



FEBS Letters 340 (1994) 185–188

FEBS 13731

**FEBS  
LETTERS**

# Proteolytic modification of membrane-associated phospholipase C- $\beta$ by $\mu$ -calpain enhances its activation by G-protein $\beta\gamma$ subunits in human platelets

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Received 21 December 1993; revised version received 19 January 1994

## Abstract

Membrane-associated phosphoinositide-phospholipase C (PI-PLC)- $\beta$  (150 kDa) and its truncated forms (100 kDa and 45 kDa) were purified from human platelets. The 100 kDa PI-PLC- $\beta$  was found to be activated to a greater extent by brain G-protein  $\beta\gamma$  subunits compared to the intact 150 kDa enzyme. Furthermore, treatment with  $\mu$ -calpain of the intact PI-PLC- $\beta$  (150 kDa) caused a marked augmentation of its activation by  $\beta\gamma$  subunits. This enhanced PLC activation by  $\beta\gamma$  subunits was due to truncation by  $\mu$ -calpain, producing a 100 kDa PI-PLC, but not by another protease, thrombin.

**Key words:** Phospholipase C activation; G-protein  $\beta\gamma$  subunit; Proteolysis by calpain; Human platelet

## 1. Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) is a key signal transducing enzyme generating two second messengers, inositol trisphosphate and diacylglycerol [1]. There are three types ( $\beta$ ,  $\gamma$ ,  $\delta$ ) containing nine distinct subtypes [2,3]. It is established that the PI-PLC- $\gamma$  type is activated by tyrosine kinases [2,4], while the PI-PLC- $\beta$  type is regulated by interaction with G-proteins in two distinct ways; one through  $\alpha$  subunits of the Gq family, which is insensitive to pertussis toxin (PT), and the other through the  $\beta\gamma$  subunits of PT-sensitive G-proteins [5–8].

It has been shown in human platelets that multiple forms of PI-PLC are present in cytosol and membrane fractions containing PLC- $\beta$ ,  $\gamma 1$ ,  $\gamma 2$  and  $\delta$  [9,10]. We previously indicated that membrane-associated platelet PI-PLC activity was stimulated by either GTP $\gamma$ S-activated Gi or Go to nearly the same extent [11]. This and other findings [7,8] lead us to suggest that  $\beta\gamma$  subunits of heterotrimeric G-proteins may be involved in the PI-PLC activation in human platelets. In order to gain further insight into the PI-PLC activation by G-protein in

human platelets, we have purified the membrane-associated PI-PLC that was activated by brain G-protein  $\beta\gamma$  subunits and demonstrated that truncation of the PI-PLC by  $\mu$ -calpain much enhanced its activation by G-protein  $\beta\gamma$  subunits.

## 2. Materials and methods

### 2.1. Materials

PIP<sub>2</sub> (bovine brain) and phosphatidylethanolamine (bovine brain) were obtained from Sigma Chemical Co. [<sup>3</sup>H]PIP<sub>2</sub> (specific activity, 8.0 Ci/mmol) was from Du Pont-New England Nuclear.  $\mu$ -Calpain (porcine erythrocyte, 120 U/mg protein) and diisopropyl fluorophosphate were from Nacalai Tesque (Kyoto). E-64 was from Peptide Institute Inc. (Osaka).

### 2.2. Purification procedures

The membrane-associated PI-PLC- $\beta$  was purified from outdated human platelet concentrates as described previously [12]. The G-protein  $\beta\gamma$  subunits from bovine brain were purified as outlined in [13].

### 2.3. Assay for PI-PLC activity and activation by G-protein $\beta\gamma$ subunits

PI-PLC activity was determined as described previously [9], using [<sup>3</sup>H]PIP<sub>2</sub> (18,000 dpm)/PIP<sub>2</sub> (0.1 mM)/PE (1.0 mM) as substrate. The activation of PI-PLC by G-protein  $\beta\gamma$  subunits was evaluated as described by Smrcka and Sternweis [14]. The reaction mixture (60  $\mu$ l) contained 50 mM Na HEPES (pH 7.2), 0.17 mM EDTA, 3 mM EGTA, 1 mM dithiothreitol, 17 mM NaCl, 67 mM KCl, 0.83 mM MgCl<sub>2</sub>, 1.5 mg/ml bovine serum albumin and 1.5 mM CaCl<sub>2</sub> to give 100 nM free Ca<sup>2+</sup> concentration. The bovine brain G-protein  $\beta\gamma$  subunits were solubilized in 0.3% octyl  $\beta$ -D-glucopyranoside (final concentration, 0.05%).

