data indicate that cPLAPγ fully activates $cPLA_2$ even at sub-micromolar concentrations of Ca^{2+} (Figure 5). The effect of GTPγS on the activity of cPLAPγ may be due to its ability to enhance cPLAPγ activity rather than to increase its sensitivity to submicromolar Ca^{2+} (Figure 5). The full activity of cPLAPγ in 10^{-7} M of Ca^{2+} was also inhibited by GDPβS in a dose-dependent manner (data not shown).

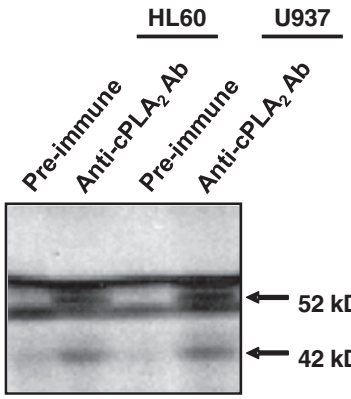

Fig. 4 Co-immunoprecipitation of cPLAPγ with $cPLA_2$ in HL60 and U937 cells metabolically labelled with L-[^{35}S]methionine. HL60 and U937 cells were labelled with [^{35}S]-methionine and treated with 1 μM A23187 as described in 'Materials and Methods' section. The cells were resuspended in an RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA for lysis and spun down to obtain the resulting supernatants, as described in 'Materials and Methods' section. The proteins of the soluble fractions were pre-cleared and incubated with anti-$cPLA_2$ antibody bound to Protein A-Sepharose beads. The $cPLA_2$ and associated proteins in the immune complexes were eluted and subjected to 10% SDS–PAGE as described in 'Materials and Methods' section. The gel was then dried at 80°C for 40 min and exposed to film overnight at −70°C for fluorography.

Discussion

AA is known to serve as a chemical mediator as well as a second messenger and is generated in membrane lipids by activation of $cPLA_2$ (*1*, *2*). The $cPLA_2$ enzyme is strictly regulated and abnormal signalling is believed to cause serious pathological disasters. The regulatory mechanisms of $cPLA_2$ have been extensively studied to understand the nature of its pathogenesis and to develop new therapy targets. However, the mechanism by which AA is released at an early stage of stimulation remains largely unknown.

G proteins have been proposed as a transducer and molecular switch for $cPLA_2$. Many phospholipases have been shown to be regulated by directly interacting with specific G proteins (*16–18*). There have been many attempts to discover a corresponding G protein that regulates $cPLA_2$ (*24*). Several lines of evidence are accumulating that AA release in various cells and tissues is regulated by G proteins using guanine nucleotide analogues and bacterial toxins such as cholera and pertussis toxin (*25*). It has also been suggested that a Rac1 G protein may serve as a mediator of $cPLA_2$ activation (*27*, *28*). However, at present, there is no direct evidence for the existence of a G protein coupled with $cPLA_2$ either *in vivo* or *in vitro*.

The present study reports a G protein that may interact with $cPLA_2$ to mediate its activation based on the following results: (i) cPLAPγ selectively increased $cPLA_2$ activity among various mammalian types of PLA_2; (ii) cPLAPγ activity was enhanced by GTPγS and decreased by GDPβS in a dose-dependent manner, and this effect required Mg^{2+}; (iii) ATP and other purine nucleotides such as AppNHp had no effect under the same conditions; (iv) cPLAPγ was

