FEBS Letters 454 (1999) 42–46 FEBS 22199

Selective activation of phospholipase D2 by unsaturated fatty acid

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Received 31 March 1999

Abstract Although oleate has been implicated in the regulation of phospholipase D (PLD) activity, the molecular identity of the oleate-stimulated PLD is still poorly understood. We now report that oleate selectively stimulates the enzymatic activity of PLD2 but not of PLD1, with an optimal concentration of 20 μM in vitro. Intriguingly, phosphatidylinositol 4,5-bisphosphate (PIP_2) synergistically stimulates the oleate-dependent PLD2 activity with an optimal concentration of 2.5 μM. These results provide the first evidence that oleate is a PLD2-specific activating factor and PLD2 activity is synergistically stimulated by oleate and PIP_2.

Key words: Phospholipase D2; Oleic acid; Phosphatidylinositol 4,5-bisphosphate

1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. PA has a number of biological activities including direct regulation of a range of cell-specific target proteins in vitro [1–3]. PA can be further metabolized by PA phosphohydrolase to yield diacylglycerol which may function as an activator of protein kinase C (PKC) isoforms [4]. In the context of these specific roles, it has been proposed that PLD functions in a variety of physiological processes including inflammation, secretion, mitogenesis, neural and cardiac stimulation, diabetes, and senescence [4].

Several factors were reported to stimulate PLD activity in vitro. These include phosphatidylinositol 4,5-bisphosphate (PIP_2), monomeric GTP-binding proteins such as ADP-ribosylation factor 1 (ARF1) and RhoA, and protein kinase C [1]. Hammond et al. [5] showed that the first cloned human PLD, hPLD1, is regulated by PIP_2, ARF, RhoA, and protein kinase Cα. The enzyme has low basal activity and is synergistically activated by ARF1, RhoA, and PKCα in the presence of PIP_2. A second isoform of PLD, PLD2, was recently cloned from mouse, rat, and human [6–8]. The PLD2s also essentially require PIP_2 for their enzymatic activities. They are constitutively active in the presence of PIP_2 [6]. Both PLD1 and PLD2 contain the pleckstrin-homology domain which can bind to PIP_2 [9]. Thus PIP_2 has been implicated in PLD activation as an essential PLD-activating factor [10].

In addition to the PIP_2-dependent PLD isozymes, oleate-dependent PLD activities have been reported by several research groups [11–15]. Chalifour and Kanfer [11] identified an oleate-dependent PLD activity in rat brain microsomes. The enzyme is potently activated not only by oleate but also by other unsaturated fatty acids including palmitoleate, linoleate, and arachidonate. Okamura and Yamashita [14] purified an oleate-dependent PLD with a molecular mass of 190 kDa from pig lung membranes. Moreover, Massenburg et al. [15] reported that the oleate-dependent PLD activity from rat brain could be separated from the ARF-dependent PLD activity by column chromatography. Despite these various reports, the molecular structure and the characteristics of the oleate-dependent PLD remained poorly understood.

In this study, we demonstrate for the first time that oleate specifically stimulates the enzymatic activity of PLD2, but not of PLD1, and that it synergistically potentiates the PIP_2-stimulated activity of PLD2. The data suggest that unsaturated fatty acids may play a role in the regulation of PLD2 in concert with PIP_2.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidyl[*methyl*-^{3}H]choline was purchased from Amersham International (Buckinghamshire, UK). PIP_2, dipalmitoyl-PC, dioleoyl-phosphatidylethanolamine (dioleoyl-PE), phosphatidylserine (PS), phosphatidylinositol (PI), and fatty acids were purchased from Sigma (St. Louis, MO, USA). β-Octylglucopyranoside was from Calbiochem (San Diego, CA, USA). Chelating Sepharose resin was from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.2. Measurement of PLD activity

