ISOLATION OF CYCLIC INOSITOL-1,2-PHOSPHATE FROM MAMMALIAN CELLS AND A PROBABLE FUNCTION OF PHOSPHATIDYLINOSITOL TURNOVER.

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Summary: Cyclic inositol-1,2-phosphate was found to be present in tissue culture cells -SV40 transformed- at an approximate concentration of 10^{-6} molar. Of the water soluble radioactive split products of phosphatidylinositol metabolism more than 90% represents glycerylphosphorylinositol. In pulse chase experiments with [^{3}H]glycerol and ^{32}P-phosphate changes in the ^{3}H/^{32}P ratio of phosphatidylinositol were investigated during the biphasic decomposition of this lipid. On the basis of the results obtained, phosphatidylinositol turnover and a model of information exchange between membranes are discussed.

Introduction: An increased incorporation of ^{32}P into PI in secreting pancreas slices was first described by Hokin and Hokin (1). The observation of an increased turnover of PI upon stimulation has since been extended to various other cell systems (for references see 2,3). The PI turns over in a biphasic manner (4,5,6). One of the products of PI breakdown is a cyclic phosphate-diester of inositol (7,8).

The PI turnover seems to be associated with transport processes through membranes (3). Furthermore, it has been suggested that I-1,2-cP may function as a second messenger (2).

In this communication we want to give support to this hypothesis by demonstrating that I-1,2-cP can be extracted from cells growing in tissue culture. In addition a kinetic experiment attempts to correlate the biphasic turnover of PI with its presumed functions.

Material and Methods: Suspension cultures of STU-51A/232B mouse cells were used throughout the experiments (9). Pulse chase experiments were performed and evaluated as described earlier (6). The I-2-P, which was used as reference, was purchased from Sigma Chemical Co.

Abbreviations: I, inositol; PI, phosphatidylinositol; I-1-P, inositol--1-phosphate; I-2-P, inositol-2-phosphate; I-1,2-cP, cyclic inositol--1,2-phosphate; GPI, glycerylphosphorylinositol.

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The I-1,2-cP was synthezised according to Pizer and Ballou (10). The GPI used for reference purposes was prepared as described by Dawson (11).

Radioactivity of solutions was determined with a Packard Scintillation Counter. Radioactive paper chromatograms were scanned with the Packard Radiochromatogram Scanner.

Isolation of water soluble products of PI metabolism: Cells were propagated for 24 hours in medium from which inositol, except for that present in the serum, had been omitted. To 500 ml of this suspension ($2x10^6$ cells/ml) were added 600 μCi of [$^3H_2$]inositol (Amersham). The cells were allowed to grow for a total of 48 hours. During this time they were diluted once with 300 ml of fresh medium. The labelled cells were harvested by centrifugation and washed once with 80 ml of medium containing a 10fold excess of inositol. A total of 3.5 ml of packed cells was then frozen at -20°.

The cells were extracted with 90 ml chloroform/methanol 2:1 using ultrasonication. The extract was partitioned (12) into two phases by addition of 16 ml water. The lower phase was used to determine the specific activity of PI. The chloroform/methanol insoluble material was extracted twice with 10 ml of 50% ethanol containing 1 mg of nonradioactive I-1,2-cP as carrier substance. These extracts were combined with the upper phase from the partition, concentrated to about 3.0 ml on a rotary evaporator at 40° and then lyophilized.

The lyophilized material was extracted three times with 0.5 ml 50% ethanol. The combined extracts were further studied by descending chromatography and electrophoresis on Whatman No. 1 paper.

In the first chromatography the material was applied over a range of 40 cm and the solvent used was ethylacetate/pyridine/water 2:2:1. In chromatography system 2 the material was applied over a range of 10 cm and i-propanol/ammonia 3:2 was used (13). Electrophoresis was performed according to Dawson (13), the material was applied over a range of 4 cm.

Paper strips were scanned and radioactivity containing areas were eluted with water, the eluates were lyophilized and redissolved in small volumes of water.

Phosphorus containing compounds were detected on paper using an acid molybdate spray (14) and free inositol was stained with an alkaline silver nitrate reagent (15).

Results: When cells are grown for 48 hours in the present of 600 μCi [^3H]inositol $8.4x10^6$ dpm or about 4 μCi are recovered in PI with a specific activity of $1.1x10^4$ dpm/nmol.

