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Phosphatidylinositol hydrolysis by human plasma phospholipase D

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A phospholipase D activity able to hydrolyze phosphatidylinositol has previously been described in the cytosol of human neutrophils. The experiments reported here demonstrate that this phosphatidylinositol-hydrolyzing phospholipase D activity is also present in human plasma. This activity was assessed by free inositol release from phosphatidylinositol substrate, by phosphatidate formation and by phosphatidylethanol formation through its capacity of catalyzing a transphosphatidylation reaction. This plasma enzyme activity shows an optimum pH of 8.0 and is inhibited by EGTA.

Phosphatidylinositol; Phospholipase D; Human plasma

1. INTRODUCTION

It is becoming increasingly recognized that phospholipase D plays a role in the cell activation process [1–17]. Phospholipase D attacks the terminal phosphodiester bond of phosphoglycerides to form phosphatidic acid and appropriate bases.

Upon cell activation, phospholipase D activity seems to possess broad substrate specificity, being able to hydrolyze choline- [4–6,10,11,13,14,17], ethanolamine- [16], and inositol- [1–3] containing phosphoglycerides. However, studies conducted in cell-free systems seem to suggest the existence of different phospholipase D enzymes. Thus a Ca^{2+}-independent, membrane-bound, phosphatidylcholine-hydrolyzing phospholipase D has been described in rat brain [18–21] and endothelial cells [22], whereas human neutrophils [9,23] and monocytes [23] possess an EGTA-sensitive, soluble phospholipase D acting preferentially on phosphatidylinositol.

Recent studies have demonstrated the presence of another type of mammalian phospholipase D [24,25]. This enzyme is present in plasma and appears to hydrolyze the glycosylphosphatidylinositol anchor of a number of cell surface proteins [24–27].

Due to the considerable similarities shared by the glycosylphosphatidylinositol-specific phospholipase D and by the phosphatidylinotisol-specific phospholipase D from human phagocytes [9,23–25] in terms of Ca^{2+}-dependency, physical state and molecular size, we studied the capacity of human plasma phospholipase D to hydrolyze phosphatidylinositol. The results herein reported show conclusively that this occurred. The physiological implications of this finding are discussed.

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2. EXPERIMENTAL

2.1. *Materials*

L-3-Phosphaticyl[2-^{3}H]inositol (sp. act. 10–15 Ci/mmol) and 1-stearoyl, 2-[^{14}C]arachidonoylphosphatidylinositol (sp. act. 50–60 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK). Lipid standards were from Supelco (Bellefonte, PA, USA) or Sigma (St. Louis, MO, USA). Anion exchange AG 1-X8 resin was from Bio Rad (Richmond, CA, USA). Organic solvents were of analytical grade from Merck (Darmstadt, FRG). All other chemicals were purchased from Sigma or Scharlau (Barcelona, Spain). Phosphatidylethanol standard was kindly provided by Dr Emilio Diez (Department of Immunology, Smith Kline & French Laboratories, King of Prussia, PA, USA).

2.2. *Assay for phospholipase D activity in human plasma*

Human plasma was isolated from fresh peripheral blood samples by centrifugation at 3000 rpm for 30 min. Phospholipase D activity was measured essentially as described in [9]. Briefly, 30 nmol of radiolabeled phospholipid substrate (1500 cpm/nmol) were treated for 1 h at 37°C with 10 μl of fresh plasma, in 100 mM Tris-HCl, 1 mM $CaCl_2$, 15 mM LiCl, pH 7.5. Final volume of the reaction mixture was 250 μl. Controls without added cell protein were run in parallel. Reactions were stopped by adding 0.63 ml of ice-cold methanol/HCl (100:1) and the mixture extracted with 0.62 ml chloroform and 0.31 ml water. Radiolabeled inositol headgroups were separated by anion-exchange chromatography on AG 1-X8 resin as described [28].

Phospholipase D activity was also measured with 1-stearoyl, 2-[^{14}C]arachidonoylphosphatidylinisotol as substrate. After stopping the reactions with 0.94 ml of chloroform/methanol/HCl (10:20:1), lipids were extracted as described [9] and separated by thin-layer chromatography. For phosphatidic acid quantitation, plates were developed twice using the upper phase of a mixture consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100) [6]. Neutral lipids were separated with *n*-hexane/diethyl ether/acetic acid (70:30:1) as a solvent system [29].

When measuring phosphatidylethanol formation, the reaction mixture contained 175 mM ethanol. Phosphatidylethanol was separated by using the same system described above for phosphatidic acid separation [6].

2.3. *Data presentation*

Experiments were performed at least 3 different times. The data presented are from representative experiments.

