

Phospholipase C in rat liver plasma membranes

Phosphoinositide specificity and regulation by guanine nucleotides and calcium

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Phospholipase C activity against phosphoinositides in isolated rat liver plasma membranes has been examined using exogenous substrates. The enzyme hydrolyzed phosphatidylinositol 4,5-bisphosphate 30–40-times faster than phosphatidylinositol 4-monophosphate, while phosphatidylinositol was not a substrate. Maximum activity was observed with 1.1 mM phosphatidylinositol 4,5-bisphosphate at pH 5.0. The enzyme was stimulated by micromolar concentrations of Ca^{2+} . The GTP analogue guanylyl (β,γ -methylene)diphosphonate enhanced phospholipase C activity at and above 0.3 μM Ca^{2+} , but was inhibitory at 0.1 μM Ca^{2+} . This supports the suggestion that plasma membrane phospholipase C is regulated by guanine nucleotide-binding protein, but indicates a regulatory mechanism different from that of other enzymes regulated by such proteins.

Ca^{2+} Guanine nucleotide Inositol trisphosphate Phosphatidylinositol bisphosphate Phospholipase C
(Rat liver plasma membrane)

1. INTRODUCTION

Phospholipase C-dependent hydrolysis of PIP_2 to the intracellular messengers diacylglycerol and IP_3 appears to be a common mechanism for the transduction of hormonal and other signals over the plasma membrane in many kinds of cells [1,2]. This reaction has recently been shown to be stimulated by guanine nucleotide analogues in membranes isolated from blowfly salivary glands [3] and neutrophils [4] and in liver plasma mem-

branes [5,6], suggesting a regulatory role for guanine nucleotide-binding protein. Vasopressin has also been shown to stimulate the breakdown of PIP_2 in isolated rat liver plasma membranes [7]. These investigations were performed using endogenous substrates radiolabelled in situ. We have further characterized the reaction in rat liver plasma membranes by using exogenous phosphoinositide substrates. Our results indicate that PIP_2 is the preferred substrate and that phospholipase C activity is regulated differently from other enzymes dependent on nucleotide-binding proteins in that GTP analogues and Ca^{2+} may act in concert.

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Abbreviations: GMP-PCP, guanylyl (β,γ -methylene)diphosphonate; IP_2 , *myo*-inositol bisphosphate; IP_3 , *myo*-inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate

2. EXPERIMENTAL

Liver plasma membranes were prepared [8] from adult male Sprague-Dawley rats fed ad libitum. The N_2 fraction enriched in bile canaliculi mem-

branes [9,10] was used. Protein was determined according to Lowry et al. [11] using bovine serum albumin as standard. [^3H]PI and [^3H]PIP were purified from baker's yeast [12] after incubation with [^3H]myo-inositol (Amersham). [^3H]PIP₂ was purchased from New England Nuclear. In some experiments ^{32}P -labelled PIP₂ obtained by enzymatic phosphorylation [10] was used. Similar phospholipase C activities were obtained with either of the substrates. Non-labelled PIP and PIP₂ were isolated from a phosphoinositide-rich brain extract (Sigma).

The standard incubation mixture contained 50 mM Tris-maleate, pH 5.0, 10 μM free Ca^{2+} (EGTA- CaCl_2 mixture as in [13], assuming that the stability constant is the same at pH 5.0 as at pH 7.4), 100 μM GMP-PCP, 1.25 mM PIP₂ (micellar solution) and 10 μg of membrane protein in a final volume of 40 μl . The mixture was incubated for 10 min at 37°C before addition of PIP₂ from a micellar solution (2.5 mM) prepared as follows: PIP₂ in solvent was evaporated to dryness and taken up into the incubation buffer by sonication for 10–12 min. Incubations (in duplicate or triplicate) were for 10 min at 37°C and were stopped with 1 ml chloroform/methanol (2:1, v/v). After the addition of 250 μl of 1 M HCl, vortex-mixing and 30 s centrifugation in a Beckman microfuge, the radioactivity in the water phase was measured by scintillation counting. The reaction rate was constant for 20 min and proportional to the amount of membrane (up to 20 μg) under these conditions. Reaction products formed under various conditions were also analyzed by ion-exchange chromatography on AG 1-X8 (BioRad) columns [14]; more than 75% was IP₃, the remainder IP₂. The IP₂ was most likely formed by dephosphorylation of IP₃ after phospholipase C cleavage as no [^3H]PIP could be detected after incubation. PIP is also an inefficient substrate for the enzyme (see below).