Oleate-dependent activity was assayed by a slightly modified procedure of Massenburg et al. [15]. PC vesicles (25 μl) containing 5 nmol of dipalmitoyl-PC and 200 000 dpm of dipalmitoylphosphatidyl[*methyl*-^{3}H]choline were added to reaction mixtures (175 μl) containing 50 mM HEPES-NaOH (pH 7.0), 2 mM EGTA, 1.7 mM $CaCl_2$, 20 μM sodium oleate, and 0.1 M KCl. The final concentration of PC was 25 μM in the reaction mixtures. Assays were incubated at 30°C for 1 h before adding 1 ml of $CHCl_3$/CH_3OH/concentrated HCl, 50:50:0.3 (v/v) and 0.3 ml of 1 M HCl/5 mM EGTA. After centrifugation (5 min, 2000 rpm, 4°C, Sorvall RC3B, H2000 B swinging bucket rotor), [*methyl*-^{3}H]choline in 0.5 ml of the aqueous phase was quantified by liquid scintillation spectroscopy. PIP_2-dependent PLD activity was measured by choline release from PC essentially as described [16].

2.3. Cell culture and preparation of the membrane fraction

U937 and L1210 cells were maintained in RPMI supplemented with 10% (v/v) bovine calf serum (HyClone), 100 units of penicillin/ml, and 100 mg of streptomycin/ml at 37°C in a humidified, CO_2-controlled

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Abbreviations: PLD, phospholipase D; PIP_2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PA, phosphatidic acid; ARF, ADP-ribosylation factor; PKC, protein kinase C; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ECL, enhanced chemiluminescence

PII: S0014-5793(99)00745-0

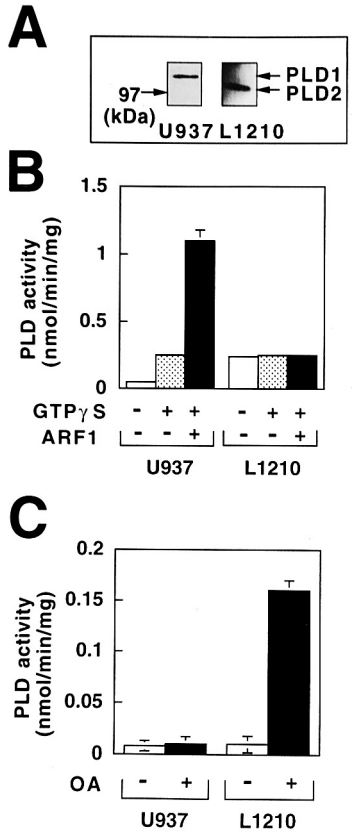

Fig. 1. Effect of PIP_2, ARF1, and oleate on the PLD activities of U937 and L1210 cells. A: Immunoprecipitation of PLD isozymes from U937 and L210 cells. Lysates (3 mg) of U937 and L1210 cells were reacted with anti-PLD antibody, and the immune complexes were subjected to SDS-PAGE followed by immunoblot analysis with anti-PLD antibody. Data are representative of two separate experiments. B: Effect of PIP_2 and ARF1 on the PLD activities in membrane fractions of U937 and L1210 cells. PIP_2-dependent PLD activities of U937 and L1210 cells were measured in the absence or in the presence of 1 μM ARF1 and 10 μM GTPγS as indicated. Aliquots (10 μg) of the membrane fractions were added to the reaction mixtures containing substrate phospholipid vesicles (PE:PIP_2:PC, 16:1.4:1) and incubated at 37°C for 15 min to measure choline-releasing PLD activities as described in Section 2. C: Effect of oleate on the PLD activities in membrane fractions of U937 and L1210 cells. Aliquots (10 μg) of the membrane fractions were added to reaction mixtures containing 25 μM PC in the absence (−) or the presence (+) of 20 μM sodium oleate (OA), and were further incubated at 30°C for 1 h as described in Section 2. Data represent the means ± S.D. from three different experiments.

