Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 220-225

ENZYMATIC SYNTHESIS AND HYDROLYSIS OF [^{32}P]PHOSPHATIDYLINOSITOL PHOSPHATE

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Received April 19, 1985

Phosphatidylinositol kinase activity in plasma membrane preparations of mouse liver was found to be comparable to that in A431 cells and higher than that in three human tumor xenografts. This activity was exploited in preparing ^{32}P-labeled phosphatidylinositol phosphate of high specific radioactivity in which approximately 4% of the radioactivity of the substrate, [γ-^{32}P]ATP, was incorporated into the lipid. The subcellular distribution of phosphatidylinositol phosphate phosphatase in a human astrocytoma xenograft was determined using [^{32}P]phosphatidylinositol phosphate as a substrate. The highest phosphatase activity was found in the plasma membranes.

Recent interest in phosphoinositide metabolism has been generated by cumulative evidence that rapid turnover of these lipids follows reception of physiological stimuli in a variety of tissues (1, 2). The enzymes which participate in these reactions include (i) kinases which phosphorylate PI and PIP, (ii) phosphodiesterases (phospholipase C's) which release diacylglycerol from PI, PIP and PIP_2, (iii) phosphomonoesterases (phosphatases) which dephosphorylate PIP and PIP_2, and (iv) phosphomonoesterases (phosphatases) which dephosphorylate inositol mono-, di- and tri-phosphates. Although the existence and properties of these enzymes have been described, very few of them have been purified, and conflicting results on the subcellular distribution of some of these enzymes were obtained (see ref. 3).

In several studies on phosphoinositide phosphatases, non-radioactive lipids were used as substrates, and Pi production by phosphatases was determined colorimetrically (4-7). These procedures require large amounts of phosphoinositides, and are further limited by the sensitivity of the spectrophotometric

ABBREVIATIONS: PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; Pi, inorganic phosphate.

0006-291X/85 $1.50

method for determining Pi. While an alternative assay method utilizing ^{32}P-labeled lipid substrates would be desirable, the existing and widely used procedure for preparing radioactive phosphoinositides (8) was judged to be unsuitable for routine preparation. The procedure employed erythrocyte ghosts in incorporating ^{32}Pi into various phospholipids, however, available data indicated that only 0.01% of the radioactivity was incorporated into PIP (8).

We have developed a procedure for obtaining [^{32}P]PIP with a greatly improved incorporation. One of the uses of [^{32}P]PIP is illustrated by the determination of subcellular localization of PIP phosphatase activity in a human astrocytoma.

MATERIALS AND METHODS

Materials: Soybean PI, brain PIP and PIP_2 were obtained from Sigma Chemical Co. [γ-^{32}P]ATP (>3000 Ci/mmol) was purchased from ICN. Silica gel H was obtained from E. Merck.

Preparation of subcellular fractions and plasma membranes: Subcellular fractions of astrocytoma (T-24) xenograft were prepared by differential and sucrose gradient centrifugation according to the procedure of Aronson and Touster (9) as modified by Knowles et al. (10). Plasma membranes of astrocytoma were purified from both microsomal and nuclear pellets.

Plasma membranes of mouse liver, human hepatoma (Li-7), and human oat cell carcinoma (T-293) were obtained from the microsomal pellet by sucrose gradient centrifugation. The purity of some of the plasma membrane preparations has been reported (10). Plasma membrane fractions of A-431 cells, a human epidermoid cell line rich in epidermal growth factor receptors, were prepared according to the procedure of Thom et al. (11).

Assay for PI kinase activity: The reaction mixture (50 µl) contained 20 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 0.2% NP-40, 50 µM vanadate, 20-50 µg protein and 0.4 mM [γ-^{32}P]ATP (20,000 cpm/nmol) with or wihtout added PI. After 5 min at 37°, the reaction was terminated by addition of 1 ml of 1 N HCl followed by the addition of 2 ml $CHCl_3$:CH_3OH (1:1). After mixing and a brief centrifugation to separate the two phases, the chloroform layer was removed, and radioactivity in a 0.5 ml aliquot of the chloroform extract was determined by scintillation counting.