### 2.4. Western blot analysis

Western blot analysis of the PI-PLC was performed using polyclonal antisera raised against PI-PLC- $\beta$  which was kindly supplied by Dr. T.

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**Abbreviations:** PI-PLC, phosphoinositide-specific phospholipase C; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; G-protein, guanine nucleotide-binding regulatory proteins; PT, pertussis toxin.

Takenawa (Institute of Medical Science, University of Tokyo) and monoclonal antibody of PL-PLC- $\beta$  by Dr. S.G. Rhee (NIH, Bethesda)

### 2.5. Proteolytic truncation of platelet PI-PLC- $\beta$ by $\mu$ -calpain

The purified membrane-associated platelet PI-PLC- $\beta$  (150 kDa) was incubated with 0.2 U of  $\mu$ -calpain in a 30  $\mu$ l reaction mixture containing 50 mM HEPES (pH 7.2), 1 mM dithiothreitol, 3 mM EGTA, and 4 mM  $\text{CaCl}_2$  at 30°C. At the indicated times, the reaction mixture (5  $\mu$ l) was removed and added to 50  $\mu$ l of 3 mM EGTA solution containing 1  $\mu$ g of E-64 in order to terminate proteolysis.

## 3. Results

A membrane-associated PI-PLC was purified from human platelets as described previously [12]. When subjected to five steps of column chromatographies (Q-Sepharose, heparin-Sepharose, Ultrogel AcA-44, hydroxyapatite, and Mono Q), a PI-PLC preparation thus obtained revealed two major polypeptide bands (150 kDa and 60 kDa) on SDS-polyacrylamide gel by silver stain (Fig. 1A, lane 1). The polypeptide with 150 kDa cross-reacted with the polyclonal antibody raised against PI-PLC- $\beta$  fragments, but 60 kDa polypeptide did not (Fig. 1B, lane 1). Further purification of the 150 kDa PI-PLC- $\beta$  fraction by TSKgel-3000 column chromatography produced a single protein peak corresponding to PLC activity. But when this PLC activity peak was subjected to SDS-PAGE, it ran as 100 kDa and 45 kDa polypeptides (Fig. 1A, lane 2). These results suggest that nicking occurred at the region between the 100 kDa and 45 kDa polypeptides of the purified 150 kDa PI-PLC- $\beta$ . These 100 kDa and 45 kDa polypeptides cross-reacted with the polyclonal anti-PI-PLC- $\beta$  antibody (Fig. 1B, lane 2), which were thought to be produced by truncation of the intact 150-kDa polypeptide. The 150 kDa and 100 kDa PI-PLC- $\beta$  forms showed similar catalytic properties in substrate specificity for PI- and  $\text{PIP}_2$ -hydrolysis, but the 45 kDa polypeptide showing cross-reactivity with the polyclonal anti-PI-PLC- $\beta$  antibody had no catalytic activity (data not shown). Furthermore, effects of brain G-protein  $\beta\gamma$  subunits on the  $\text{PIP}_2$ -hydrolyzing activities of the intact and the truncated PI-PLC- $\beta$  were examined. As shown in Fig. 1C, two PI-PLC- $\beta$  fractions (PLC-1 with intact 150-kDa polypeptide; PLC-2 with truncated 100-kDa polypeptide) were activated to different extents by the brain G-protein  $\beta\gamma$  subunits. At 400 nM of  $\beta\gamma$  subunits, the truncated 100 kDa PI-PLC- $\beta$  activity was enhanced to a much greater extent (27-fold) compared to the intact 150 kDa enzyme exhibiting only a 6-fold increase.

To determine whether proteolytic cleavage of the 150 kDa PI-PLC- $\beta$  to the 100 kDa form was responsible for the marked activation by  $\beta\gamma$  subunits, the PI-PLC- $\beta$  (150 kDa) was subjected to proteolysis catalyzed by  $\mu$ -calpain. Upon incubation with various concentrations of  $\mu$ -calpain, the extents of activation of the PI-PLC- $\beta$  by

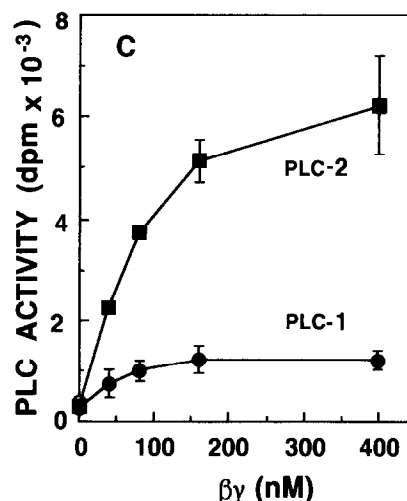
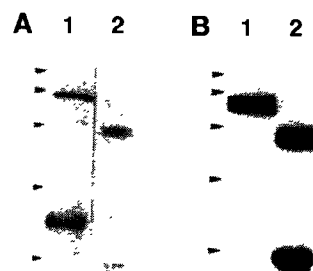


