Non-enzymic phosphorylation of polyphosphoinositides and phosphatidic acid is catalysed by bivalent metal ions

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Phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate and phosphatidic acid undergo non-enzymic phosphorylation by ATP in the presence of bivalent metal ions. The non-enzymic reaction is more rapid in a mixture of water, chloroform and methanol than in water alone. Chemical evidence indicates that the product formed from phosphatidylinositol 4-phosphate is the corresponding 4-pyrophosphate. This product shows an R_F value very close to that of phosphatidylinositol 4,5-bisphosphate on t.l.c. with an acidic solvent commonly used to characterize and measure the latter; however, it can be separated readily with an alkaline solvent. Chemical evidence indicates that the products formed from phosphatidylinositol 4,5-bisphosphate and phosphatidic acid are also pyrophosphates.

INTRODUCTION

The action of many hormones and some neurotransmitters is expressed, among other things, through an increase in the turnover of phosphoinositides (Michell *et al.*, 1981; Berridge, 1984). One of the earliest events during this increased turnover is the cleavage of PIP₂ (Michell *et al.*, 1981; Weiss *et al.*, 1982; Creba *et al.*, 1983) to inositol 1,4,5-trisphosphate and diacylglycerol (Streb *et al.*, 1983; Burgess *et al.*, 1984; Prentki *et al.*, 1984; Joseph *et al.*, 1984). The turnover sequence includes the phosphorylation of PIP to PIP₂, a reaction catalysed by PIP kinase. This enzyme has been reported to be stimulated by calcium ions (Van Dongen *et al.*, 1984). PIP kinase activity has been reported to reside in the purified Rous sarcoma virus transforming gene product pp60^{v-src} (Sugimoto *et al.*, 1984). We report here that bivalent metal ions catalyse the non-enzymic phosphorylation of PIP, PIP₂ and PA by ATP. Ca²⁺ and Mn²⁺ are particularly effective in catalysing the non-enzymic reaction.

METHODS

Each reaction mixture contained 20 µg of PIP, 0.1 µmol of [γ -³²P]ATP (1.5×10^7 c.p.m.), and 2 µmol of Hepes/NaOH buffer, pH 7.5. Where indicated, the reactions also contained the following additions: 0.5 µmol of CaCl₂ or MnCl₂ or MgCl₂. The reaction was started by adding [γ -³²P]ATP and was run at 25 °C for 5 min. All samples were sonicated during the incubation. Three different reaction systems were employed: A, an aqueous system in which the reaction was run in a final volume of 0.1 ml of water; B, a two-phase system which was the same as A except that 0.5 ml of chloroform/methanol (2:1, v/v) was added to the reaction; and C, a one-phase aqueous organic system in which the reactants without ATP were in 0.019 ml of water, 0.561 ml of chloroform/methanol (2:1, v/v) was added, and the reaction was started by adding 0.020 ml of [γ -³²P]ATP.

The reactions were stopped by adding 0.25 ml of 1.2 M-HCl; this was followed by 0.5 ml of chloroform/methanol (2:1, v/v) and 0.25 ml of chloroform for system A, or by only 0.25 ml of chloroform for systems B and C. The samples were mixed vigorously and the phases were allowed to separate. The lower, organic layer was washed twice with 0.5 ml of 1.2 M-HCl/methanol (1:1, v/v). The organic layer was removed, dried under N₂, and dissolved in a small volume of chloroform. T.l.c. of the resulting solution was performed according to Jolles *et al.* (1981), except that the following solvent systems were used: acidic, chloroform/acetone/methanol/acetic acid/water (40:15:18:12:10, by vol.) or basic, chloroform/methanol/4 M-NH₄OH (90:70:23, by vol.). Phospholipids were visualized by I₂ vapour staining. Radioactive spots were located by autoradiography using Kodak X-Omat XAR-5 film.

Phosphatidylinositol was obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other lipids were from Sigma Chemical Co. [γ -³²P]ATP was obtained from ICN Biochemicals, Inc. All other chemicals used were of analytical grade. Catalytic subunit of cyclic AMP-dependent protein kinase was a gift from Dr. Susan Taylor.

RESULTS AND DISCUSSION

During studies of the PIP kinase reaction we observed that Ca²⁺ ions catalyse the non-enzymic transfer of the γ -phosphoryl group from [γ -³²P]ATP to PIP. T.l.c. in the acidic solvent system shows a radioactive product with R_F values of 0.38 and 0.35 in the absence and presence of added standards, respectively, compared with an R_F of 0.38 for authentic PIP₂ (Fig. 1a, lanes 5 and 6). However, t.l.c. in the basic solvent system shows a radioactive product that is readily distinguishable from PIP₂ (R_F values of 0.35 and 0.27, respectively) (Fig. 1b). Note that the addition of unlabelled standards again results in a slight shift of the R_F value of the radioactive

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid.

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Fig. 1. Products of the non-enzymic phosphorylation of PIP after their separation by t.l.c.

