Human neutrophil cytosolic phospholipase C: partial characterization

Anat Faber and Irit Aviram

Department of Biochemistry, Tel Aviv University, Tel Aviv (Israel)

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The activity of neutrophil cytosolic phospholipase C on PIP₂ and PI was compared employing [³H]inositol-labeled heat-inactivated membranes of differentiated HL-60 cells, into which tracer [³²P]PIP₂ was incorporated. Hydrolysis of PIP₂ did not require Ca²⁺ and was stimulated when the content of PIP₂ in the membrane was increased by incorporation of unlabeled inositol lipid. At equal concentrations of PI and PIP₂ in the membrane, hydrolysis of PIP₂ was faster and no evidence of competition between the two substrates was obtained. Incorporation of PI into PE-[³²P]PIP₂ vesicles, accelerated PIP₃ hydrolysis also at conditions that favor hydrolysis of PI. Partial purification of neutrophil cytosolic PLC on Q Sepharose, phenyl Sepharose and heparin-Agarose columns is described. From heparin-Agarose column, two PLC activity peaks exhibiting different substrate specificities were eluted. The elution profile of the main PLC species from Superose 12 gel filtration column was compatible with an approx. 150 kDa protein.

Introduction

Phosphoinositide-specific phospholipase C (PLC) participates in signal transduction initiated by hormones, neurotransmitters and growth factors [1,2]. Following cell stimulation, PLC catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two second messengers, sn-1,2-diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP₃) [3]. Activation of PLC may be mediated by receptor-coupled G proteins or by the tyrosine kinase activity of receptors to growth factors [4]. Mammalian PLC in their membrane-bound or cytosolic forms consist of at least four isoforms, designated α, β, γ and δ. The isoforms differ in their structure, molecular mass and activity.

Surface-receptors for chemotactic ligands expressed by neutrophils are coupled to PLC-mediated phosphoinositide hydrolysis. Due to this, neutrophils and the related leukemia HL-60 cell line cells, have been extensively used as models for studies of signal transduction pathways involving inositol lipid turnover [5–8]. Participation of G proteins in the activation of PLC in intact and permeabilized neutrophils and in neutrophil membranes has been documented [9,10]. Only few studies, however, have been devoted to the characterization of neutrophil PLC at the molecular level [11,12]. In this present report, a partial characterization of cytosolic neutrophil PLC with respect to its activity and structure, is presented. Preliminary data of this study have been published [13].

Materials and Methods

Materials. Dowex-AG-X8 (100–200 mesh, formate form) was purchased from Bio-Rad. Culture media RPMI-1640 and M-199 were obtained from Biological Industries (Beth Haemek). All other materials were from Sigma.

Fractionation of neutrophils. Human neutrophils were isolated from fresh buffy coats by standard procedures of dextran sedimentation, Ficoll density gradient centrifugation and hemolysis. The cells were broken and fractionated as described elsewhere [14].

Synthesis of [³²P]PIP₂ by human red blood cell PIP kinase [15]. PIP (0.6 mg/ml) was suspended by bath sonication in 50 mM Mes (pH 6.5) 2 mM EGTA/2 mM EDTA. Red blood cell membranes were suspended at 1.5 mg protein/ml in 10 mM Tris–HCl (pH
7.4) 1 mM EGTA/250 mM sucrose/0.5% CHAPS. 25 μCi of [γ-32P]ATP (Amersham, 3000 Ci/mmol) were mixed with 100 μM ATP solution in 100 μM KP (pH 7.5) 10 mM MnCl2/20 mM MgCl2 (final volume of 10 μl). 1 μl aliquots of PIP2, ATP and red blood cell membr.anes were mixed and incubated at 30°C for 3.5 h. The reaction was terminated by the addition of 30 μl of 5 mM KP and 2 ml of chloroform/methanol/HCl (800:800:4, v/v). After vortexing and incubation with shaking (20 min at 37°C), 0.4 ml of 0.6 M HCl was added and phases were separated by centrifugation. The lower phase was washed twice with 1 ml chloroform/methanol/HCl (3:48:47, v/v) and its Cerenkov radiation was estimated. The radiolabeled lipid was stored at −20°C.

