Volume 331, number 1,2, 38–42 FEBS 13001 September 1993

A G-protein-coupled 130 kDa phospholipase C isozyme, PLC-β4, from the particulate fraction of bovine cerebellum

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Received 26 May 1993

A 130 kDa PLC isozyme was purified from the particulate fraction of bovine cerebellum. This PLC was recognized by a polyclonal antiserum generated against the purified 97 kDa PLC-β4 Reconstitution of the purified 130 kDa PLC with the membranes of C_6 Bu-1 cells in the presence of GTPγS or AlF_4^- resulted in PLC activation as well as the association of PLC with the membranes. Both the association and activation were revoked when the membrane was washed with 2 M KCl. The 97 kDa PLC-β4 did not associate with membranes. These data suggest that the 130 kDa PLC is the intact form of PLC-β4 the activity of which is likely to be regulated by a G-protein on the membrane.

Phospholipase C isozyme, Bovine cerebellum; G-protein-dependent activation

1. INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in receptor-mediated signal transduction. This enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) in response to the occupancy of receptors by many calcium-mobilizing agonists to generate two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate [1–4]. PLCs can be classified into three principal types of isozymes (PLC-β, -γ and -δ) on the basis of sequence similarity and the relative locations of the putative catalytic domains, and each type includes several distinct subtypes [7,10,18]. At least five PLC-β type isozymes, three mammalian enzymes (PLC-β1, PLC-β2 and PLC-β3) and two *Drosophila* enzymes (PLC-*norpA* and PLC-*p21*), have been identified by protein purification and/or by molecular cloning [5,8–10,19–21,27]. Three mammalian β-type isozymes, PLC-β1, PLC-β2 and PLC-β3, were recently shown to be activated by the α subunits of the G_q class G proteins or by the βγ subunits of G-proteins in different order [11–15,22–24].

Recently, we purified a 97 kDa PLC isozyme from bovine cerebellum cytosol and designated it PLC-β4 by comparing partial amino acid sequences with the corresponding sequences of other PLCs [25]. The molecular weights of the intact forms of PLC-β type isozymes are in the range of 130–150 kDa [8,20,26,27]. Here, we report the purification of a 130 kDa PLC isozyme showing immunological cross-reactivity with 97 kDa PLC-β4. Reconstitution of the purified 130 kDa PLC isozyme with the membranous fracation of C_6Bu-1 cells indicates that this protein can be activated by a G-protein and that it associates with the membrane via a peripheral protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

3-Phosphatidyl[2-^{3}H]inositol (17.9 Ci/mmol) and [2-^{3}H]inositol were purchased from Amersham International, UK. Wheat germ phosphatidylinositol (PI) was obtained from Calbiochem Corp., San Diego. Butyl-Toyopearl 650M gel matrix, TSK Phenyl-5PW and TSK DEAE-5PW HPLC column were purchased from Tosoh, Tokyo. The Mono S column was obtained from Pharmacia, Sweden. PLC isozymes and the polyclonal antiserum against each PLC isozyme were prepared as described [25].

2.2. *Purification procedure*

Two kg of frozen bovine cerebellum was homogenized in a homogenizer (Brinkman, Westbury, NY.) with 6 liter of buffer A (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, and 0.1 mM dithiothreitol) containing 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 13,000 × *g* for 40 min and the precipitate was washed with another 6 liter of homogenation buffer by homogenation followed by centrifugation at 13,000 × *g* for 40 min. The washed precipitate was homogenized with 4 liter of homogenation buffer containing 2 M KCl and centrifuged at 13,000 × *g* for 1 h. The supernatant was adjusted to 3 M by adding solid KCl and centrifuged at 13,000 × *g* for 1 h to remove the turbid debris. The clear supernatant was applied to a Butyl-Toyopearl column (10 × 16 cm) preequilibrated with buffer B (20 mM HEPES, pH 7.0, 1 mM EGTA, and 1 mM EDTA) containing 3 M KCl. The column was eluted with 4 liter of a decreasing KCl gradient from 3 to 0 M. A major and a minor peak of activity eluted at 2.1 and 1.3 M KCl, respectively, and only the major peak fractions were pooled. The pooled fractions were further purified by sequential uses of TSK Phenyl-5PW (21.5 × 150 mm), TSK DEAE-5PW (21.5 × 150 mm) and Mono S (5 × 50 mm) HPLC with the same eluent as described before [25].