Before the first chromatography the water/ethanol extracts contained

about 100 uCi of radioactivity. By far the largest part of this material was free [^{3}H]inositol which could be separated from a small amount of radioactive material moving much slower (Fig.1A). In the presence of

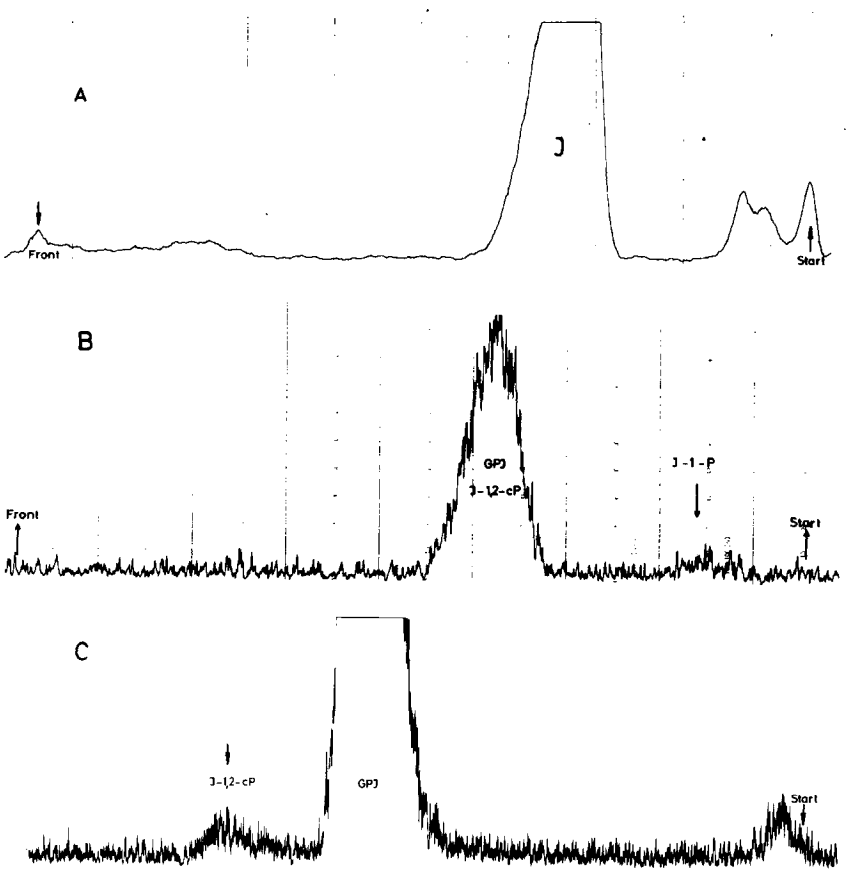

Fig.1 Separation of [^{3}H]inositol labelled water soluble cell constituents. Radioscans of A) chromatogram after separation of [^{3}H] inositol from phosphorylated compounds in system 1; B) chromatogram after separation of GPI and I-1,2-cP from I-1-P in system 2; and C) electropherogram after separation of I-1,2-cP from GPI.

cell extract the reference substances I-1,2-cP, I-2-P and GPI move to the same position. The slow moving material was eluted and yielded about $2x10^6$ dpm. Rechromatography of 1/10 of this material in the same system showed that it was free of [^{3}H]inositol.

Chromatography system 2 separated I-1-P from GPI and I-1,2-cP (13). Since in the systems used I-1-P behaves similar to I-2-P the latter compound was used as reference. Figure 1B shows that the majority of the

radioactivity is present in the area of GPI and I-1,2-cP, only traces of radioactivity are found in the area of I-1-P.

The GPI and I-1,2-cP were separated by electrophoresis (13) as shown in Fig. 1C. From the area of GPI were eluted 1.4×10^6 dpm, from that of I-1,2-cP were eluted 3.8×10^4 dpm. Rechromatography of the eluted GPI showed that I-1,2-cP is not formed from GPI during electrophoresis.