Preparation of PE-[32P]PIP2 vesicles. Aliquots of [32P]PIP2 (about 15 000 cpm) were mixed with 2.5 μg unlabeled PIP2 dissolved in chloroform. After evaporation of the solvent under a stream of nitrogen, the lipids were suspended by bath sonication (20 min) in the reaction buffer.

Preparation of heat-inacivated, [3H]inositol- and [32P]PIP2-labeled membranes. HL-60 cells were labeled with [3H]-myo-inositol (Amersham, 0.75-0.9 μCi/ml) and isolated as described [16]. The membranes were heat treated for 10 min at 100°C to inactivate intrinsic membrane-bound PLC. For an assay, membranes (about 15 μg protein) were added to a test tube containing 100 000 cpm of dried [32P]PIP2 and the components were sonicated in a bath sonicator (10 min) to permit incorporation of PIP2 [7]. For the preparation of PIP2-enriched membranes, unlabeled PIP2 was pre-mixed with [32P]PIP2 to give a final 1:1 molar ratio of PI:PIP2.

Enzyme activity of cytosolic PLC. Neutrophil cytosol (about 240 μg protein/ml) was incubated with one of the labeled substrates (6 min, 37°C) in 0.05 ml (for PE-PIP2 vesicles) or 0.25 ml (for labeled membranes) reaction mixtures containing 50 mM Hapes (pH 6.7) 5 mM MgCl2/10 mM LiCl/3 mM EGTA. CaCl2 was added to the desired concentration calculated according to Schatzman [18]. Activities of column fractions were determined at 2.3 μM free Ca ++ plus 340 μM arachidonate [16]. Hydrolysis was terminated by the addition of chloroform/methanol (2:1, v/v). Inositol phosphates were isolated on Dowex AG1-X8-formate columns eluted with 0.2 M ammonium formate/1.0 M formic acid and their radioactivities were determined in liquid scintillation counter [19].

Gel filtration of cytosolic PLC on Superose-12. Cytosol (1 mg in 0.25 ml) was fractionated on a Superose-12 column equilibrated with 10 mM KP, (pH 7.0) 131 mM NaCl/0.5 mM EGTA/0.5 μM PMSF/1 μg/ml leupeptin employing an HPLC-system of Waters, Milford, MA. Fractions of 0.3 ml were collected (flow rate was 0.2 ml/min) and their enzymic activities were determined.

Chromatography of cytosolic PLC on a Q Sepharose column. Cytosol (ca. 12 mg protein) was diluted with 2 vol of 10 mM Hepes (pH 7.5) and loaded at a flow rate of 12 ml/h onto a Q Sepharose column (1.5 ml), equilibrated with 10 mM Hepes (pH 7.5) 43 mM NaCl. The column was washed and the proteins were eluted with a linear gradient of 0.043-0.6 M NaCl (20 ml). Fractions of 1 ml were collected and stored at −20°C up to 6 months without loss of activity.

Chromatography of PLC on a phenyl-Sepharose column. Active fractions from Q Sepharose were concentrated by ultrafiltration (Diaflo membrane XM-100, Amicon, MA), mixed with 0.8 M ammonium sulfate applied (12 ml/h) to a phenyl-Sepharose column (2 ml) equilibrated with 0.8 M ammonium sulfate/10 mM KP (pH 6.7). The column was washed and proteins were eluted with 8 ml of KP-buffered 0.3 M ammonium sulfate followed by 8 ml of 40% (v/v) KP-buffered ethylene glycol. Fractions of 1 ml were collected and stored at −20°C.