3. RESULTS

Initial experiments indicated that plasma membrane-bound phospholipase C could utilize exogenous PIP₂ as substrate, perhaps due to the fact that approx. 35% of the phospholipid became associated with the membrane under the experimental conditions. We took advantage of this

property to characterize the reaction. The phospholipase C had a rather low pH optimum at pH 5.0 (fig.1); at this pH the activity was more than 10-fold higher than at pH 7.4 (not shown).

Phospholipase C activity increased linearly with increasing substrate concentration reaching a maximum around 1.1 mM PIP₂ (fig.2). The GTP analogue GMP-PCP at 100 μM enhanced PIP₂ hydrolysis by approx. 50%, and the enhancement was constant up to saturating substrate concentration.

Micromolar concentrations of Ca^{2+} also stimulated phospholipase C activity (fig.3). Maximum activity was observed at 0.1 and 0.3 μM free Ca^{2+} in the absence and presence of 100 μM GMP-PCP, respectively. This corresponded to 4- and 7-fold increases of the basal activities observed without addition of Ca^{2+} when 1 mM EGTA was present. Interestingly, GMP-PCP inhibited phospholipase C activity by approx. 40% at 0.1 μM free Ca^{2+} , but stimulated the enzyme by 50% at 0.3 μM Ca^{2+} and above (fig.3). The effect of 1 and 10 μM GMP-PCP was similar to that of 100 μM GMP-PCP at 10 μM free Ca^{2+} .

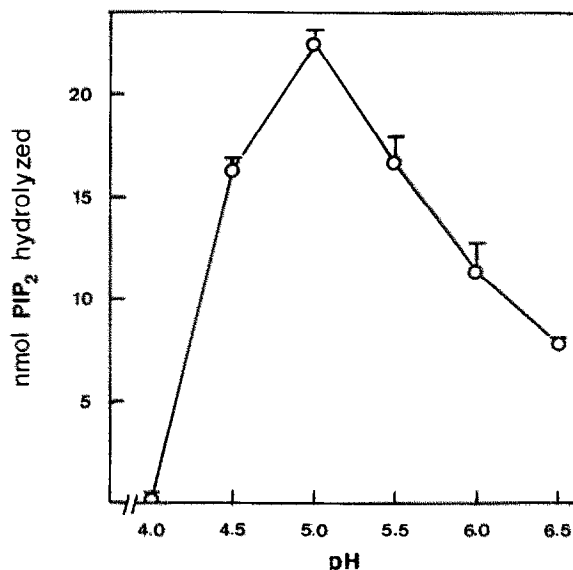


Fig.1. PIP₂ phospholipase C activity as a function of pH. Incubations were performed for 15 min with 15 μg of membrane protein. 50 mM (final concentration) Tris-maleate buffers were used. Each point represents the mean of duplicate incubations with deviations from the mean indicated.

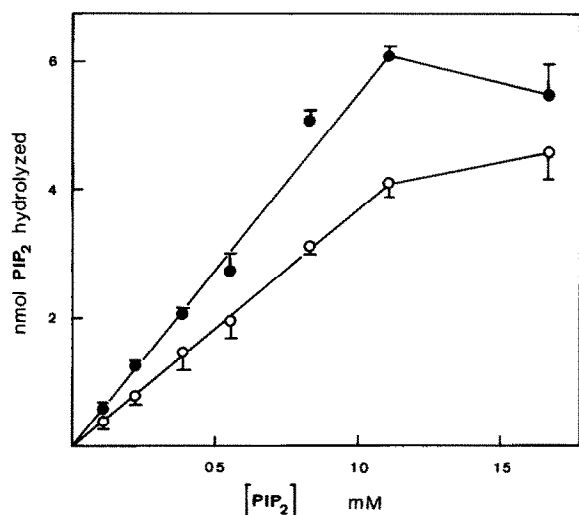


Fig. 2. PIP₂ phospholipase C activity as a function of substrate concentration. Incubations in duplicate were performed as described under section 2 with (●) or without (○) 100 μ M GMP-PCP.