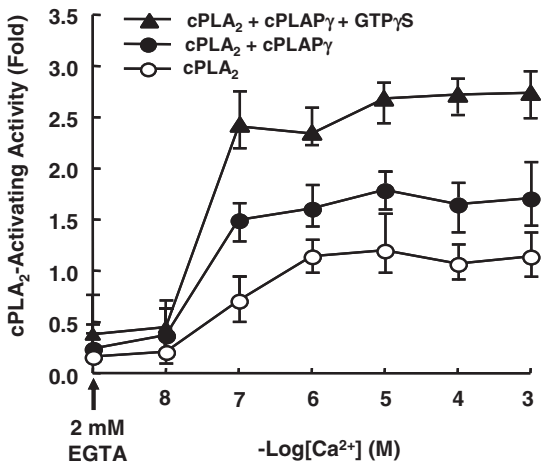

Fig. 5 Effects of cPLAPγ on Ca^{2+}-dependency of the purified $cPLA_2$. cPLAPγ activity was assayed in 75 mM Tris–HCl buffer (pH 7.5) containing Ca^{2+} ions at the indicated concentrations. The absolute concentrations of free Ca^{2+} were calculated using an equation based on the stability constant of the EGTA/$CaCl_2$ system (5 mM EGTA and 5 mM $CaCl_2$). The $cPLA_2$ enzyme was purified from porcine spleen. The data represent three independent experiments, showing the mean ± SEM of three measurements for each sample.

labelled with [α-^{32}P]GTP through photoaffinity; and (v) metabolic labelling with [^{35}S]methionine and co-immunoprecipitation by anti-cPLA$_2$ antibody revealed that cPLAPγ binds cPLA$_2$.

Two major peaks associated with cPLAP activity were detected, peaks α and γ, from the Superose 12-gel filtration FPLC column (Figure 1E). Peak α of ~200 kDa was not responsive to GTPγS or GDPβS (data not shown). Peak γ, termed cPLAPγ, was further purified with a Mono S cation exchange FPLC column to near homogeneity (Figure 1F). Based on SDS–PAGE analysis, photoaffinity labelling of cPLAPγ, and the migration profile in gel filtration chromatography, the molecular weight of cPLAPγ was estimated 42 kDa. Because the molecular mass of the α-subunit of the heterotrimeric G protein is 42 kDa and $AlF4^-$ is an activator of the α-subunit, the effect of $AlF4^-$ on the cPLAPγ activity was examined. Unfortunately, the effect could not be evaluated because $AlF4^-$ significantly inhibited cPLA$_2$ activity in a dose-dependent manner (data not shown). Thus the cross-reactivity with antibodies against previously known six types of G proteins, Gs, Gi, Gq, Go, Gi$_2$ and Gi$_3$ proteins, was also examined through the immunoblot analysis on fraction of final purification step, but the 42 kDa cPLAPγ did not react with any of them (data not shown).

Further study should also be carried out to examine whether the 42-kDa band as shown by fluorography in Figure 4 is cPLAPγ. Metabolic labelling analysis suggested that cPLAPγ may associate with cPLA$_2$. However, neither cPLA$_2$ activity nor cPLAPγ activity was detected in the immunoprecipitates obtained from the metabolic labelling cells (data not shown), suggesting that the RIPA buffer may denature the proteins, with consequent loss of their activities. The authors' previous study showed that considerable cPLA$_2$ activity was detected in immunoprecipitates obtained using an anti-cPLA$_2$ antibody (*31*).

In the rat brain, cPLA$_2$ was ubiquitously expressed throughout the brain (*37*). Although cPLA$_2$ activity comprises a small amount of the total PLA$_2$ activity of the brain tissue, its specific activity is high. Such a small amount of cPLA$_2$ activity may have important biological and pathological functions (*38*). cPLA$_2$ is functionally linked with both COX-1 and COX-2 during immediate and delayed eicosanoid production (*39*). Coordinated up-regulation of cPLA$_2$ and COX-2 activities is also thought to contribute to brain damage following cerebral ischaemia and neuroinflammation (*40*). On the other hand, it was demonstrated that PLA$_2$ activating proteins occur in Aplysia neurons and rat cerebral cortex (*41*). In the context of our results and previous reports, we hypothesize that cPLAPγ is important in cPLA$_2$ regulation and may be involved in inflammatory response induced by G protein coupled receptors. G protein coupled receptors in the brain may activate cPLAPγ, in turn, trigger increase of cPLA$_2$ activity that results in neuronal apoptosis or pathological changes. Although this is an appealing possibility, we do not currently have data to support this. Further study should also be carried out to identify and elucidate its primary structure and more precise mechanism for the cPLA$_2$ activation.