Separation of cytosolic PLC forms by heparin-Agarose. Cytosol (0.4 ml, 1.65 mg protein) was diluted with an equal volume of 10 mM Hepes (pH 7.5) and applied (10 ml/h) to a column pre-equilibrated with 10 mM Hepes (pH 7.5) 65 mM NaCl. After washing, proteins were eluted stepwise with 0.2 M, 0.5 M and 0.8 M Hepes-buffered NaCl solutions. 1 ml fractions were collected.

Protein determination. The method of Bradford was employed, with bovine serum albumin as a standard [20].

Results

In the first part of this study, neutrophil cytosol was employed as a source of PLC to follow hydrolysis of PIP2 in PE-[32P]PIP2 vesicles. Formation of the products proceeded linearly with time (up to 6 min) and with the concentration of cytosolic proteins. At concentrations exceeding about 240 μg protein/ml deviations from linearity were observed (data not shown).

PIP2 hydrolysis did not require Ca2+ and proceeded also in the presence of 3 mM EGTA (Table 1); rates of substrate breakdown were augmented by arachidonate and Ca2+. Arachidonate and other cis-unsaturated fatty acids, in the presence of Ca2+, were previously shown to dramatically stimulate PI hydrolysis by neutrophil cytosolic PLC [16].

Incorporation of PI into the PE-[32P]PIP2 vesicles at a 1:1 molar ratio to PIP2 was expected to inhibit PIP2 hydrolysis by competition between the two substrates for the available enzyme [21]. PI, however, markedly augmented rates of PIP2 breakdown, also at conditions previously shown by us to stimulate PI breakdown, i.e.,...
TABLE I
The effect of PI on the hydrolysis of [32P]PIP2 in PE-PIP2 vesicles a

<table>
<thead>
<tr>
<th>Additions</th>
<th>[32P]PIP2 hydrolyzed (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-PIP2 vesicles</td>
<td>PE-PIP2-PI vesicles</td>
</tr>
<tr>
<td>None</td>
<td>1.29</td>
</tr>
<tr>
<td>Ca2+ (2.3 μM)</td>
<td>2.26</td>
</tr>
<tr>
<td>Arachidonate (340 μM)</td>
<td>1.58</td>
</tr>
<tr>
<td>Ca2+ plus arachidonate</td>
<td>6.41</td>
</tr>
</tbody>
</table>

a PE-PIP2 vesicles contained 1 μg PE, 2.5 μg unlabeled PIP2, and [32P]PIP2 (15000 cpm). When indicated, 2.5 μg PI was also incorporated into the vesicles.

in the presence of Ca++ and arachidonate [16] (Table I).

The opposite situation, namely the effect of PIP2 on the hydrolysis of PI, was investigated in heat-inactivated membranes which may be considered closer to the natural milieu of cellular phospholipids than PE-PIP2 vesicles (see Discussion). Since labeling of PIP2 in [3H]inositol membranes is very low [16], tracer [32P]PIP2 was incorporated into [3H]inositol membranes to tag endogenous PIP2 [17]. In some of the experiments, [32P]PIP2 was supplemented with unlabeled PIP2 to match the level of endogenous membrane PI (PIP2-enriched membranes). Table II summarizes activities of cytosolic PLC on the two substrates in both types of membrane. In regular membranes, with PI as the predominant inositol lipid, in the absence of Ca2+ and arachidonate as well as in the presence of either one, rates of hydrolysis of both substrates by cytosolic PLC were low. Simultaneous presence of Ca2+ and arachidonate enhanced hydrolysis of both PI and PIP2, the effect on PI being much more pronounced. Enrichment of the membrane by PIP2 to a PI:PIP2 molar ratio of 1:1, substantially increased rates of PIP2 hydrolysis even in the absence of activators, indicating that in regular membranes, PIP2-PLC operated below Vmax. Hydrolysis could be further stimulated by the addition of Ca2+ and arachidonate. Breakdown of PI in membranes and its stimulation by Ca2+ and arachi-

TABLE II
PLC-catalyzed hydrolysis of [3H]PI and [32P]PIP2: effect of PI:PIP2 ratio a