Assay for PIP phosphatase activity: The reaction mixture (50 µl) contained 100 mM Na-acetate (pH 6.5), 2 mM EDTA, 0.4% Triton X-100, 0.5-10 µg protein and 10 µM [^{32}P]PIP. Reaction was terminated after 2 min at 37° by addition of 150 µl of 20% trichloroacetic acid. Denatured protein and unhydrolyzed [^{32}P]PIP were sedimented by centrifugation. ^{32}Pi in the supernatant solution was extracted by 2.5 ml of isobutanol-benzene (12), and radioactivity was determined by scintillation counting.

RESULTS AND DISCUSSION

Phosphatidylinositol kinase activities of various plasma membranes. We have previously established optimal conditions for endogenous PI phosphorylation in plasma membranes (13). These studies have been extended to show that exogenously

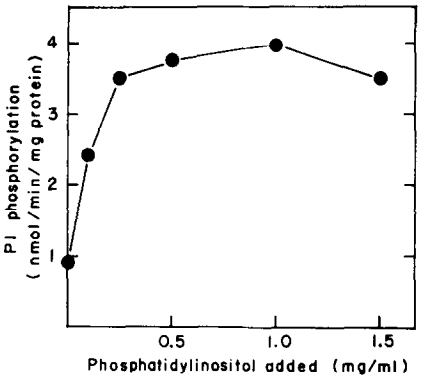

Figure 1. Stimulation of phosphatidylinositol phosphorylation in mouse liver plasma membranes by addition of exogenous PI.

added PI can also be phosphorylated, maximal phosphorylation being obtained at a PI concentration of 0.25-1 mg/ml (Fig. 1).

PI kinase activity was determined in a variety of plasma membranes with and without added PI. Results in Table I show that, among the five tissues and cells examined, the membrane fraction from A-431 cells has the highest activity. Plasma membranes from mouse liver have significatntly greater activity than plasma membranes from three human tumor xenografts. Figure 2 shows that the major product of lipid phosphorylation in these membrane fractions is PIP, the amount of [^{32}P]PIP_2 obtained is usually less than 10% of that of [^{32}P]PIP.

Preparation of [^{32}P]phosphatidylinositol phosphate: For the development of a generally useful method for the enzymatic preparation of [^{32}P]PIP, we used plasma membranes from mouse liver due to its ready availability and high kinase activity. Phosphorylation of PI was carried out in 10 ml reaction mixture

TABLE I. KINASE ACTIVITIES FOR ENDOGENOUS AND EXOGENOUS PHOSPHATIDYLINOSITOL IN VARIOUS PLASMA MEMBRANE PREPARATIONS

Plasma membranes from	Lipid kinase activity	
	-PI	+PI
	nmol/min/mg protein	
Astrocytoma (T-24)	0.40	0.94
Oat cell carcinoma (T-293)	0.78	1.28
Hepatoma (Li-7)	0.34	0.56
A431 cells	2.54	3.80
Mouse liver	1.42	2.54

When exogenous PI was added, the concentration was 0.5 mg/ml.

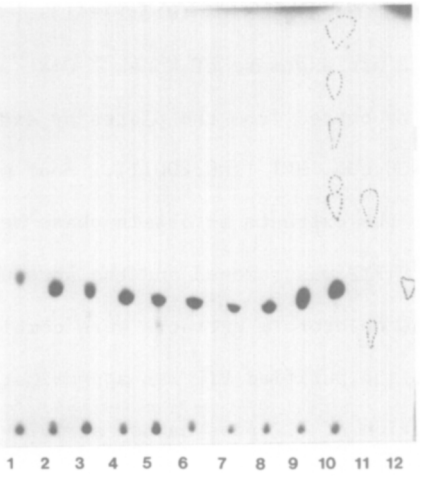

Figure 2. Autoradiogram of thin layer chromatogram of lipids phosphorylated by [γ-^{32}P]ATP in various plasma membrane fractions. Phosphorylation of membranes was carried out under conditions established for PI kinase determination. Phosphorylated lipids were extracted by acidified chloroform-methanol, concentrated and approximately 5000-50000 cpm were applied to Silica gel H plates. Lanes 1, 3, 5, 7, 9 contained samples from membranes phosphorylated in the absence of PI. Lanes 2, 4, 6, 8, 10 contained samples from membranes phosphorylated in the presence of PI. Plasma membranes used were astrocytoma (lanes 1, 2), mouse liver (lanes 3, 4), oat cell carcinoma (lanes 5, 6), hepatoma (lanes 7, 8) and A431 cells (lanes 9, 10). Lane 11 contained PI and PIP_2 standards, and lane 12 contained PIP standard. The phosphoinositide standards, as well as non-radioactive lipids in membrane extract (only shown for lipids in lane 10), were detected after exposure of the plate to iodine vapor (circles enclosed by dotted lines).