To confirm that the radioactivity present in the area of I-1,2-cP is actually contained in this compound one half of the eluted material was treated with 1 N HCl for 4 min. at 80°. This converts I-1,2-cP to I-1-P (10). The hydrolized and the untreated sample were lyophilized and re-chromatographed in system 2 (Fig.2). From Fig. 2B it can be seen that

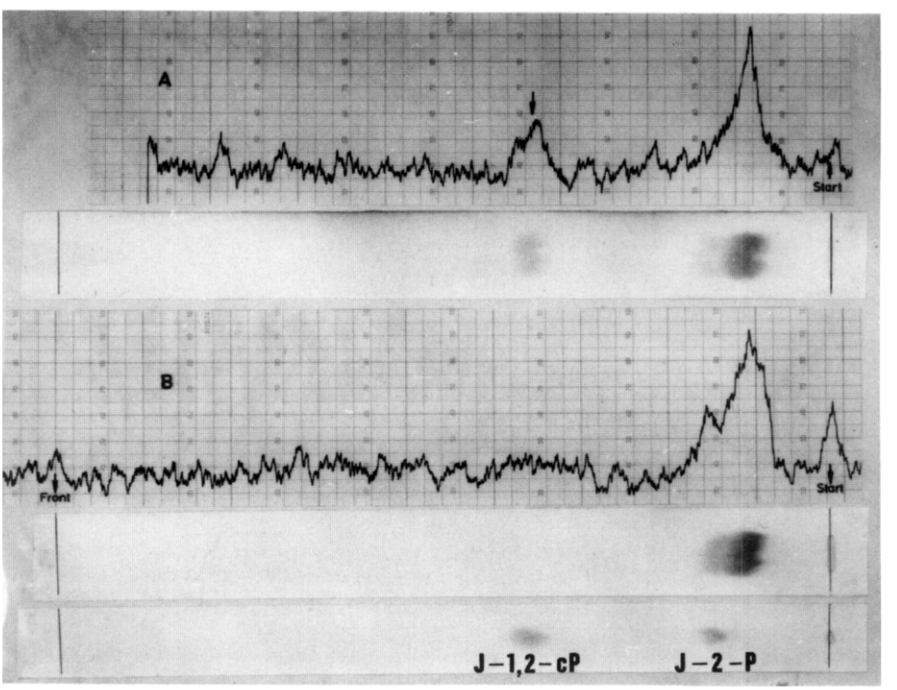

Fig.2 Characterization of I-1,2-cP. Radioscans and chromatograms stained for phosphorous containing substances before (A) and after hydrolysis (B). The bottom chromatogram shows the reference substances.

after hydrolysis neither in the stained chromatogram nor in the radio-chromatogram material is present which corresponds to I-1,2-cP. Only in the area corresponding to I-1-P or I-2-P radioactive phosphorus containing material is present. In the unhydrolized sample (Fig.2A) such

material is present in both positions. The amount of radioactive material corresponding to I-1-P in the unhydrolized sample however is less than in the hydrolized one. Control experiments with a pure sample of I-1,2-cP showed that conversion of I-1,2-cP to its noncyclic derivatives can occur in our hands during drying of the electropherogram. This observation offers an explanation for the detection of radioactive, phosphorus containing material in the area of I-1-P, even when the sample is not hydrolized.

When the experiment was repeated with cells exposed for a 12 hour period to [^{3}H]inositol essentially the same results were obtained.Again more than 90% of the water soluble radioactive products of inositol metabolism behaved like GPI, only about 5% like I-1,2-cP and I-1-P.

If one assumes that the material identified as I-1,2-cP has the same specific activity as its precursor PI (1.1×10^4 dpm/nmol) the isolation of 3.8×10^4 dpm in I-1,2-cP after electrophoresis indicates a concentration of this material in the cells (3.5 ml) of about 10^{-6} molar or the presence of 10^5-10^6 molecules of I-1,2-cP per cell.

The finding that I-1,2-cP can be isolated from logarithmically growing cells prompted further investigations of the turnover of PI, its presumed precursor. Previously we have reported that in pulse chase experiments with [^{3}H]glycerol and ^{32}P-orthophosphate the ^{3}H/^{32}P ratio of PI increases during the early chase period (6). During the same time this ratio decreases in the other phospholipids. Using the same technics, the observation period was now extended up to 120 hours. The results from one of these experiments are depicted in Fig.3 . The ratio of ^{3}H/^{32}P in PI increases rapidly up to about 40 hours. Thereafter, however, it declines, and again rapidly when compared to the other phospholipids represented in Fig. 3 by the ^{3}H/^{32}P ratio for PC.