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity (pmol/min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca2+ arachid.</td>
<td>Membrane a</td>
</tr>
<tr>
<td>PL</td>
<td>PIP2</td>
</tr>
<tr>
<td>−   −</td>
<td>≤ 2</td>
</tr>
<tr>
<td>−   +</td>
<td>≤ 2</td>
</tr>
<tr>
<td>+   −</td>
<td>≤ 2</td>
</tr>
<tr>
<td>+   +</td>
<td>165.1</td>
</tr>
</tbody>
</table>

a Tracer [32P]PIP2 was incorporated into [3H]PI-labeled inactivated neutrophil membranes without affecting the PI:PIP2 ratio. b [32P]PIP2 plus unlabeled PIP2 were incorporated into [3H]PI-labeled inactivated neutrophil membranes resulting in a PI:PIP2 ratio of 1:1. c The concentrations of Ca2+ and arachidonate were 2.3 μM and 340 μM, respectively.

TABLE III
Effects of guanine nucleotides and NaF on cytosolic PLC activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity (cpm) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>PIP2</td>
</tr>
<tr>
<td>None</td>
<td>n.d. b</td>
</tr>
<tr>
<td>GTPyS</td>
<td>n.d. b</td>
</tr>
<tr>
<td>GDPyS</td>
<td>n.d. b</td>
</tr>
<tr>
<td>NaF</td>
<td>n.d. b</td>
</tr>
</tbody>
</table>

a Activity was determined using double-labeled membranes [3H]inositol-5000 cpm/assay; [32P]PIP2-10000 cpm/assay; 2.3 μM Ca2+ and 80 μg cytosolic protein. A representative experiment carried out in duplicate and repeated five times with essentially similar results.

b Not detected.

Fig. 1. (A) Chromatography of cytosolic PLC on a Q Sepharose column. Neutrophil cytosol (4 mg/ml, 3 ml) was applied to a Q Sepharose column (1.5 ml), equilibrated with 10 mM Hepes (pH 7.5), 43 mM NaCl and eluted with a linear gradient of NaCl. (o) A280 (△) PI hydrolysis. In three similar experiments, the mean recovery of enzymatic activity equaled 110%. (B) Chromatography of PLC on a phenyl-Sepharose column. Active pooled fractions from Q Sepharose column were applied to a phenyl-Sepharose column (2 ml) equilibrated with 0.8 M ammonium sulfate/10 mM KP4 (pH 6.7) (a). Elution with (b) 0.3 M ammonium sulfate (c) 40% ethylene glycol. 82% of the loaded activity was recovered.
The NaCl content of the eluting solutions is indicated. Hydrolysis of the substrates was not reduced by enrichment with PIP₂ in spite of simultaneous hydrolysis of the latter. Thus, no inhibition of P₁ hydrolysis by the competing substrate, PIP₂, was observed.

GTPγS (1–100 μM) was unable to enhance activity of cytosolic PLC when tested in either PE-PIP₂ vesicles or in the double labeled membranes (Table III). This result was obtained at different concentrations of calcium ions. GDPβS (400 μM) inhibited hydrolysis of PIP₂ in both forms of substrate presentation and unexpectedly NaF (10 mM) also reduced activity in both cases (Table III).

The molecular mass of the main peak of cytosolic PLC was estimated on Superose 12-HPLC column (Fig. 3). The enzyme coeluted with alcohol dehydrogenase (molecular mass 150 kDa). A similar molecular mass was detected in sucrose gradients (data not shown).

Discussion

Most studies on inositol lipid-specific PLC in neutrophils dealt with membrane-associated enzyme in intact, permeabilized or broken cell preparations [5–9]. The neutrophil enzyme was stimulated by binding of chemotactic ligands to surface receptors coupled to G proteins [7–10].