On the other hand, cPLA$_2$ activity is known to be regulated by the intracellular concentration of Ca^{2+} (*9*, *42*). Activity was not detected at concentrations of 1 to 5×10^{-7} M Ca^{2+} observed in the resting cells, but cPLA$_2$ was fully activated at a concentration of ~10^{-6} M Ca^{2+} enhanced by stimulation (*9*, *42*). Interestingly, cPLAPγ fully activated cPLA$_2$ at sub-micromolar concentration of Ca^{2+} (Figure 5), suggesting that cPLA$_2$ may become sensitive to lower concentrations of Ca^{2+} through cPLAPγ activity. This finding strongly supports the possibility that cPLA$_2$ may be activated irrespective of the PI-PLC pathway, which is known to be activated through G protein-coupled receptor activation to produce IP$_3$ and DAG (*43*). It has been unclear whether cPLA$_2$ activation is dependent on the activation of PI-PLC, which could trigger a sharp increase in intracellular Ca^{2+} via the role of IP$_3$ in Ca^{2+} mobilization (*44*). Many lines of evidence indicate that the release of AA from membrane lipids could occur without the generation of IP$_3$ (*45*), suggesting activation of PLA$_2$ prior to activation of PI-PLC. However, no evidence has been provided to explain this phenomenon. The present results may shed light on this longstanding and controversial finding that cPLA$_2$ can be activated without the involvement of PI-PLC (*46*, *47*).

At present, the biochemical mechanism by which cPLAPγ enhances cPLA$_2$ activity and allows the enzyme to fully activate at sub-micromolar concentrations of Ca^{2+} remains unknown. It has been reported that cPLA$_2$ contains a pleckstrin homology (PH) domain and interacts with multi-modular membrane-binding proteins that play key roles in signal transduction (*8*). The PH domain is known as a site of action for certain G proteins, and thus may be a site of action for cPLAPγ.

In summary, cPLAPγ was identified as a 42-kDa G protein that specifically enhances the *in vitro* activity of cPLA$_2$. cPLAPγ associates with cPLA$_2$ and allows the enzyme to fully activate at sub-micromolar concentrations of Ca^{2+}. In addition, cPLAPγ is suggested to play a role in the release of AA by activating cPLA$_2$ at an early stage prior to activation of PI-PLC, which is known to provide the micromolar concentrations of Ca^{2+} required for full activation of cPLA$_2$. To verify this, the precise nature of cPLAPγ should be dissected through sequencing of its primary structure and a study of the interaction between cPLAPγ and cPLA$_2$.

Funding

This work was supported by the National Research Foundation of Korea Grant funded by the Korea Government (NRF-313-2008-2-E00485).

Conflict of interest

None declared.

