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Generation of phosphatidylinositol 4,5-bisphosphate proceeds through an intracellular route in rat hepatocytes

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The subcellular distribution in rat hepatocytes of enzymes participating in the entire generation cycle of phosphatidylinositol 4,5-bisphosphate, and phosphorylated intermediates of this pathway, has been examined by Nycodenz gradient centrifugation. Our results indicate that the synthesis of phosphatidylinositol takes place in the endoplasmic reticulum, and that its phosphorylation to phosphatidylinositol 4-phosphate occurs intracellularly in low-density membranes before translocation to the plasma membrane, where it is further phosphorylated to phosphatidylinositol 4,5-bisphosphate. The intracellular formation of PIP implies a vesicular transport to the plasma membrane.

Phosphoinositide turnover; Subcellular localization; Phosphoinositide phosphorylation; Polyphosphoinositide; (Rat hepatocyte)

1. INTRODUCTION

The receptor-dependent transducing mechanism involving hydrolysis of PIP_2 [1] is dependent on efficient regeneration of this phospholipid as it is present only in small amounts in the plasma membrane [2]. The regeneration proceeds through CDP-diglyceride, presumably in part formed from the hydrolysis product DAG via PA, and PI. It is generally thought that these intermediates are synthesized in the endoplasmic reticulum. PI is then transported to the plasma membrane, presumably by exchange proteins [3], before phosphorylation in two steps to PIP_2.

In order to control the regeneration of PIP_2 two functionally distinct pools of phosphoinositides, one of which might respond to stimulation, would be advantageous. A plasma membrane location of a responsive pool, and indeed of the entire regeneration pathway, has been suggested [2]. Accordingly, there is evidence for a distinct PI synthase activity in plasma membranes of GH_3 cells [4]. However, the distribution of PI kinase and PIP kinase is not entirely clear. In rat liver, we have found these enzymes to be differently located, in Golgi and plasma membranes, respectively [5,6], whereas others have found both enzymes in plasma membranes [7,8].

We have now examined, by Nycodenz gradient centrifugation, the subcellular distribution of enzymes and newly phosphorylated intermediates of the entire phosphoinositide turnover cycle in order to resolve the question of where the different steps required for PIP_2 synthesis take place in rat hepatocytes.

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

2. MATERIALS AND METHODS

2.1. *Chemicals*

PIP and PIP_2 were purified [6,9] and [^{32}P]ATP synthesized [10] as described. Radiolabelled compounds were from Amersham or New England Nuclear. PI (soybean or liver), DAG (stearoyl-arachidonylglycerol) and CDP-dipalmitoyl glycerol were from Serdary Research Laboratories (Ontario), polyethylene glycol (Carbowax 3350) from Union Carbide, col-

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lagenase (Type IV), PA and other chemicals for enzyme analyses from Sigma and Nycodenz from Nycomed (Oslo).

2.2. *Isolation and subcellular fractionation of hepatocytes*

Hepatocytes were prepared from adult male Sprague-Dawley rats (250–300 g) [11]. Cells (viability above 90%) suspended in perfusion buffer (117 mM NaCl, 5.35 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 9.5 mM NaH_2PO_4, 15 mM glucose, 40 mM Hepes, pH 7.4) were sedimented at $75 \times g$ for 2 min. This and the following steps were performed at 0–4°C. The cells were resuspended in 25 mM Tris-HCl, pH 9.0 ($20–30 \times 10^6$ cells/ml), homogenized by N_2 cavitation (100 bar, 10 min) and centrifuged at $1000 \times g$ for 5 min. The pellet was resuspended in half the volume buffer, N_2 cavitated (5 min) and centrifuged as above. The combined supernatants were hand-homogenized by 3 strokes with a teflon pestle and centrifuged for 5 min at $4400 \times g$. 1 ml supernatant (approx. 15 mg protein) was layered on top of a 12 ml of 7.5–30% linear Nycodenz gradient in 25 mM Tris-HCl, pH 9.0, and centrifuged at $100000 \times g$ for 90 min (Beckman SW40 rotor). Fractions (0.65 ml) were collected by upward displacement and stored in portions at −20°C. PIP kinase and galactosyltransferase were assayed immediately.

