CHARACTERIZATION AND PROPERTIES OF A PHOSPHATIDYLINOSITOL PHOSPHODIESTERASE (PHOSPHOLIPASE C) FROM PLATELET CYTOSOL

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1. Introduction

It has been reported that phospholipase C from *Clostridium welchii* induces aggregation and release reaction of human blood platelets [1–3]. During these processes, the diacylglycerols generated in the platelet membrane are phosphorylated into phosphatidic acids [4]. Similar changes occur in platelets activated by physiological agents [4–7] and in many other stimulated cells (reviewed in [8]). According to [8], this 'phospholipid effect' might be due to the activation of a PI-phosphodiesterase (phospholipase C) leading to an increased turnover of PI. We report here the presence of such an enzyme in human platelet cytosol and describe some of its properties.

2. Experimental

2.1. *Substrate preparation*

^{32}P-labelled PI was isolated from baker's yeast grown in the presence of ^{32}P-orthophosphate (CEA, Gif sur Yvette, France) [9]. Lipids were extracted as in [9], a PI-rich fraction was precipitated by propanol-2 [10] and was further purified by thin-layer chromatography [11]. The PI (spec. act. ~1200 dpm/nmol) was chromatographically pure [12] and devoid of any ninhydrin-positive material.

Non-labelled PI was prepared in the same way and was deacylated with *Crotalus adamanteus* phospholipase A_2 [13]. The lysophosphatidylinositol was reacylated with [5,6,8,9,11,12,14,15(*n*)-^{3}H]-arachidonic acid (Radiochemical Centre, Amersham) using rat liver microsomes [14]. The 1-acyl, [2-^{3}H]arachidonoyl-PI (spec. act. ~600 dpm/nmol) was purified as above.

Abbreviation: PI, phosphatidylinositol

2.2. *Enzyme preparation*

Human blood platelets were prepared according to [15] and finally suspended in 0.025 M Tris–maleate–NaOH buffer (pH 6.0) containing 0.125 M KCl. Platelet count was adjusted to 2×10^9 cells/ml and microscopic observation showed no contamination by other blood cells. Platelets were lysed by sonication (3 times, 10 s) using an MSE sonifier at maximum output. The lysates were kept at 0–4°C or centrifuged at 105 000 × *g* for 60 min at 4°C in order to obtain a particulate fraction and a soluble supernatant.

2.3. *Incubation procedures*

Appropriate amounts of labelled PI were evaporated to dryness under nitrogen and sonicated in water just prior to use. The standard incubation medium contained in 0.5 ml : 200 nmol substrate, Tris–maleate–NaOH buffer (final conc. 0.0125 M, pH 6.0) and 0.1 ml of the enzyme preparation. After 5 min at 37°C, the reactions were stopped by adding 1.9 ml chloroform/methanol, 1/2 (v/v) and partitioned as in [16].

2.4. *Analysis of incubation products*

Upper aqueous phase was routinely counted for total radioactivity. Identification of the hydrosoluble products was performed by thin-layer chromatography [17] before and after acidic hydrolysis [18]. In the incubations performed with [2-^{3}H]arachidonoyl-PI, the lower organic phases were analysed by thin-layer chromatography [19].

2.5. *Standards*

A mixture of inositol-1-phosphate and inositol-1,2-cyclic phosphate was prepared from ^{32}P-labelled PI using a crude phospholipase C from *Bacillus cereus* (Sigma, St Louis, MO). Glycerylphosphorylinositol was obtained by alkaline hydrolysis [20] and lysophosphatidylinositol as described above. Lipid standards are currently available from our laboratory.

2.6. *Calcium concentration*

Ionized Ca^{2+} concentration was adjusted with Ca-EGTA buffer [21], taking in account the amounts of endogenous Ca^{2+}, which were determined in platelet extracts as in [22].