2.3. *Preparation of [^{3}H]inositol labeled C_6Bu-1 cell membrane*

Cultures of C_6Bu-1 cells were maintained in Dulbecco's Modified

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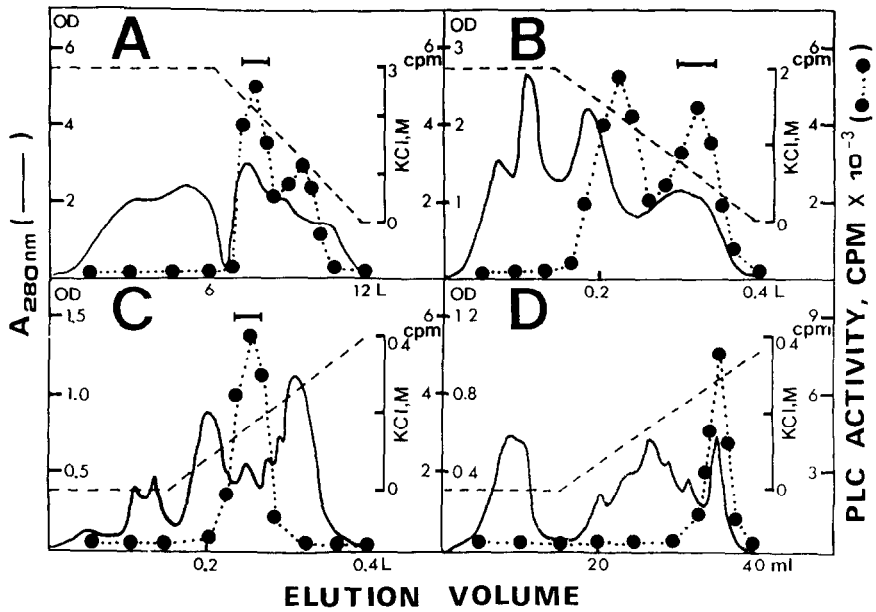

Fig. 1. Purification of a new 130 kDa PLC isozyme (PLC-β4) from 2 M KCl extracts of the particulate fraction of bovine cerebellum by sequential chromatography on Butyl-Toyopearl (A), TSK Phenyl-5PW (B), TSK DEAE-5PW (C), and Mono S (D) columns. PLC activity of each fraction (●) was assayed for PI hydrolysis and the active fractions pooled at each step for the purification of 130 kDa PLC-β4 were indicated (|—|). The solid line indicates the absorbance at 280 nm. The dashed line indicates the salt concentration

Eagle's Medium (DMEM) supplemented with 10% bovine calf serum and grown in 150 mm dishes until approximately 80% confluent. Membrane inositol phospholipids were labeled by incubation of the cells in inositol-free DMEM supplemented with 0.5% dialyzed fetal bovine serum, [^{3}H]inositol (1 μCi/ml), for 24 h. Cells were harvested by scraping followed by centrifugation at 500 × g for 10 min. Membrane fractions were obtained by the following procedures. (1) homogenation of C_6Bu-1 cells by sonication with buffer A containing 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 150 mM NaCl, (2) centrifugation of the homogenate at 500 × g for 5 min to remove the nucleus and large cell debris, (3) centrifugation of the supernatant at 100,000 × g for 1 h, (4) washing of the pellets twice by resuspension in the same buffer as step (1) and centrifugation at 100,000 × g for 1 h. The procedure to obtain KCl-washed membranes differed from the above only in the use of a buffer containing 2 M KCl in the first wash of step 4.

2.4. *Reconstitution of PLC with C_6Bu-1 cell membrane*

Each purified PLC enzyme (3 μg) was incubated with a cell membrane fraction (300,000 cpm, 200 μg protein) prepared in a 80 μl reaction mixture containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 10 μM free Ca^{2+} (EGTA-Ca^{2+} buffer system) with or without 10 μM GTPγS and AlF_4^- (10 mM NaF/10 μM $AlCl_3$). After 30 min of incubation at 37°C, half (40 μl) of the reaction mixture was taken out to measure the production of inositol phosphates by the procedure described previously [6]. To ascertain the distribution of the PLC in the membrane fraction, we spun the rest of the incubation mixture at 100,000 × g for 1 h in a refrigerated ultracentrifuge. Laemmli buffer was added to each supernatant and pellet for electrophoresis on an 8% SDS-polyacrylamide gel and immunoblotting with anti-PLC-β4 antibody.