The release of ^{32}P as well as of [^{3}H]glycerol from PI is biphasic (Fig.3A). The ^{32}P is lost more rapidly than [^{3}H]glycerol from PI during the early chase period, while the loss of [^{3}H]glycerol is more marked during the late chase period.

Discussion: The data presented in Fig. 3 show that some PI molecules turn over fast, some slow. When the rapidly metabolized radioactive molecules are predominant, ^{32}P disappears from PI somewhat faster than [^{3}H] glycerol. The ratio of ^{3}H/^{32}P consequently increases. This has been interpreted as being due to the reutilization of the glycerol moiety for the synthesis of PI (6). From the bulk of the rapidly metabolized PI molecules, however, ^{32}P and [^{3}H]glycerol are lost together, most prob-

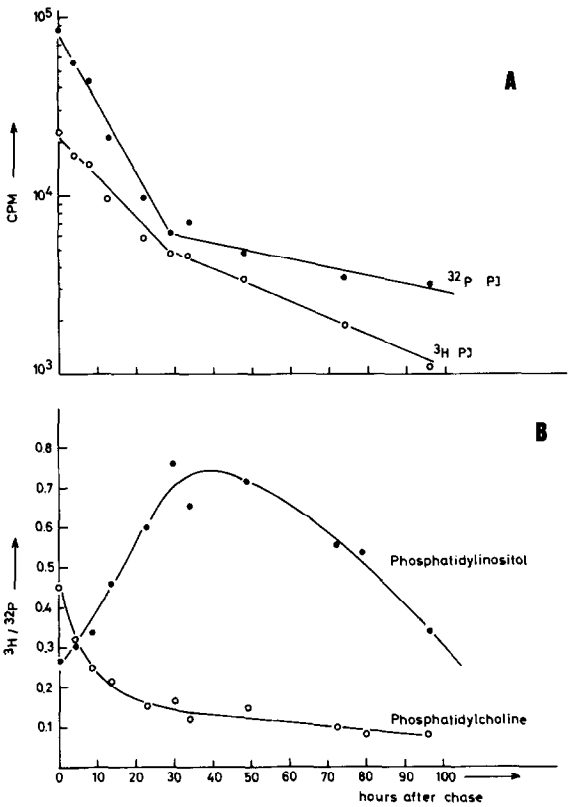

Fig.3 Time course of A) release of ^{32}P and [^{3}H]glycerol from prelabelled cellular PI during the chase period. B) ^{3}H/^{32}P ratio in PI and phospatidylcholine prelabeled with ^{32}P-phosphate and [^{3}H] glycerol.

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able as GPI which we detected in high amounts chromatographically in extracts from [^{3}H]inositol labelled cells.

When the breakdown of labelled PI has levelled off, the ^{3}H/^{32}P ratio decreases. We attribute this to a reutilization of the phosphorylinositol moiety of PI for the resynthesis of PI. One can envision that these two processes of reutilization of PI breakdown products occur at different places and/or membranes. In a model based on this hypothesis the water soluble I-1,2-cP would be liberated in membrane I and reutilized in membrane II for the synthesis of PI. The amount of I-1,2-cP arriving at membrane II could signal events taking place at or in membrane I.

Unexpected was the detection of large amounts of radioactive GPI in cells prelabelled with [^{3}H]inositol. From Fig. 3 it can be deduced that

from most of the rapidly metabolized PI molecules ^{3}H and ^{32}P are lost together. It has been demonstrated that PI metabolism is linked to the transport of material across the cell membrane, possibly by removal of the polar group (3). Openings in the cell membrane could be accomplished more effectively by removal of the whole lipid molecule as free fatty acids and GPI. The high amount of radioactive GPI found, would then be an indicator for the extensive transport across the cell membrane taking place in our exponentially growing cells.

It appears not unreasonable to assume that structures, i.e. PI, which are involved in transport mechanisms are at the same time generators of signals which may cooperate in the regulation of cell growth, e.g. from the plasma cell membrane to the nuclear membrane.

In preliminary experiments we have attempted to explore whether the PI turnover in the plasma cell membrane and in the nuclear membrane exhibit differences. For this purpose we studied the PI turnover of whole cells and of nuclei isolated either by the method of Blobel and Potter (16) or of Hershey et al. (17). Only with the first method differences in the $^{3}H/^{32}P$ ratio of nuclear PI as compared to the PI of the whole cell were observed.

Experiments designed to show that I-1,2-cP can be used for the synthesis of PI are in progress.

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