(5%) incubator. The cells were centrifuged at 500×*g* for 5 min into a pellet, washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and resuspended in ice-cold hypotonic buffer (20 mM HEPES-NaOH (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 2 mM leupeptin, 1 mM pepstatin). The cells were then disrupted by sonication using an ultrasonicator (10-s burst; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT) and centrifuged at 500×*g* to remove intact cells. The cell homogenate was then centrifuged at 100 000×*g* for 1 h using a TLA-100 ultracentrifuge to separate membrane and cytosolic fractions. The membrane fractions was resuspended in the hypotonic buffer at a concentration of 2 mg protein/ml.

2.4. Immunoprecipitation and Western blotting

For immunoprecipitation, the membrane fractions of U937 and L1210 cells were solubilized with ice-cold lysis buffer (20 mM HEPES-NaOH (pH 7.5), 2% cholic acid, 1 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and precleared by centrifugation at 15 000×*g* for 10 min at 4°C. Immunoprecipitations were performed by addition of 30 μl of protein A agarose (Pierce) which was coupled with 2 μg anti-PLD antibody as previously reported [17].

3. Results

3.1. Characterization of PLD isozymes in U937 and L1210 cells

We examined the cellular distribution of PLD1 and PLD2 in several cell lines including U937 promonocytic leukocytes and L1210 lymphocytic leukemia cells by immunoprecipitation of PLD isozymes and subsequent immunoblot analysis using anti-PLD antibody which was raised against the C-terminal dodecapeptide of hPLD1 as previously described [18]. The antibody specifically recognizes both PLD1 and PLD2, and we found that U937 and L1210 cells exclusively contained PLD1 and PLD2, respectively (Fig. 1A). U937 cells have a low PIP_2-dependent PLD activity which can be stimulated up to 10-fold by the addition of 1 μM ARF1 and 10 μM guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) in the presence of PIP_2 (Fig. 1B). On the other hand, L1210 cells have high PIP_2-dependent PLD activity which is not affected by the addition of ARF1 and GTPγS. These biochemical properties of U937 and L1210 cells coincide with the properties of PLD1 and PLD2 as previously reported by Colley et al. [6].

In addition to the PIP_2- or ARF1-stimulated PLD activities, the existence of an oleate-dependent PLD activity has been suggested in various reports [11–15]. We looked for oleate-dependent PLD activity in the absence of PIP_2 and ARF1 and found that the membrane fraction of the L1210 cells indeed contained oleate-stimulated PLD activity (Fig. 1C). In contrast, U937 cells did not show any oleate-stimulated PLD activity. These results raised the possibility that PLD2 activity may be stimulated by oleate.

3.2. Expression and affinity purification of hPLD1 and hPLD2

hPLD1 and hPLD2 were expressed in a baculovirus expression system and purified from detergent extracts of baculovi-

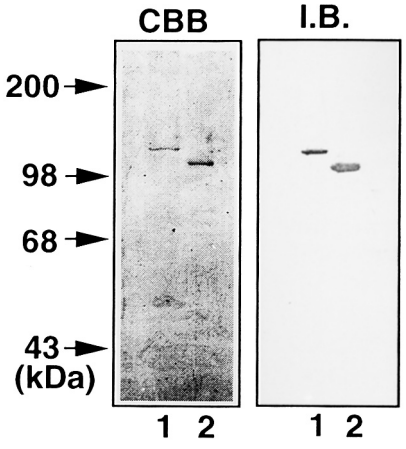

Fig. 2. Analysis of purified hPLD1 and hPLD2 by SDS-PAGE and Western blotting. Recombinant hPLD proteins were expressed by baculovirus in Sf9 cells and affinity-purified using chelating Sepharose column chromatography. The purified 0.2 μg hPLD1 (1) and 0.5 μg hPLD2 (2) were subjected to SDS-PAGE followed by Coomassie brilliant blue staining (CBB) and Western blotting (I.B.) using anti-PLD antibody.