2.5. *Other methods*

PLC activity was quantitated by the same method as described previously [25]. SDS-PAGE was performed by the method of Laemmli [16], and the protein bands were stained with Coomassie brilliant blue. Electrophoretic transfer of protein from gels to nitrocellulose sheets and subsequent immunoblotting using alkaline phosphatase-conjugated goat anti-rabbit IgG were performed as described [17].

3. RESULTS

Chromatography of the KCl-extract from the 13,000 × g pellet of bovine cerebellum on a Butyl-Toyopearl column yielded two peaks of PLC activity, which were eluted at 2.1 and 1.3 M KCl, respectively (Fig. 1A). The first, major peak was subjected to chromatography on a Phenyl-5PW HPLC column and activity was resolved into two peaks (Fig. 1B). The first peak proved to be PLC-β1 upon subsequent purification and immunoblot analysis with monoclonal anti-PLC-β1 antibody (data not shown). The second peak was further purified by sequential use of DEAE-5PW and Mono S HPLC (Fig. 1C and D). The final preparation (510 μg of protein) obtained from the Mono S HPLC column appeared to be a single band on a SDS-polyacrylamide gel (Fig. 2A). The specific activity of the purified enzyme was 2.1 μmol PI hydrolyzed/min/mg of protein.

The purified PLC showed a single band with an apparent molecular weight of 130 kDa on a SDS-polyacrylamide gel, and it was recognized by polyclonal rabbit anti-97 kDa PLC-β4 antiserum (Fig. 2B). This protein was also recognized by the sequence-specific antibodies generated against the X-domain and the Y-domain (anti-X and anti-Y, respectively) of PLC (Fig. 2C and D). This protein, however, was not recognized by any of the polyclonal antibodies generated against

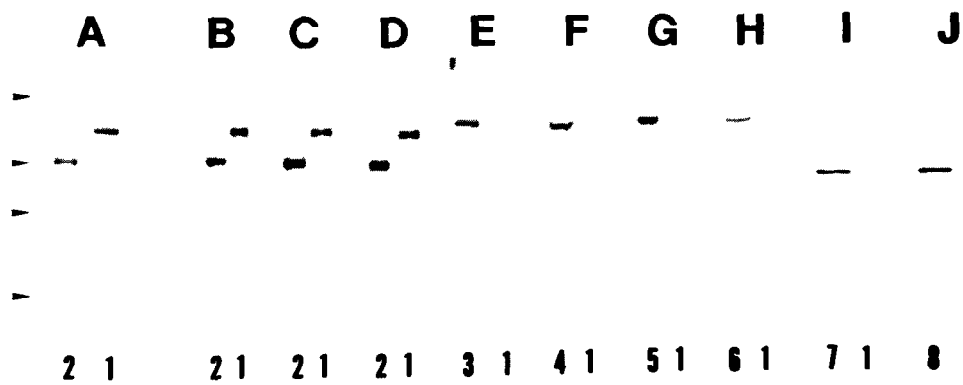

Fig. 2. SDS-PAGE and immunoblot analysis of the purified PLC. Purified PLC from bovine cerebellum was subjected to 8% SDS-PAGE and either stained with Coomassie blue (A) or immunoblotted with anti-97 kDa-PLC-β4 (B), anti-X (C), anti-Y (D), anti-PLC-β1 (E), anti-PLC-β2 (F), anti-PLC-γ1 (G), anti-PLC-γ2 (H), anti-PLC-δ1 (I), or anti-PLC-δ2 (J) antibodies. Lanes: 1, purified 130 kDa PLC-β4; 2, 97 kDa PLC-β4; 3, PLC-β1; 4, PLC-β2; 5, PLC-γ1; 6, PLC-γ2; 7, PLC-δ1; 8, PLC-δ2. Arrows indicate molecular weight standards from top to bottom, myosin (200,000), phosphorylase *b* (97,000), bovine serum albumin (68,000) and ovalbumin (43,000)

PLC-β1, -β2, -γ1, -γ2, -δ1 and -δ2 (Fig. 2E to J). The N-terminal sequence of this 130 kDa protein was not amenable to analysis by Edman degradation, suggesting that the N terminus is blocked as in the case of the 97 kDa enzyme [25].