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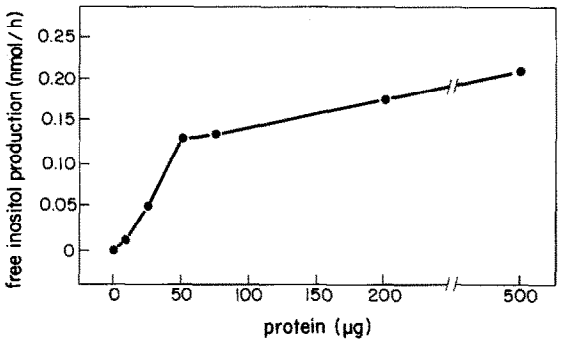

Fig.1. Effect of protein concentration on free inositol release from phosphatidylinositol substrate. Reactions were carried out at pH 7.5, during 1 h as described in section 2.

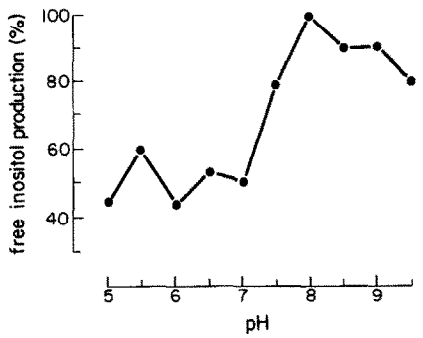

Fig.3. pH dependence of the free inositol-producing activity of human plasma. Reactions were carried out during 1 h and contained 10 μl plasma. The buffering systems for each pH were as follows: pH 5.0–6.0, 100 mM sodium acetate; pH 6.0–7.0, 100 mM L-histidine; pH 7.0–9.0, 100 mM Tris-HCl; pH 9.0–10.0, 100 mM glycine. All reactions were performed in the presence of 1 mM $CaCl_2$.

3. RESULTS

Incubation of phosphatidyl[^{3}H]inositol with increasing concentrations of human plasma in the presence of 1 mM $CaCl_2$ resulted in an appreciable release of radioactive water-soluble compound(s) into the reaction mixture (fig.1). The hydrolytic activity was shown to be linear with respect to the quantity of protein assayed up to 75 μg/assay. However, a concentration of 500 μg protein/assay (approx. 10 μl plasma) was chosen to be used in all subsequent experiments in order to improve sensitivity. The water-soluble labeled compound was identified as free inositol by anion-exchange chromatography (fig.2). Neither glycerophosphoinositol nor inositol phosphate was recovered, suggesting that the enzyme activity responsible for free inositol production was a phospholipase D.

Fig.3 shows the effect of pH on the release of free inositol from phosphatidylinositol substrate. The hydrolytic reaction showed a broad pH dependence, with optimal activity at neutral-alkaline pH values. We examined next the Ca^{2+} requirement of this reaction. When incubations were conducted in the presence of 2 mM EGTA, no release of free inositol was observed, indicating that this reaction was Ca^{2+}-dependent.

To assess the phospholipase D specificity, reactions were conducted with [^{14}C]arachidonoylphosphatidylinositol as substrate. Analyses of chloroform-soluble compounds after the hydrolytic reaction by thin-layer chromatography revealed that only spots migrating as phosphatidic acid and arachidonic acid were labeled.

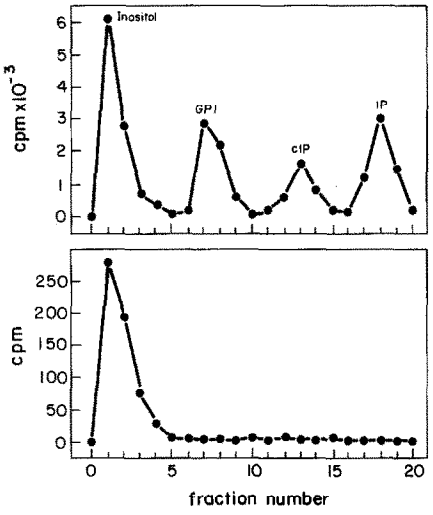

Fig.2. Anion-exchange chromatography of water-soluble inositol compounds. Reactions were carried out at pH 7.5 during 1 h and contained 10 μl of fresh plasma. Reactions were stopped and water-soluble inositol compounds were extracted as described in section 2. Samples were applied to a column of AG 1-X8 resin (1 ml). The column was first eluted with water (fractions 1–5, containing free inositol) and then with a stepwise gradient of ammonium formate in water as follows: 0.025 M (fractions 6–10, containing glycerophosphoinositol, GPI), 0.1 M (fractions 11–15, containing inositol *cyclic*-phosphate, cIP), and 0.2 M (fractions 16–20, containing inositol phosphate, IP) [28]. Upper panel shows the elution profile of authentic labeled standards.