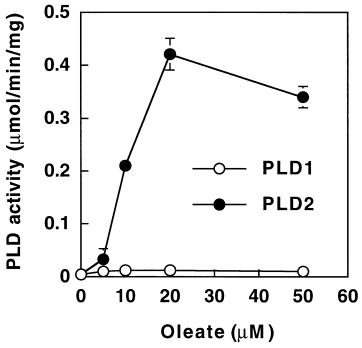

Fig. 3. Effect of oleate on the PLD activities of hPLD1 and hPLD2. Aliquots (5 ng) of purified hPLD1 and hPLD2 were assayed for oleate-dependent PLD activity in the presence of the indicated concentrations of sodium oleate at 30°C for 1 h as described in Section 2. Data represent the means ± S.D. from three different experiments.

rus-infected Sf9 cells using chelating Sepharose affinity column chromatography as previously described [19]. From the ten 150-mm dishes of Sf9 cells infected with recombinant baculovirus we obtained about 20 μg of hPLD1 and hPLD2, respectively. The purified protein preparations were analyzed by SDS-PAGE and Western blotting. As shown in Fig. 2, hPLD1 and hPLD2 appeared to be purified to homogeneity as estimated by the staining with Coomassie brilliant blue, and the purified proteins were specifically recognized by anti-PLD antibody. The molecular masses of hPLD1 and hPLD2 were estimated to be approximately 120 kDa and 100 kDa, respectively.

3.3. Selective activation of hPLD2 by oleate

In order to clarify whether PLD2 is specifically activated by oleate, we examined the effect of sodium oleate on the enzymatic activity of purified hPLD1 and hPLD2 in the absence of PIP_2 and ARF1. As shown in Fig. 3, neither hPLD1 nor PLD2 showed PLD activity in the absence of sodium oleate. Intriguingly, hPLD2 was specifically stimulated by the addition of sodium oleate. The optimal concentration of sodium oleate was about 20 μM. In contrast, hPLD1 was not affected by the presence of sodium oleate.

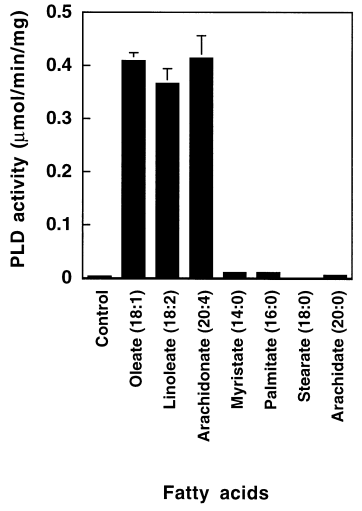

Fig. 4. Effect of fatty acids on the enzymatic activities of hPLD2. Aliquots (5 ng) of hPLD2 were added to the reaction mixtures containing the sodium salt form of indicated fatty acid at a final concentration of 20 μM as described in Fig. 3. Data represent the mean ± S.D. from three different experiments.

3.4. Effect of fatty acids on PLD2 activity

The oleate-induced PLD2 activation may be due to a structural change in the substrate phospholipid vesicles caused by oleate acting as a non-specific detergent. To exclude this possibility, we next examined the effect of other fatty acids on the PLD activity and found that, in addition to oleate, other unsaturated fatty acids including linoleate (18:2) and arachidonate (20:4) also stimulated PLD2 activity, while the saturated fatty acids including myristate (14:0), palmitate (16:0), stearate (18:0), and arachidate (20:0) were inactive (Fig. 4).

3.5. Synergistic activation of PLD2 by PIP_2 and oleate

PIP_2 has been described as an essential activating factor for PLD2 activation [10]. To explore the effect of PIP_2 on the requirement of oleate for PLD2 activation, we tested the effect of PIP_2 on the oleate-dependent PLD2 activity. As shown in Fig. 5A, PLD2 was maximally active in the presence of 2.5 μM PIP_2 in the absence of oleate. Intriguingly, PIP_2 synergistically stimulated PLD2 activity in the presence of 20 μM oleate. We next examined the optimal concentration of sodium oleate for PLD2 activation in the absence and presence of 2.5 μM PIP_2.