3. Results

3.1. *Identification of platelet phospholipase C activity*

Upon incubation with ^{32}P-labelled PI, platelet lysates released 2 hydrosoluble radioactivity compounds. These had the same chromatographic behaviour as inositol-1-phosphate and inositol-1,2-cyclic phosphate (R_F 0.22 and 0.52, respectively) and were separated from glycerylphosphorylinositol and lysophosphatidylinositol standards (R_F 0.42 and 0.81, respectively). Furthermore, upon acidic hydrolysis, only inositol monophosphate was detected on the chromatograms of the assays, whereas the glycerylphosphorylinositol and lysophosphatidylinositol standards remained unchanged. On the other hand, incubation of [2-^{3}H]arachidonoyl-PI led to the appearance of radioactive diacylglycerols. This indicates that platelets contain a typical phosphatidylinositol phosphodiesterase.

3.2. *Subcellular distribution of platelet phospholipase C*

When measured under optimal conditions of pH and substrate concentration (see below), 90% of the total phospholipase C activity was found in the soluble supernatant. Noteworthy, the sum of enzyme activity recovered in both soluble and particulate fractions represented 100–105% of the lysate activity (spec. act. 15–20 nmol/min/10^9 cells).

3.3. *Properties of the phospholipase C activity*

3.3.1. Influence of pH

Maximal hydrolysis was measured with little variation at pH 6.0–7.0, the optimal conditions being obtained at pH 6.0 (fig.1).

3.3.2. Time course of PI hydrolysis

As shown in fig.2, PI hydrolysis was linear for at least 5 min and the ratio inositol-1-phosphate/inositol-1,2-cyclic-phosphate remained constant during the incubation.

3.3.3. Influence of substrate concentration

No hydrolysis was detectable at < 0.040 mM PI (fig.3). However, this limit-value is 20-times higher than critical micellar concentration of PI (measured under our incubation conditions). Phospholipase C activity increased rapidly afterwards and reached a plateau at $\geqslant 0.200$ mM substrate. No significant inhibition by substrate excess was observed until 2 mM PI.

3.3.4. Influence of divalent cations

The phospholipase C activity could be determined without any added divalent cation. However, it must

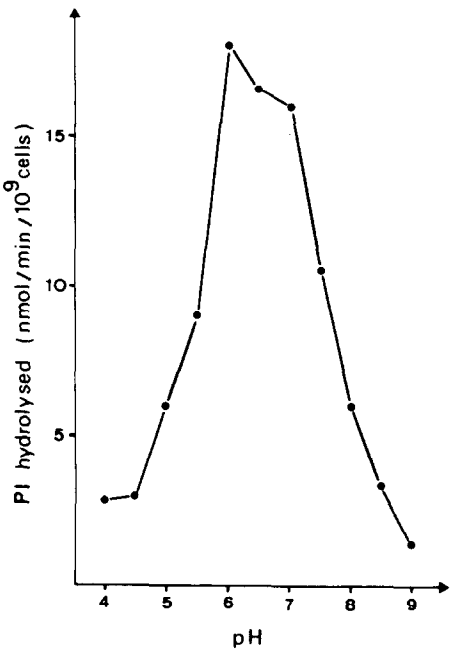

Fig.1. pH dependence of platelet phospholipase C. PI was incubated with platelet lysates under standard incubation conditions, each pH being adjusted using Tris–maleate–NaOH buffer.

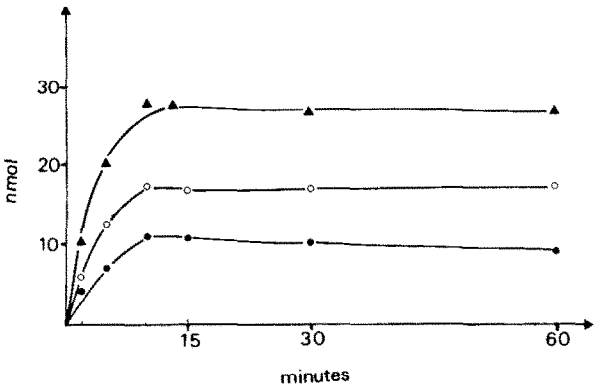

Fig.2. Time course of PI hydrolysis. Ordinates represent the amounts of hydrosoluble compounds released under standard incubation conditions, using 0.2 × 10^9 cells: total hydrosoluble compounds (▲——▲); inositol-1,2-cyclic phosphate (○——○); inositol-1-phosphate (●——●).