Fig. 1. SDS-PAGE and immunoblot of platelet membrane-associated PI-PLC. The purified membrane-associated PI-PLC fractions were subjected to SDS-polyacrylamide gel (8%) electrophoresis and proteins were detected by silver staining (A) and by immunostaining with polyclonal anti-PLC- $\beta$  antibody (B). Lanes 1, membrane PI-PLC obtained by Mono Q column chromatography; lanes 2, membrane PI-PLC obtained by TSKgel 3000 column chromatography. Arrows indicate  $M_r$  standards (from top); 212 kDa (myosin), 170 kDa ( $\alpha_2$ -macroglobulin), 116 kDa ( $\beta$ -galactosidase), 76 kDa (transferrin), 53 kDa (glutamic dehydrogenase). (C) Activation of the purified membrane-associated PI-PLCs by brain G-protein  $\beta\gamma$  subunits. The PI-PLC activities were measured with different amounts of brain G-protein  $\beta\gamma$  subunits under the conditions described in section 2. PLC-1, intact PI-PLC fraction shown in Fig. 1A, lane 1; PLC-2, truncated PI-PLC fraction shown in Fig. 1A, lane 2. Results represent mean  $\pm$  S.D. from three determinations.

$\beta\gamma$  subunits were elevated in dose- (Fig. 2A) and time- (Fig. 2B) dependent manner. When the 150 kDa PI-PLC- $\beta$  preparation was subjected to gel filtration on a Superose 12 column, a single  $\text{PIP}_2$ -hydrolyzing activity peak was eluted at the position of approx. 290 kDa, indicating a dimer form in the native state. After treatment with  $\mu$ -calpain (0.2  $\mu$ g) for 30 min, the activity was eluted at the position of approx. 110 kDa, suggesting that the higher  $M_r$  form of PI-PLC- $\beta$  (150 kDa) was converted to the lower  $M_r$  form (100 kDa) by  $\mu$ -calpain. The augmented activation of PI-PLC- $\beta$  by  $\beta\gamma$  subunits was due to proteolytic modification by  $\mu$ -calpain, but not by another protease thrombin (Fig. 3).

#### 4. Discussion

In this paper, we have demonstrated that the truncated 100 kDa PI-PLC- $\beta$  form purified from human platelet membranes was activated to a much higher extent by brain G-protein  $\beta\gamma$  subunits compared to the intact PI-PLC- $\beta$  (150 kDa). It has been reported that the PI-PLC- $\beta$ 1 was activated by Gq $\alpha$  family and its truncated 100 kDa enzyme produced by calpain could not be activated by Gq $\alpha$  [15]. Furthermore, most recently, Blank et al. [16] showed that a 110 kDa PI-PLC purified from brain cytosol which was identified to be a truncated form of PI-PLC- $\beta$ 3, showed extremely higher activation by  $\beta\gamma$  subunits but was not affected by Gq $\alpha$ . Lee et al. [17] indicated that the PI-PLC- $\beta$ 2 mutants which lack the carboxyl-terminal were activated by  $\beta\gamma$  subunits but not by Gq $\alpha$ . These results suggest that  $\beta\gamma$  subunits could activate PI-PLC- $\beta$  by interacting at distinct site from that of the Gq $\alpha$ . We have previously purified a cytosolic 100 kDa PI-PLC- $\beta$  which exhibited similar catalytic properties to those of the membrane 100 kDa PI-PLC- $\beta$  [12]. Moreover, it was demonstrated that the truncated 100 kDa PI-PLC- $\beta$ 1 purified from bovine brain membrane was activated by the brain G-protein  $\beta\gamma$  subunits to a lower extent (two-fold) as compared to the platelet 100 kDa PI-PLC- $\beta$  showing a 30- to 40-fold activation (data not shown). Therefore, it might be predicted that the human platelet PI-PLC- $\beta$  enzymes responsible for activation by  $\beta\gamma$  subunits could be of the  $\beta$ 3 subtype.