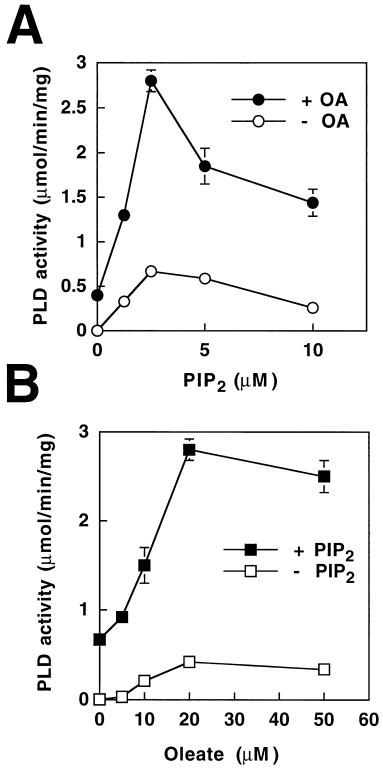

Fig. 5. Effect of PIP_2 on the oleate-dependent PLD activity. A: Effect of varying the concentration of PIP_2 on the basal and oleate-stimulated PLD2 activity. The amount of PIP_2 was varied as indicated while maintaining the amount of PC at 25 μM. Oleate-dependent PLD2 activity was measured in the absence (−OA) or presence (+OA) of 20 μM sodium oleate as in Fig. 3. B: Effect of varying the concentration of oleate on the PIP_2-stimulated PLD activity of PLD2. The amount of oleate was varied as indicated, and the oleate-dependent PLD2 activity was measured in the absence ($-PIP_2$) or presence ($+PIP_2$) of 2.5 μM PIP_2. Data represent the means ± S.D. from three different experiments.

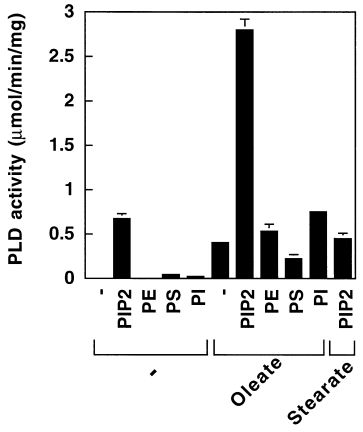

Fig. 6. Effect of phospholipids on the oleate-dependent PLD2 activity. PLD2 activity was measured in the absence (−) or presence (+) of 20 μM oleate and 2.5 μM phospholipids, including PIP_2, PE, PS, and PI as in Fig. 5. As a control, 20 μM stearate and 2.5 μM PIP_2 were added to the reaction mixture in the absence of oleate. Data represent means ± S.D. from three different experiments.

As shown in Fig. 5B, sodium oleate synergistically stimulated PLD2 activity at an optimal concentration of 20 μM in the presence of PIP_2, suggesting that the synergistic activation of PLD2 by PIP_2 and oleate may be mediated by the specific interaction of PLD2 with PIP_2 and oleate.

3.6. *Effect of phospholipids on the oleate-dependent PLD activity*

PIP_2 is an acidic phospholipid. Therefore it is tempting to speculate that the PIP_2-induced PLD2 activation could be caused by a change in the surface charge or structure of the substrate PC vesicles caused by the negative charge of PIP_2. In order to be able to exclude this possibility, we examined the effect of other phospholipids including PE, PS, and PI on the oleate-dependent PLD2 activity. As shown in Fig. 6, the three phospholipids had a small effect on the oleate-dependent PLD activity at 2.5 μM concentrations. PS and PI, the acidic phospholipids, did not stimulate the oleate-dependent PLD2 activity as well as the basal PLD2 activity at concentrations ranging from 0 to 25 μM (data not shown). This suggests that the PIP_2-induced PLD2 activation does not merely derive from the change in structure and surface charge of the substrate PC vesicles. In contrast, stearate, which is the corresponding saturated fatty acid of oleate, did not stimulate the PIP_2-dependent activity of PLD2. Moreover, PLD1 activity was not affected by the addition of various concentrations of both PIP_2 and sodium oleate (data not shown).