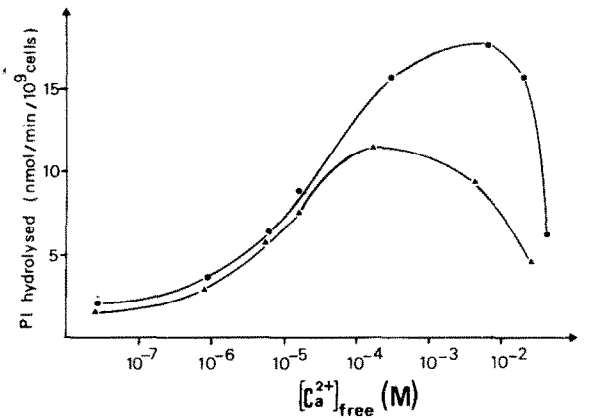

Fig.4. Influence of $[Ca^{2+}]_{free}$ on phospholipase C activity. Platelet lysates were incubated at varying $[Ca^{2+}]_{free}$ with either 0.2 mM (●——●) or 0.4 mM (▲——▲) PI.

be emphasized that such divalent cations were present in the platelet extracts. For instance, Ca^{2+} in the incubation medium as 0.3 × 10^{-4} M and 0.8 × 10^{-4} M when using soluble supernatant or total lysate, respectively. The addition of 2 mM EDTA inhibited the enzyme by 70%. This inhibition was overcome by 2 mM $CaCl_2$, while $MgCl_2$ was inefficient up to 5 mM. As shown in fig.4, the phospholipase C activity increased in a range of calculated $[Ca^{2+}]_{free}$ from 10^{-6}–10^{-3} M. Optimal Ca^{2+} concentrations were 3 × 10^{-4} M and 8 × 10^{-3} M when using 0.2 mM and 0.4 mM PI, respectively. In both cases, a dramatic inhibition occurred at higher Ca^{2+} concentration.

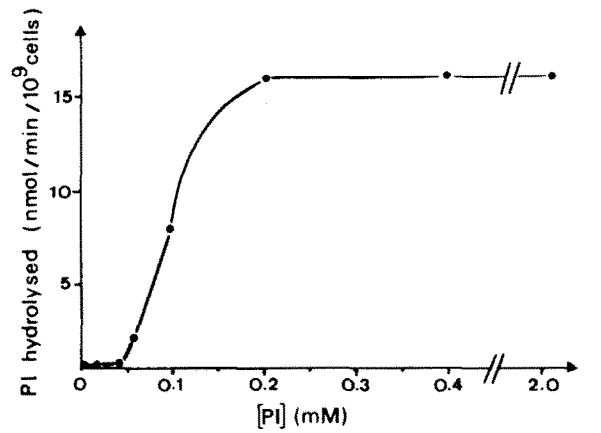

Fig.3. Variation of PI hydrolysis with substrate concentration.

4. Discussion

We report here evidence that human platelets contain a soluble, Ca^{2+}-dependent PI phosphodiesterase. Most of the mammalian phospholipases C described so far are cytosolic [16,23,24] or membrane-bound [25] and require Ca^{2+} for activity. A second group of PI phosphodiesterases of lysosomal origin possesses more acidic optimal pH and is not inhibited by EDTA [26]. From this study, it is evident that the platelet phospholipase C belongs to the former group. Furthermore, the enzyme is almost totally soluble upon cell lysis. It should be noted that 90% of lactate dehydrogenase, a cytoplasmic marker, and only 10% of *N*-acetyl-β-D-glucosaminidase, a lysosomal marker, are solubilized by our lysis procedure [27]. So the platelet enzyme might be exclusively located in the cytosol.

The presence of such an enzyme in platelet cytosol might explain the phosphatidic acid production occurring in stimulated platelets [4–7]. But the mechanism by which the enzyme could be activated remains still unknown. A role for a raised cytoplasmic Ca^{2+} concentration during the course of platelet activation could be questioned. However, like in other cells [28], physiological agents are more efficient than Ca^{2+} ionophore in triggering phosphatidic acid production by platelets [7]. In this case, the phosphatidic acid accumulation could precede the

Ca^{2+} availability, since this phospholipid has been reported to behave as a Ca^{2+} ionophore [29,30].

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