2.3. *Determination of enzymes*

Galactosyltransferase was assayed in 100 mM Tric-HCl, pH 8.0, 10 mM $MnCl_2$, 1% Triton X-100 (w/v), 30 mg/ml ovalbumin, 1 mM UDP[^{3}H]galactose (3000 dpm/nmol) and sample protein in a total volume of 50 μl. Incubations were for 30 min at 37°C. The mixture was transferred to filter paper discs (Munktell 00), which were washed 3 times for 10 min each in 15% trichloroacetic acid before liquid scintillation counting. Alkaline phosphodiesterase I [12], arylesterase 13, NADPH-cytochrome *c*-reductase [14], phosphoinositidase C [15], PI kinase [5,6] and PIP kinase [6] (both with exogenous substrate) and DAG kinase (stearoyl-arachidonylglycerol as substrate) [16] were analyzed as described. PI synthase was assayed in 100 mM Tric-HCl, pH 7.6, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.2 mM CDP-dipalmitoyl glycerol, 0.1 mM [^{3}H]inositol (80–100 dpm/pmol) and membrane protein in a volume of 50 μl. After 30 min at 37°C the reaction was terminated with 1 ml $CHCl_3/CH_3OH$, 2:1 (v/v) and 250 μl of 1 M HCl added. After mixing, 30 s centrifugation in a Beckman Microfuge and washing the lower phase with 0.6 ml $CHCl_3$-saturated CH_3OH/50 mM HCl, 1:1 (v/v), the final chloroform phase was dried under N_2 before counting the radioactivity. Control thin-layer chromatography showed that >94% of the radioactivity co-migrated with authentic PI. Inositol incorporation by base exchange [2] was less than 5%. The CTP/PA cytidylyltransferase reaction mixture (100 μl) contained 100 mM Tris-HCl, pH 7.2, 80 mM KCl, 50 μg bovine serum albumin, 8 mM 2-mercaptoethanol, 1 mM PA (sonicated in the buffer), 2 mM GTP, 5% polyethylene glycol (w/v), 1 mM [^{3}H]CTP (1000 dpm/nmol), membrane protein and 20 mM $MgCl_2$ to start the reaction. It was stopped after 20 min at 37°C as for PI synthase and 500 μl of 2 M KCl containing 0.1 M HCl added. The lower phase, obtained as above, was washed once with 1 ml of 2 M KCl. The final chloroform phase was used for scintillation counting. All enzyme analyses were performed under saturating conditions and were linear with time and amount of sample, except for cytidylyltransferase which was linear above 16 μg sample protein after a lag at low protein concentrations. Protein was determined according to Bradford [17].

2.4. *^{32}P-labelled phospholipid turnover experiments*

Hepatocytes were sedimented at $50 \times g$ for 1 min and resuspended ($\sim 7 \times 10^6$ cells/ml) in a low phosphate buffer (122 mM NaCl, 5.35 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 4.5 mM Na_2SO_4, 10 mM glucose, 4.2 mM $NaHCO_3$, 1.5 mM $CaCl_2$ and 20 mM Hepes, pH 7.4). After preincubation for 5 min at 37°C under gentle shaking, 25 μCi/ml [^{32}P]P_i was added. The incubation was stopped with 6 vol perfusion buffer at 0°C. Cells were disrupted and processed, and the $4400 \times g$ supernate centrifuged in Nycodenz gradients, as described above. Phospholipids were extracted from 250 μl of each gradient fraction by adding 1 ml $CHCl_3/CH_3OH$, 1:1 (v/v). Lipid standards were added as carriers to aid in identification. Phase separation was effected by addition of 300 μl of 2 M HCl and centrifugation. The lower phase was washed once with 1 ml CH_3OH/1 M HCl, 1:1 (v/v), and then with 1 ml CH_3OH/0.1 M HCl, 1:1 (v/v). The final chloroform phase was dried under N_2 before thin-layer chromatography. PI and PA were separated using system A as in [18] and PIP and PIP_2 as in [6]. Appropriate bands were scraped off before scintillation counting.

3. RESULTS

3.1. *Separation of marker enzymes in Nycodenz gradients*

Centrifugation of postmitochondrial supernatant of rat hepatocytes in a 7.5–30% Nycodenz gradient (fig.1A) showed that galactosyltransferase, a Golgi marker, was recovered well above the plasma membrane marker alkaline phosphodiesterase I. The endoplasmic reticulum marker arylesterase focussed bimodally, with one peak overlapping the denser part of the plasma membrane marker and another peak having higher density. Thus, the resolution of these markers, particularly the Golgi and plasma membrane ones, was better in Nycodenz gradients than earlier obtained in Percoll gradients [19].