4. Discussion

PLD2 has been known to be activated by PIP_2, and the PIP_2-dependent activity of PLD2 is not affected by PLD1-activating factors such as ARF, small G proteins of the Rho family, and PKC [6]. However, PIP_2 is also essential for PLD1 activation, thus it remained unclear how PLD2 activity is specifically regulated. In the current study, for the first time, we show evidence that oleate is a PLD2-specific activating factor, and that the oleate-dependent activity of PLD2 is synergistically up-regulated by PIP_2.

The possibility that oleate may be acting as a non-specific detergent that changes the structure of the substrate phospholipid vesicles enhancing PLD activity needed to be considered. Alterations in the structure of the phospholipid vesicles may affect the access of PLD to the vesicles, thus apparently stimulating PLD activity. However, this can be ruled out based on the following findings. First, saturated fatty acids (Fig. 3) and detergents including Triton X-100 and sodium cholate (data not shown) were ineffective towards PLD2 activation, which is in agreement with previous observations [12,20]. Second, PLD2, but not PLD1, is specifically stimulated by oleate. The selective activation of PLD2 by oleate suggests that oleate stimulates PLD2 activity through a specific interaction with PLD2 rather than acting as a non-specific detergent on the lipid vesicles.

An interesting finding is the synergism between oleate and PIP_2 with respect to the PLD2 activation. In the present study, we showed that 20 μM oleate and 2.5 μM PIP_2 synergistically stimulated the PLD2 activity. Oleate has been reported to inhibit the PIP_2-stimulated PLD2 activity [7,8] as well as PLD1 activity stimulated by PIP_2 and ARF [19,21]. However, we observed that at concentrations over 100 μM, oleate strongly inhibits the PIP_2-stimulated PLD2 activity and is thus less effective in PLD2 activation (data not shown), consistent with the previous reports showing an inhibitory effect of oleate on PLD activities. Moreover, the basal and oleate-stimulated PLD2 activities were optimally stimulated by 2.5 μM PIP_2, and concentrations higher than 5 μM PIP_2 inhibited PLD2 activity (Fig. 5A), which is in agreement with a previous report [22]. At high concentrations, PIP_2 and oleate may affect the structure of substrate vesicles, therefore it is possible to speculate that a structural alteration of the vesicles may be the cause of the apparent inhibition of the enzymatic activity of PLD2, and the molar ratios of oleate and PIP_2 may, therefore, be critical for the synergistic activation of PLD2.

PIP_2 has been implicated as a PLD-activating factor essential for the activation of both PLD1 and PLD2. Both PLD isozymes have the pleckstrin-homology domain which can bind to PIP_2 [9]. The interaction of the two PLD isozymes with PIP_2 helps in the access to the substrate vesicles containing PIP_2 as previously reported [23]. Thus, the PLD2-specific activation by oleate raises the possibility that the PLD2 activation may be induced by direct interaction with oleate. It will be necessary to find out whether PLD2 directly interacts with oleate and which domain of PLD2 is implicated in this interaction.

Oleate and other unsaturated fatty acids have been proposed to act as second messengers in signal transduction [24]. Oleate and arachidonate regulate the activities of a great number of signaling enzymes including PKC [25], guanylate cyclase [26], Ca^{2+}-calmodulin-dependent kinase [27], and phospholipase Cγl [28]. In the present report we show evidence that unsaturated fatty acids including oleate specifically up-regulate the PLD2 activity and synergistically stimulate PLD2 activity in concert with PIP_2. We are currently investigating whether the PLD2 activity is regulated by oleate and PIP_2 in cells in vivo.

Acknowledgements: This work was supported in part by the program of the Korea Science and Engineering Foundation, the '97 Good Health R&D Project of the Ministry of Health and Welfare, and the Center for Cell Signaling Research in the Republic of Korea.

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