In a previous communication, we described a remarkable enhancement of the activity of neutrophil cytosolic PLC by unsaturated fatty acids and calcium ions [16]. Stimulating effects of anionic phospholipids and amphiphiles and their ability to relieve P₁ hydrolysis from inhibition by long-chain phosphatidylcholine molecules, were demonstrated in other systems by Irvine et al. [22] and Hofmann and Majerus [23]. These authors attributed alterations in the activity of PLC caused by incorporation of certain lipids into the membrane, to the effects of the lipids on the physical structure of the substrate.

In the present report, we extended our earlier studies on P₁-hydrolyzing activity of neutrophil cytosolic PLC [16] to hydrolysis of PIP₂ in membranes and PE-PIP₂ vesicles. The activity of cytosolic PLC on PIP₂ in PE-PIP₂ vesicles was also augmented by arachidonate and calcium; unlike P₁ breakdown, however, PIP₂ hydrolysis proceeded also in the absence of both activating agents (Table I). The remarkable potentiation of PIP₂-hydrolyzing activity by incorporation of P₁ in the absence of activators, namely at conditions unfavorable for P₁ hydrolysis, may be attributed to alterations in the physicochemical structure of the vesicles [22–24]. Alternatively, it can be suggested that P₁ as well as arachidonate interact with a resting or turning-over PLC at a site distinct from the active site of the

**Physicochemical properties of cytosolic PLC**

The main P₁-hydrolyzing activity peak of cytosolic PLC eluted from Q Sepharose anion-exchange column at 0.15 M NaCl concentration (Fig. 1A). On phenyl Sepharose, the single activity peak was eluted at 40% ethylene glycol (Fig. 1B). On both columns, PIP₂ and P₁-hydrolyzing activities coeluted (data not shown).

When cytosol was fractionated on a heparin-Agarose column (Fig. 2), two activity peaks were detected: the enzyme present in the first peak, eluting at 0.2 M salt, acted on both substrates, PIP₂ and P₁. The second activity peak, eluted with 0.5 M NaCl, exhibited mainly PIP₂-hydrolyzing activity. This finding suggested the presence of at least two different isozymes in the cytosol.

The molecular mass of the main peak of cytosolic PLC was estimated on Superose 12-HPLC column (Fig. 3). The enzyme coeluted with alcohol dehydrogenase (molecular mass 150 kDa). A similar molecular mass was detected in sucrose gradients (data not shown).
enzyme. In a single case, binding of PLC to PI vesicles was indeed described [25].

In cellular signalling processes,PIP$_2$ is hydrolyzed preferentially to PI, to give two second messengers, IP$_3$ and diacyl glycerol [1,2]. Potentiation of PIP$_2$ hydrolysis by PI demonstrated in the present study (Table I), may constitute a factor that contributes to the preferential cleavage of PIP$_2$ in biological membranes. It should be pointed out, however, that our data were obtained employing lipid vesicles and not membranes as a substrate.

Experiments conducted with membranes (Table II) permitted direct comparison of activities of neutrophil cytosolic PLC on PI and PEP$_2$ in a milieu which may be considered as close as possible to the natural milieu in the cell. Although the heat pretreatment of the membranes, employed by us to inactivate intrinsic PLC, might have introduced alterations in their topography, the overall composition of the membrane was retained. Since in vivo the negatively charged inositol lipids and particularly PIP$_2$ are bound to membrane proteins [26], the presence of proteins in the microenvironment of the substrate may represent an advantage over pure lipid or lipid-detergent vesicles.