There are many studies indicating that calpain activation and cleavage of specific endogenous protein substrates, such as cytoskeletal proteins, are correlated with physiological events including microvesicle shedding, cytoskeletal rearrangement, secretion or clot retraction in

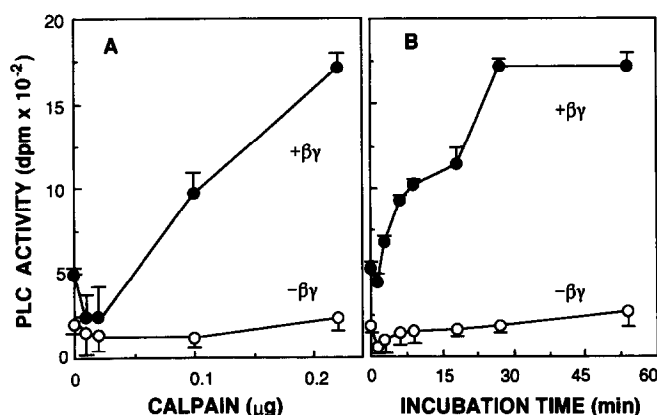


Fig. 2. G-protein  $\beta\gamma$  subunits-dependent activation of  $\mu$ -calpain-treated PI-PLC- $\beta$ . The 150 kDa PI-PLC- $\beta$  fraction was incubated for 30 min with different amounts (A) of  $\mu$ -calpain under the conditions as described in section 2. At the indicated times (B), 5  $\mu$ l of reaction mixture were removed and added to 50  $\mu$ l of a 3 mM EGTA solution containing 1  $\mu$ g E-64. Aliquots (20  $\mu$ l) of this reaction mixture were then incubated at 37°C for 10 min in a final volume of 60  $\mu$ l of PI-PLC assay mixture with or without G-protein  $\beta\gamma$  subunits (200 nM). Results represent mean  $\pm$  S.D. from three determinations from two similar experiments.

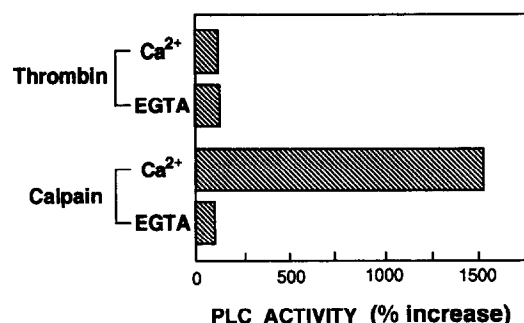


Fig. 3. Enhancement of  $\beta\gamma$ -dependent activation of PI-PLC- $\beta$  by proteolytic truncation. The 150 kDa PI-PLC- $\beta$  fraction was incubated with 0.2  $\mu$ g  $\mu$ -calpain or 1  $\mu$ M thrombin in the reaction mixture (30  $\mu$ l) containing 3 mM EGTA or 1 mM Ca<sup>2+</sup> for 30 min at 30°C and then added to 50  $\mu$ l of 3 mM EGTA solution containing 1  $\mu$ g E-64 and 0.1 mM diisopropyl fluorophosphate. Aliquots (20  $\mu$ l) of this reaction mixture were incubated in the PI-PLC assay mixture (total 60  $\mu$ l) with or without G-protein  $\beta\gamma$  subunits (200 nM) at 37°C for 10 min. The results are expressed as % increase in PI-PLC activity obtained in the presence of G-protein  $\beta\gamma$  subunits. Results are mean of three determinations from two similar experiments.

human platelets [18,19]. Furthermore, recent reports have shown that pp60<sup>src</sup> [20] and protein phosphotyrosine phosphatase 1B [21] were also endogenous substrates for calpain in platelet activation. It has also been reported that calpain translocates from cytosol to membrane and activates itself via autolysis which is promoted by polyphosphoinositides at physiological calcium concentration [22]. Moreover, the results of calpain inhibition in thrombin-activated human platelets implied that calpain may play a role in platelet activation through a PI-PLC activation [23]. Since platelet PI-PLC is known to be a good substrate for calpain [24] it is conceivable that membrane-associated PI-PLC- $\beta$  is positively regulated by  $\beta\gamma$  subunits via a limited proteolysis by calpain. To prove this hypothetical view, further investigations are required to examine whether truncation of PI-PLC- $\beta$  by calpain occurs in platelet activation in vivo.

**Acknowledgements:** We thank Dr. T. Takenawa (Institute of Medical Science, Tokyo University) and Dr. S.G. Rhee (National Institutes of Health, Bethesda, MD, USA) for the supply of anti-PI-PLC antibodies. This study was supported by a Grant-in-Aid from the Ministry of Science, Culture and Education of Japan.

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