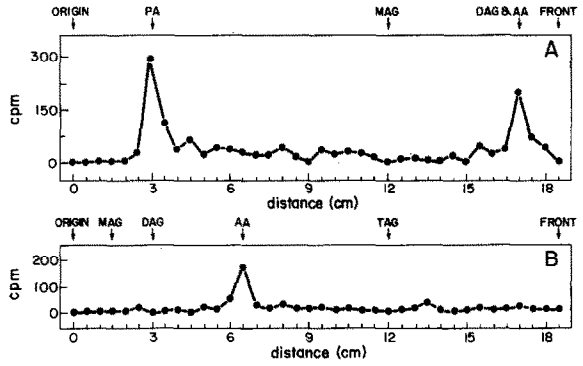

Fig.4. Thin-layer chromatography analyses of chloroform-soluble compounds produced after incubation of phosphatidylinositol with 10 μl of human plasma for 1 h. Plates were chromatographed as described in section 2. Plates were scraped in 5-mm slices and assayed for radioactivity. (A) Separation of phosphatidic acid. (B) Separation of neutral lipids. Arrows at the top of the figures show the position of authentic standards. Radioactivity values corresponding to blanks without added cell protein have been subtracted. AA, arachidonic acid; PA, phosphatidic acid; MAG, monoacylglycerol (monoolein); DAG, diacylglycerol (1-stearoyl, 2-arachidonoyl-*sn*-glycerol); TAG, triacylglycerol (triolein).

Neither diacylglycerol nor monoacylglycerol became labeled (fig.4). These results show conclusively that the plasma activity responsible for free inositol and phosphatidic acid formation is a phosphodiesterase of the phospholipase D-type. It is interesting to recall that human plasma lecithin:cholesterol:acyltransferase has phospholipase A_2 activity [30]. This activity could be responsible for the release of arachidonic acid.

To further assess the phospholipase D activity, we studied the formation of phosphatidylethanol through the transphosphatidylating activity of phospholipase D [31]. When assays were conducted with [^{14}C]-arachidonoylphosphatidylinositol in the presence of 175 mM ethanol, a product co-migrating with authentic phosphatidylethanol became labeled, and the radioactive content of the phosphatidic acid spot slightly decreased (results not shown).

4. DISCUSSION

Previous studies have established the presence in human plasma of an enzyme activity capable of hydrolyzing the glycosylphosphatidylinositol membrane anchor of variant surface glycoprotein from *Trypanosoma brucei* [24], human decay-accelerating factor [24] and human placental alkaline phosphatase [25]. This enzyme activity was identified as a Ca^{2+}-dependent phospholipase D [24,25], exhibiting a strict substrate specificity for glycosylphosphatidylinositol and not for phosphatidylcholine, phosphatidylethanolamine or phosphatidylinositol [24]. Nevertheless, in this paper we have demonstrated that plasma phospholipase D is able to hydrolyze phosphatidylinositol to a significant extent. This apparent discrepancy could be explained by the different acyl chain composition of the substrate's lipid moiety employed by Davitz et al. [24] and by us in the present study. Davitz et al. [24] assayed phospholipase D activity toward dimyristoylphosphatidylinositol, a minor phosphatidylinositol class in mammalian membranes, where the predominant class is 1-stearoyl, 2-arachidonoylphosphatidylinositol [32], just the substrate used in the present study. As has been suggested previously [33], it is possible that variations in the fatty acid composition of phosphatidylinositol are not relevant to its protein-anchoring function, but may profoundly influence the sensitivity of phosphatidylinositol to phospholipase D.

Interestingly, the results of the present study strongly suggest that both phospholipases D from human plasma and phagocytic cells [9,23] behave similarly in the basis of their common biochemical characteristics and substrate specificity. Furthermore, phosphaditylinositol-hydrolyzing phospholipase D activities from human plasma and phagocyte cytosol elute in similar fractions by Sephacryl S-200 gel-filtration chromatography (J. Balsinde and F. Mollinedo, unpublished observation).

The physiological role of this human inositol lipid-hydrolyzing phospholipase D is uncertain. The identification of this enzyme activity in plasma and in the cytosol of peripheral blood phagocytes indicates that, potentially, all phosphatidylinositol-anchored proteins could be released by a phospholipase D activity, irrespective of their subcellular localization. This is of particular interest if considered that certain glycosylphosphatidylinositol-anchored proteins are intracellularly located [33], and that a glycosylated inositol lipid has been implicated in the signal transduction mechanisms of the hormone insulin [34,35].

The activity of the phagocytic cell phospholipase D is increased upon cell activation [9,23]. It will be of interest to investigate how the plasma phospholipase D activity is regulated.

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