References

1. Rosenberger, T.A., Villacreses, N.E., Contreras, M.A., Bonventre, J.V., and Rapoport, S.I. (2003) Brain lipid

metabolism in the cPLA2 knockout mouse. *J. Lipid Res.* **44**, 109–117
2. Farooqui, A.A., Ong, W.Y., and Horrocks, L.A. (2006) Inhibitors of brain phospholipase A2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. *Pharmacol. Rev.* **58**, 591–620
3. Phillis, J.W. and O'Regan, M.H. (2004) A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. *Brain Res. Brain Res. Rev.* **44**, 13–47
4. Nitsch, R.M., Blusztajn, J.K., Pittas, A.G., Slack, B.E., Growdon, J.H., and Wurtman, R.J. (1992) Evidence for a membrane defect in Alzheimer disease brain. *Proc. Natl Acad. Sci. USA* **89**, 1671–1675
5. Kalyvas, A. and David, S. (2004) Cytosolic phospholipase A2 plays a key role in the pathogenesis of multiple sclerosis-like disease. *Neuron* **41**, 323–335
6. Kish, S.J., Robitaille, Y., Ball, M., Gilbert, J., Deck, J.H., Chang, L.J., and Schut, L. (1990) Glycerophosphoethanolamine concentration is elevated in brain of patients with dominantly inherited olivopontocerebellar atrophy. *Neurosci. Lett.* **120**, 209–211
7. Ross, B.M. (2003) Phospholipid and eicosanoid signaling disturbances in schizophrenia. *Prostaglandins Leukot. Essent. Fatty Acids* **69**, 407–412
8. Burke, J.E. and Dennis, E.A. (2009) Phospholipase A2 structure/function, mechanism, and signaling. *J. Lipid Res.* **50 (Suppl)**, S237–S242
9. Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N., and Knopf, J.L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043–1051
10. Hannigan, G.E. and Williams, B.R. (1991) Signal transduction by interferon-alpha through arachidonic acid metabolism. *Science* **251**, 204–207
11. Piomelli, D., Pilon, C., Giros, B., Sokoloff, P., Martres, M.P., and Schwartz, J.C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. *Nature* **353**, 164–167
12. Borsch-Haubold, A.G., Bartoli, F., Asselin, J., Dudler, T., Kramer, R.M., Apitz-Castro, R., Watson, S.P., and Gelb, M.H. (1998) Identification of the phosphorylation sites of cytosolic phospholipase A2 in agonist-stimulated human platelets and HeLa cells. *J. Biol. Chem.* **273**, 4449–4458
13. Huwiler, A., Johansen, B., Skarstad, A., and Pfeilschifter, J. (2001) Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. *FASEB J.* **15**, 7–9
14. Subramanian, P., Stahelin, R.V., Szulc, Z., Bielawska, A., Cho, W., and Chalfant, C.E. (2005) Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2 alpha and enhances the interaction of the enzyme with phosphatidylcholine. *J. Biol. Chem.* **280**, 17601–17607
15. Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M. (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanylnucleotides in glucagon action. *J. Biol. Chem.* **246**, 1877–1882
16. Smrcka, A.V., Hepler, J.R., Brown, K.O., and Sternweis, P.C. (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* **251**, 804–807
17. Taylor, S.J., Chae, H.Z., Rhee, S.G., and Exton, J.H. (1991) Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. *Nature* **350**, 516–518
18. Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C., and Sternweis, P.C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* **75**, 1137–1144
19. Lazarewicz, J.W., Wroblewski, J.T., Palmer, M.E., and Costa, E. (1988) Activation of N-methyl-D-aspartate-sensitive glutamate receptors stimulates arachidonic acid release in primary cultures of cerebellar granule cells. *Neuropharmacology* **27**, 765–769
20. Dumuis, A., Pin, J.P., Oomagari, K., Sebben, M., and Bockaert, J. (1990) Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. *Nature* **347**, 182–184
21. Bruner, G. and Murphy, S. (1993) Purinergic P2Y receptors on astrocytes are directly coupled to phospholipase A2. *Glia* **7**, 219–224
22. Vial, D. and Piomelli, D. (1995) Dopamine D2 receptors potentiate arachidonate release via activation of cytosolic, arachidonate-specific phospholipase A2. *J. Neurochem.* **64**, 2765–2772
23. Stella, N., Estelles, A., Siciliano, J., Tence, M., Desagher, S., Piomelli, D., Glowinski, J., and Premont, J. (1997) Interleukin-1 enhances the ATP-evoked release of arachidonic acid from mouse astrocytes. *J. Neurosci.* **17**, 2939–2946
24. Lin, L.L., Lin, A.Y., and Knopf, J.L. (1992) Cytosolic phospholipase A2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl Acad. Sci. USA* **89**, 6147–6151
25. Cockcroft, S., Nielson, C.P., and Stutchfield, J. (1991) Is phospholipase A2 activation regulated by G-proteins? *Biochem. Soc. Trans.* **19**, 333–336
26. Takai, Y., Sasaki, T., and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol. Rev.* **81**, 153–208
27. Woo, C.H., Lee, Z.W., Kim, B.C., Ha, K.S., and Kim, J.H. (2000) Involvement of cytosolic phospholipase A2, and the subsequent release of arachidonic acid, in signalling by rac for the generation of intracellular reactive oxygen species in rat-2 fibroblasts. *Biochem. J.* **348 (Pt 3)**, 525–530
28. Yoo, M.H., Woo, C.H., You, H.J., Cho, S.H., Kim, B.C., Choi, J.E., Chun, J.S., Jhun, B. H., Kim, T. S., and Kim, J. H. (2001) Role of the cytosolic phospholipase A2-linked cascade in signaling by an oncogenic, constitutively active Ha-Ras isoform. *J. Biol. Chem.* **276**, 24645–24653
29. You, H.J., Woo, C.H., Choi, E.Y., Cho, S.H., Yoo, Y.J., and Kim, J.H. (2005) Roles of Rac and p38 kinase in the activation of cytosolic phospholipase A2 in response to PMA. *Biochem. J.* **388**, 527–535
30. Clark, M.A., Conway, T.M., Shorr, R.G., and Crooke, S.T. (1987) Identification and isolation of a mammalian protein which is antigenically and functionally related to the phospholipase A2 stimulatory peptide melittin. *J. Biol. Chem.* **262**, 4402–4406
31. Kim, D.K. and Bonventre, J.V. (1993) Purification of a 100 kDa phospholipase A2 from spleen, lung and kidney: antiserum raised to pig spleen phospholipase A2 recognizes a similar form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase A2 activity. *Biochem. J.* **294 (Pt 1)**, 261–270
32. Dennis, E.A. (1997) The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends Biochem. Sci.* **22**, 1–2