(a) Reaction conditions and extraction procedures were as described in the Methods section. The acidic solvent system was used to develop the thin layer plates. In lane 6 only, the reaction products were run with standards. (b) Reaction system C was employed and the basic solvent system was used to develop the thin layer plates. Lane 1, reaction products run without standards; lane 2, reaction products run with standards. Autoradiography was followed by I₂ vapour staining. Further abbreviation: PS, phosphatidylserine.

product. We conclude that the radioactive product is not PIP₂ and presume that it is phosphatidylinositol 4-pyrophosphate. When chloroform/methanol (2:1, v/v) is added to the reaction mixture, as is routinely done during the extraction of polyphosphoinositides, more product is formed (Fig. 1a; compare lanes 1 and 2). The rate of the reaction increases further if the amount of water in the reaction is decreased and that of chloroform/methanol (2:1, v/v) is increased so as to form a single phase (compare lanes 2 and 5). The reaction is linear with time for at least 5 min. The order of effectiveness in stimulating the non-enzymic phosphoryl-transfer reaction is Mg²⁺ < Mn²⁺ < Ca²⁺ (compare lanes 3, 4, and 5). Table 1 shows a quantitative comparison of the effect of changing the composition of the solvent mixture as well as the requirements of the reaction. Bivalent metal ions also catalyse the hydrolysis of ATP (Tetas & Lowenstein, 1963). Some of the orthophosphate that results is extracted into or remains in the moist chloroform/methanol layer during the analytical procedure and is not removed completely from this layer by the two washes with 1.2 M-HCl/methanol. Repeated washings of the chloroform/methanol layer result in complete removal of the orthophosphate.

PA and PIP₂ also act as phosphoryl acceptors in the non-enzymic phosphorylation reaction; in the acidic solvent system their products show *R_F* values of 0.59 and

Table 1. Requirements for the non-enzymic phosphorylation of PIP

Reaction conditions, extraction procedures, and t.l.c. in the acidic solvent system were as described in the Methods section. Radioactive spots were scraped from the thin layer plates and counted in Ready-Solv (Beckman) scintillation fluid. Results are means of two separate experiments.

Conditions	Reaction system	Product formed (pmol)
Complete reaction mixture with Ca ²⁺	A (aqueous)	0.1
	B (two-phase aqueous/organic)	1.2
	C (one-phase aqueous/organic)	2.2
PIP omitted	C	0
Ca ²⁺ omitted	C	0

0.39, respectively (Fig. 2). In the basic solvent system, the product derived from PA shows an *R_F* of 0.44 and the products derived from PIP₂ run as an unresolved streak (Fig. 2). The presumed product of PA phosphorylation

Fig. 2. Products of the non-enzymic phosphorylation of PIP_2 and PA

Reaction conditions and extraction procedures were as described in the Methods section for reaction system C except that the phospholipids used were as indicated. When the acidic solvent system was used to develop the thin layer plates, the reaction mixtures contained: lane 1, 20 μg of PIP_2 ; lane 2, a mixture of phospholipids containing approx. 30 μg of PIP_2 , 25 μg of PIP, 5 μg of PI and 20 μg of phosphatidylserine; lane 3, 20 μg of PA. When the basic solvent system was used to develop the thin layer plates, the reaction mixtures contained: lane 1, 20 μg of PIP_2 ; lane 2, 20 μg of PA.

is diacylglycerol 3-pyrophosphate. The possible products of the non-enzymic phosphorylation of PIP_2 are phosphatidylinositol 4-phosphate 5-pyrophosphate and phosphatidylinositol 4-pyrophosphate 5-phosphate. Another possible product is phosphatidylinositol 4-pyrophosphate 5-pyrophosphate. The unresolved streak of products of this reaction mixture, seen upon t.l.c. with the alkaline solvent, is consistent with the high polarity of these presumed products. Phosphatidylinositol, phosphatidylglycerol, dipalmitin and phosphatidylserine do not act as phosphoryl acceptors under these conditions.

Hydrolysis of the non-enzymic reaction products in

0.05 M- H_2SO_4 at 100 $^\circ\text{C}$ for 7 min results in the release of > 99% of the radioactivity as orthophosphate. This is consistent with pyrophosphate formation, but not with the formation of primary phosphate esters. The non-enzymic phosphorylation product of PIP in the basic solvent shows a greater mobility than that of the enzymic product, which would be expected of an inositol pyrophosphate³⁻ as compared with an inositol 4,5-bisphosphate⁴⁻ or similar compound. The observation that substances that possess a primary phosphomonoester group act as phosphoryl acceptors, whereas substances that possess alcoholic hydroxyls and phosphodiester groups do not, is also consistent with the formation of pyrophosphate.

Sugimoto *et al.* (1984) reported that the catalytic subunit of cyclic AMP-dependent protein kinase possesses PIP kinase activity. In our experiments, the catalytic subunit of cyclic AMP-dependent protein kinase showed no significant increase in phosphorylation of PIP over the non-enzymic reaction.

We showed previously that bivalent metal ions catalyse a non-enzymic phosphoryl transfer from ATP to orthophosphate with the production of ADP and inorganic pyrophosphate (Lowenstein, 1958, 1960a, b).

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