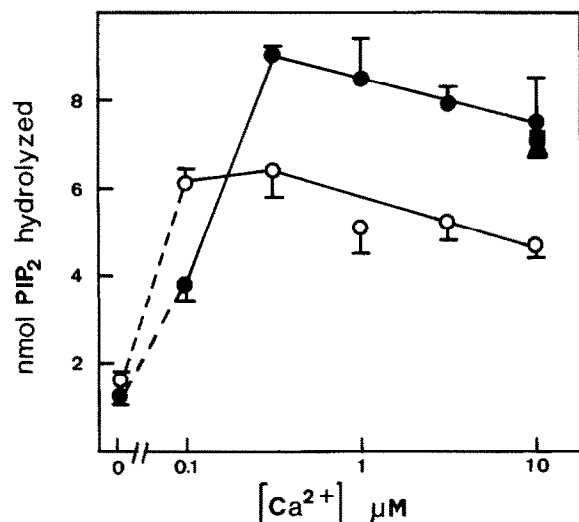


Fig. 3. PIP₂ phospholipase C activity as a function of Ca²⁺ concentration. Various concentrations of free Ca²⁺ were obtained from EGTA-Ca²⁺ mixtures [13]. 1 mM EGTA was present in the incubation without Ca²⁺. Incubations in duplicate were performed as described in section 2 in the absence of GMP-PCP (○) and in the presence of 100 μ M (●), 10 μ M (▲) or 1 μ M (■) GMP-PCP.

The phosphoinositide specificity of plasma membrane phospholipase C was examined under standard incubation conditions. While the specific activity with PIP₂ as substrate in 3 different membrane preparations was 56.2 ± 17.4 and 80.9 ± 17.4 nmol/min per mg membrane protein without and with GMP-PCP, respectively, PIP was hydrolyzed at a 30–40-fold lower rate (1.9 ± 0.3 nmol/min per mg protein both in the presence and absence of the GTP analogue). PI did not serve as a substrate. The substrate specificity was the same in the presence of 0.05% deoxycholate, indicating that the results were not due to varying degrees of incorporation of the substrate into the membrane.

Plasma membranes enriched in the blood sinusoidal region (the P₂ fraction of [8]) had a similar specific activity of PIP₂ phospholipase C as the fraction enriched in bile canaliculi membranes. Also in this case the enzyme was stimulated approx. 50% by 100 μ M GMP-PCP.

4. DISCUSSION

Plasma membrane-bound phosphoinositide phospholipase C utilized exogenous substrate while retaining its dependency on GTP analogues and/or micromolar concentrations of Ca²⁺. The enzyme preferred PIP₂ as substrate and hydrolyzed PIP slowly in accordance with the results of Uhing et al. [6] who studied the hydrolysis of endogenous phosphoinositides radiolabelled in situ. Wallace and Fain [5], using similarly labelled membranes, in addition observed a high rate of PIP degradation.

Micromolar concentrations of Ca²⁺ stimulated the hydrolysis of PIP₂ several-fold. GMP-PCP enhanced the enzyme activity except at free Ca²⁺ concentrations, estimated to be around 0.1 μ M when it was inhibitory. Such a dual effect of GTP analogues in the crucial concentration range of cytosolic free Ca²⁺ has not been reported before. The precise role of guanine nucleotides and Ca²⁺ in the regulation of phospholipase C remains to be defined.

The properties of the plasma membrane enzyme differed in several respects (substrate specificity, Ca²⁺ dependency) from two soluble phospholipase C forms partly purified from rat liver [15]. It is therefore likely that the membrane-bound enzyme

is distinct from these soluble forms. The specific activity towards PIP₂ was more than 7-fold higher in the plasma membrane than in the cytosol, further indicating that the enzyme had not become membrane-bound adventitiously.

In preliminary experiments we have solubilized the membrane-bound phospholipase C in a catalytically active form. It will be of particular interest to purify the enzyme and study interactions with the putative guanine nucleotide-binding protein. Such studies have been difficult to perform with adenylate cyclase, a guanine nucleotide-dependent enzyme, due to its lability.

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