containing 20 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 50 µM vanadate, 0.2% NP-40, 7.5 mg mouse liver plasma membranes, 5 mg PI (dispersed in 2% NP-40) and 0.4 mM [γ-^{32}P]ATP (2 x 10 cpm/nmol). The total amount of radioactivity was 0.4 mCi. After 60 min at 37°, the reaction was stopped by the addition of 1 ml 50% trichloroacetic acid. The denatured membranes which contained the ^{32}P-labeled lipids were sedimented by centrifugation. The precipitated membranes were resuspended in 1 ml of water to which was added 3.6 ml of $CHCl_3$:CH_3OH:con. HCl (100:200:2) (14). After thorough mixing, 1.2 ml $CHCl_3$ and 1.2 ml 2 M KCl were added to obtain phase separation. The organic phase was removed, and the aqueous phase was washed twice with 2.5 ml of $CHCl_3$. The chloroform extracts were combined and dried under nitrogen. Estimation of radioactivity indicated 5-7% incorporation of ^{32}P into the lipids.

The dried residue was dissolved in 0.5 ml $CHCl_3$:CH_3OH (1:1), applied to two plates coated with silica gel H (0.25 mm thickness) impregnated with oxalate,

and developed with $CHCl_3$:CH_3OH: 4 N NH OH (9:7:2) (15). [^{32}P]PIP was located by autoradiography after 2 min exposure of Kodak X-Omat AR films.

Radioactive PIP was recovered from the plates by extracting the silica gel twice with 6 ml $CHCl_3$:CH_3OH:con. HCl (100:200:1). Four milliters of $CHCl_3$ and 4 ml of H_2O were added to the extracts to obtain phase separation. The chloroform phase containing [^{32}P]PIP was removed and the aqueous phase was washed once with 8 ml $CHCl_3$. The chloroform extracts were combined and stored at -20°. Incorporation of ^{32}P into the purified PIP was approximately 4%.

Subcellular distribution of PIP phosphatase activity in astrocytoma. Using [^{32}P]PIP as the substrate, the PIP phosphatase activities in the cellular fractions of an astrocytoma were determined. More than 90% of the total phosphatase activity is associated with the various membrane components. The highest specific activity was found in the plasma membrane fractions obtained from both the crude microsomal and nuclear fractions (Table 2).

It was previously shown that astrocytoma plasma membranes contained phosphatase activities toward both phosphoseryl- and phosphotyrosyl-protein substrates (16), these activities being attributed to separate enzymes (17).

TABLE II. SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYLINOSITOL PHOSPHATE PHOSPHATASE IN A HUMAN ASTROCYTOMA

Cellular fraction	Protein (mg)	PIP phosphatase (nmol/min/mg protein)	Total activity (nmol/min)
Homogenate	2060	9.6	19776
Nuclei	341	7.6	2592
Mitochondria (heavy)	42.4	20.1	853
Mitochondria (light)	45.0	31.5	1418
Plasma membranes			
M_1	25.5	94.1	2400
N_1	6.5	202.0	1321
Endoplasmic reticulum			
M_2	24.4	48.5	1183
N_2	6.3	59.3	372
Cytosol	942	1.0	942

Subcellular fractions were prepared as described in "MATERIALS AND METHODS". M_1 and M_2 were purified from the microsomal pellet. N_1 and N_2 were purified from the nuclear pellet. M_1 and N_1 were recovered from the interface of 8.5% and 37% sucrose, and M_2 and N_2 were recovered from the interface of 37% and 47% sucrose from a three-layer discontinuous sucrose gradient.

We have noticed much similarity in the properties of the phosphotyrosyl protein phosphatase and PIP phosphatase (18). The possibility that these two phosphatases are identical is currently under investigation.

ACKNOWLEDGMENTS: This research was supported by National Institutes of Health grants CA-27117 and CA-23052 to the Athymic Mice Facility at UCSD. We thank the staff of the Facility for providing the human tumor xenografts, and Sue Shoemaker for providing the A431 cell membranes.

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