Purified 130 kDa PLC was reconstituted with the membrane fraction from C_6Bu-1 rat glioma cell. As a result, more than 2.5 fold PLC activation was obtained upon the simultaneous addition of both GTPγS and AlF_4^-, while individual addition showed less activation (Fig. 3). GDPβS, on the other hand, did not influence the PLC activity. The 97 kDa PLC-β4 was not activated by GTPγS/AlF_4^- (Fig. 4A, upper panel). The 130 kDa enzyme could be detected in the precipitated membrane fraction of C_6Bu-1 while the 97 kDa one remained in the supernatant fraction (Fig. 4A, lower panel). The association of the 130 kDa enzyme with membrane was achieved even in the absence of GTPγS/AlF_4^-. Washing of the membrane with 2 M KCl-buffer abolished both the association and the activation (Fig. 4B).

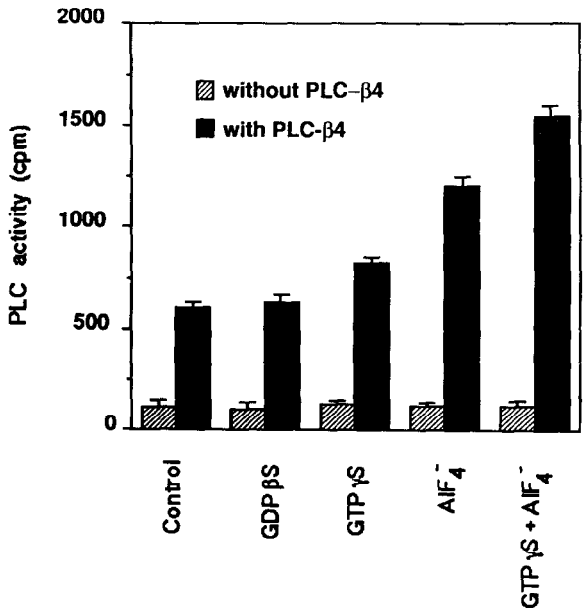

Fig. 3. Reconstitution of the membrane fraction of C_6Bu-1 rat glioma cells with the 130 kDa PLC-β4. [^{3}H]Inositol-labeled C_6Bu-1 membrane was incubated with or without the purified 130 kDa PLC-β4 as described in section 2.

4. DISCUSSION

We have purified a 130 kDa PLC isozyme from the particulate fraction of bovine cerebellum. From the results of immunological cross-reaction and reconstitution, the purified 130 kDa PLC appears to be the intact form of PLC-β4, which can associate with the membrane.

Purified 130 kDa PLC was not recognized by the polyclonal antibodies generated against PLC-β1, -β2, -γ1, -γ2, -δ1 and -δ2, but by anti-97 kDa PLC-β4 antiserum as well as by anti-X and anti-Y (Fig. 2). N-terminal sequencing by the Edman procedure failed for both the 130 kDa and 97 kDa PLC-β4 enzymes. This suggests that the 97 kDa enzyme might be generated from the 130 kDa enzyme by the removal of the C-terminal region.

The 130 kDa PLC was not detected in the cytosolic fraction [25] and was liberated from the particulate fraction of bovine cerebellum by 2 M KCl extraction. Immunohistochemical staining of brain tissue with anti 97-kDa PLC-β4 antibody revealed that the majority of PLC-β4 was localized in the glial cells of rat cerebellum (data not shown). When each of the purified PLC-β4 was reconstituted with the [^{3}H]inositol labeled C_6Bu-1 rat glioma membrane, only the 130 kDa enzyme was shown to be bound to the membrane fraction and activated by the addition of GTPγS/AlF_4^- (Fig. 4). Removal of some peripheral component(s) by washing the membrane with 2 M KCl buffer eliminated both the associa-