In HL-60 cell membranes in which the ratio of PI:PIP$_2$ is approx. 93.8:2.3 [27], hydrolysis of both inositol substrates by cytosolic PLC proceeded at low and similar rates (Table II). The presence of arachidonate and calcium rendered PI the preferable substrate, although cleavage of PIP$_2$ was also enhanced. Hydrolysis of PIP$_2$ was stimulated by the enrichment of the membranes with exogenous PIP$_2$. Assuming that the presentation of membrane-incorporated PIP$_2$ and intrinsic PIP$_2$ to the enzyme are similar [17], these data imply that prior to the enrichment, the rate of PIP$_2$ breakdown was below $V_{\text{max}}$. The experiments summarized in Table II indicate also that at equal PI and PIP$_2$ concentrations, neutrophil cytosolic PLC acts preferentially on PIP$_2$, in the presence as well as in the absence of Ca$^{2+}$. In spite of this preferential hydrolysis of PIP$_2$, excess of PIP$_2$ incorporated into the membrane did not competitively inhibit the cleavage of PI stimulated by Ca$^{2+}$/arachidonate (Table II). This result may be attributed to dual and opposing effects of PIP$_2$ acting both as a competing substrate that inhibits PI hydrolysis [21] and as a positive modulator which augments its breakdown. In membranes in which the effect of PIP$_2$ on PI hydrolysis was tested (Table II) these opposing effects were of similar magnitude; in the reciprocal situation represented by PE-PIP$_2$ vesicles enriched by PI (Table I), the positive modulation by PI was stronger resulting in a net stimulation of PIP$_2$ hydrolysis. An alternative explanation for the lack of mutual inhibition of PIP$_2$ cleavage by PI (Table I) or PI cleavage by PIP$_2$ (Table II) may implicate PLC isozymes that differ in their kinetic parameters (affinity, $V_{\text{max}}$) with respect to the two substrates. This possibility is indeed implied by chromatography on heparin-Agarose which revealed only one activity peak exhibiting substantial activity on PI (Fig. 2).

The elution profile of PLC activity from a heparin-Agarose column is consistent with the presence of at least two isozymes (Fig. 2). Most living cells contain several isozymes of PLC which differ in their structure and activity [1,2,4]. Three species of PLC were previously detected by gel filtration of neutrophil cytosol [28]. The size of one of them resembled the main PLC species of neutrophil PLC reported in this study (Fig. 4) and was compatible with the size of $\beta$ or $\gamma$ isozymes of PLC. PLC$_{\gamma 1}$ which undergoes phosphorylation on tyrosyl residues by the growth factor-stimulated kinase activity of the receptor has been detected in most living cells [4]. Moreover, in the promyelocytic leukemia HL-60 cells, which upon appropriate induction undergo maturation into cells that resemble neutrophils, PLC$\gamma$ and PLC$\beta$ forms have been identified [4,27].

The deviations from linearity of PLC activity observed by us at high concentrations of cytosol, cannot be attributed to exhaustion of the substrate (less than 15% was hydrolysed) nor to accumulation of products of the reaction. They may reflect the presence in the cytosol of an endogenous inhibitor loosely associated with the enzyme and its dissociation upon dilution of cytosol.

Stimulation of inositol lipid hydrolysis by guanine nucleotides compatible with involvement of G proteins in the activity of neutrophil PLC has been described by many investigators [8-10, 29-32]. In our hands, the activity of neutrophil cytosolic PLC assayed in lipid vesicles or in heat-inactivated membranes devoid of active G proteins was not affected by GTP$\gamma$S (Table III). Moreover, fluoride and GDP$\beta$S exerted an unexplained inhibition of the enzymic activity (Table III). In parallel experiments carried out on membrane PLC acting on endogenous substrate, we observed stimulation of the enzyme by GTP$\gamma$S (data not shown). These results may imply that either PLC isozymes present in neutrophil cytosol are not regulated by G proteins (e.g., PLC$\gamma$) or/and that compatible G proteins were not present in the cytosolic preparations. In the closely related permeabilized HL-60 cells, Cockroft et al. indeed described restoration of GTP$\gamma$S-elicited responses by rat brain soluble phospholipases C of $\beta$ and $\epsilon$ types, but not by the PLC$\gamma$ [26], indicating that G proteins responsible for the stimulation were present in membranes of the permeabilized cells. Contrary to this, Camps et al. [33] have recently demonstrated activation of cytosolic PLC from differentiated HL-60 cells by GTP$\gamma$S, implicating soluble G protein/s in activation of the enzyme. Further characterization and resolution of neutrophil cytosolic PLC forms will be required to clarify the mode of their regulation.
References