33. Dole, V.P. and Meinertz, H. (1960) Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.* **235**, 2595–2599
34. Linse, K. and Mandelkow, E.M. (1988) The GTP-binding peptide of beta-tubulin. Localization by direct photoaffinity labeling and comparison with nucleotide-binding proteins. *J. Biol. Chem.* **263**, 15205–15210
35. Baek, K.J., Das, T., Gray, C.D., Desai, S., Hwang, K.C., Gacchui, R., Ludwig, M., and Im, M.J. (1996) A 50 KDa protein modulates guanine nucleotide binding of transglutaminase II. *Biochemistry* **35**, 2651–2657
36. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
37. Molloy, G.Y., Rattray, M., and Williams, R.J. (1998) Genes encoding multiple forms of phospholipase A2 are expressed in rat brain. *Neurosci. Lett.* **258**, 139–142
38. Farooqui, A.A. and Horrocks, L.A. (2004) Brain phospholipases A2: a perspective on the history. *Prostaglandins Leukot. Essent. Fatty Acids* **71**, 161–169
39. Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999) Functional coupling between various phospholipase A2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J. Biol. Chem.* **274**, 3103–3115
40. Farooqui, A.A., Litsky, M.L., Farooqui, T., and Horrocks, L.A. (1999) Inhibitors of intracellular phospholipase A2 activity: their neurochemical effects and therapeutical importance for neurological disorders. *Brain Res. Bull.* **49**, 139–153
41. Calignano, A., Piomelli, D., Sacktor, T.C., and Schwartz, J.H. (1991) A phospholipase A2-stimulating protein regulated by protein kinase C in Aplysia neurons. *Brain Res. Mol. Brain Res.* **9**, 347–351
42. Sharp, J.D., White, D.L., Chiou, X.G., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P.L., Sportsman, J.R. *et al.* (1991) Molecular cloning and expression of human Ca(2+)-sensitive cytosolic phospholipase A2. *J. Biol. Chem.* **266**, 14850–14853
43. Manzoli, L., Martelli, A.M., Billi, A.M., Faenza, I., Fiume, R., and Cocco, L. (2005) Nuclear phospholipase C: involvement in signal transduction. *Prog. Lipid Res.* **44**, 185–206
44. Fayard, J.M., Tessier, C., Pageaux, J.F., Lagarde, M., and Laugier, C. (1998) Nuclear location of PLA2-I in proliferative cells. *J. Cell Sci* **111 (Pt 7)**, 985–994
45. Tsunoda, Y. and Owyang, C. (1995) The regulatory site of functional GTP binding protein coupled to the high affinity cholecystokinin receptor and phospholipase A2 pathway is on the G beta subunit of Gq protein in pancreatic acini. *Biochem. Biophys. Res. Commun.* **211**, 648–655
46. Tsien, R.W. and Tsien, R.Y. (1990) Calcium channels, stores, and oscillations. *Annu. Rev. Cell Biol.* **6**, 715–760
47. Tsunoda, Y. (1993) Receptor-operated Ca2+ signaling and crosstalk in stimulus secretion coupling. *Biochim. Biophys. Acta* **1154**, 105–156