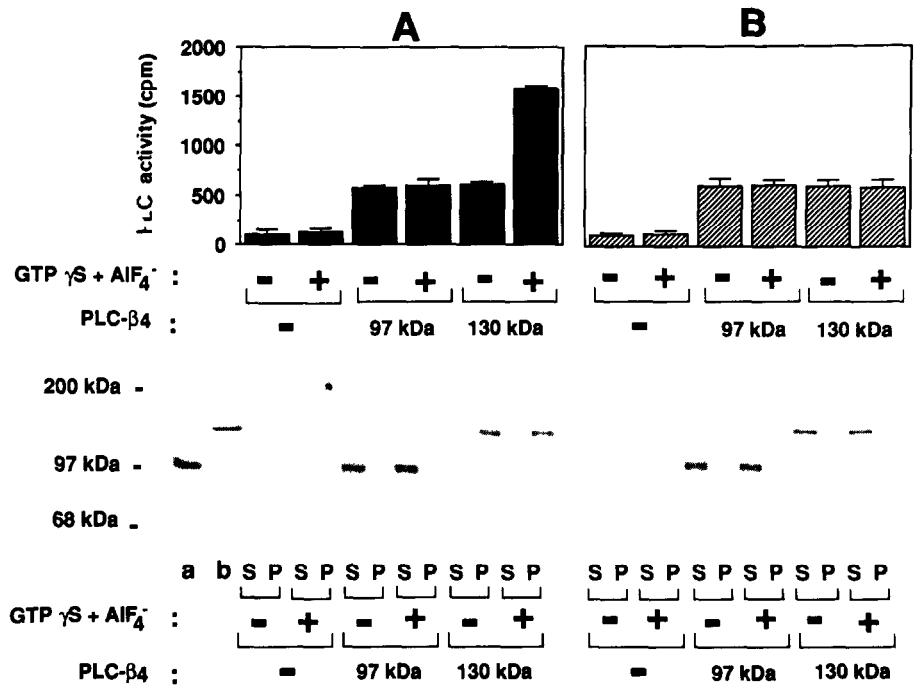

Fig. 4. Reconstitution of purified PLC-β4 with the membrane fraction of C_6Bu-1 rat glioma cells. Each purified PLC-β4 was incubated with the membrane fractions prepared by washing with the buffers containing 0 M (A) or 2 M KCl (B) for 30 min and half of the incubation mixture was used for the measurement of inositol phosphates (upper panels) while the other half was for immunoblot after resolving into supernatant (lane, S) and precipitate (lane, P) by centrifugation. Lane: a, the purified 97 kDa PLC-β4; b, the purified 130 kDa PLC-β4

tion and the activation of PLC in the reconstitution. From all of these results, we concluded that the 130 kDa enzyme is the functional form of PLC-β4, which might couple with certain G-proteins in C_6Bu-1 cells and that the C-terminal region was required for the association and the GTP-dependent activation of PLC. The importance of the carboxy-terminal domain of PLC-β isozyme on G-protein dependent activation was exemplified by the loss of $G_q\alpha$-activation of PLC-β1 which resulted from the removal of 450 carboxy-terminal residues with calpain-digestion [28] and was observed with the expression of the deleted mutant [30]. Even though both PLC-β4 and PLC-β1 are susceptible to cleavage, it is not yet clear whether calpain is involved in the generation of the 97 kDa PLC-β4 from 130 kDa enzyme as is the case with PLC-β1.

Maximal activation of 130 kDa PLC-β4 occurred in the presence of both GTPγS and AlF_4^- (Fig. 3). It is interesting that PLC-β4 was activated either by GTPγS or by AlF_4^- (Fig. 3), while significant activation of PLC-β1 by $G_q\alpha$ could be achieved only with AlF_4^- [11]. It remains to be studied whether the 130 kDa PLC could be activated by two different G-proteins, or whether the G-protein in question is activated in a cumulative manner by GTPγS and AlF_4^-.

Both the association of PLC-β4 with membrane and the activation by GTPγS/AlF_4^- were abolished by treatment of C_6Bu-1 cell membrane with 2 M KCl. Therefore, the association of the 130 kDa PLC-β4 with the membrane is likely to be mediated by certain peripheral component(s). We could not restore either the association or the activation of 130 kDa PLC-β4 by re-mixing the dialyzed KCl extract of C_6Bu-1 cell membrane fraction with KCl-washed membrane (data not shown). This might be due to change(s) in the peripheral component(s) due to the KCl treatment procedure or to the removal of certain component(s) during dialysis. Recently, the activation of PLC-β1 by the $\beta\gamma$ subunit of G-protein has been reported [24,29]. It is not clear yet whether the 130 kDa PLC-β4 can interact directly with the $\beta\gamma$ subunit of G-protein or whether an additional component is required for the association with membrane.

Acknowledgements: This work was supported in part by grants from the Korea Science and Engineering Foundation (KOSEF 89-07-02-04) and from Pohang University of Science and Technology. We would like to thank Dr. Sung Ki Jang for critical reading of the manuscript.

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