3.2. *Subcellular distribution of enzymes involved in PI turnover*

In the 7.5–30% gradient, PI kinase activity focussed at a slightly lower density than the Golgi marker (fig.1C), whereas PIP kinase sedimented with the plasma mebrane marker. By modifying the gradient a nearly complete resolution of PI kinase and galactosyltransferase could be obtained (not shown). Membrane-bound phosphoinositidase C (fig.1B), like PIP kinase, focussed

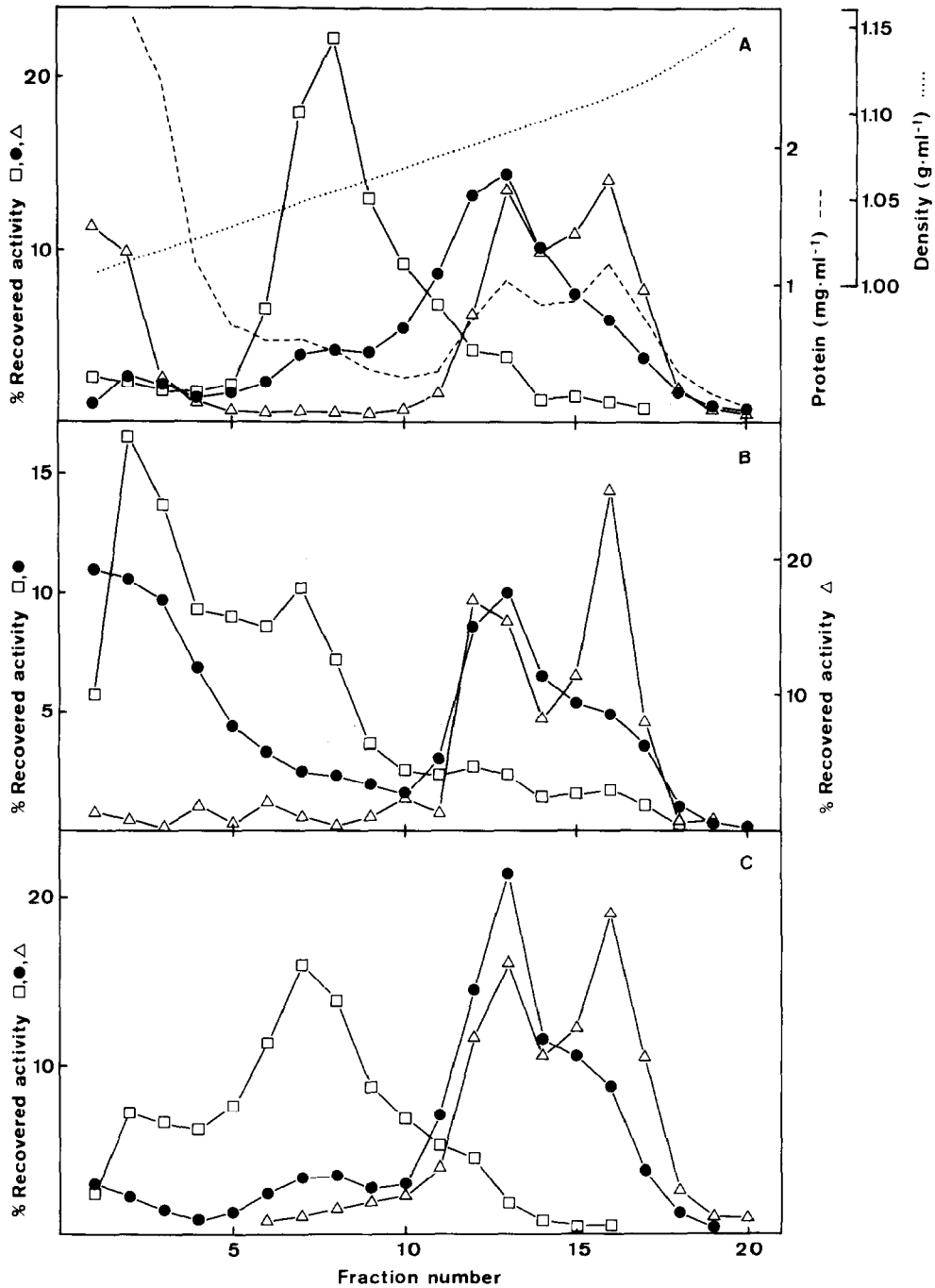

Fig.1. Distribution of enzymes of the phosphoinositide turnover cycle and various marker enzymes. The distribution of marker enzymes: (A) □, galactosyltransferase, ●, alkaline phosphodiesterase I and △, arylesterase; (B) □, DAG kinase, ●, phosphoinositidase C and △, CTP:PA cytidylyltransferase; (C) □, PI kinase, ●, PIP kinase and △, PI synthase. Recoveries of the enzyme activities in Nycodenz fractions were above 80% of those applied on the gradient, except for cytidylyltransferase which was ~40%. Recoveries in the 4400 × *g* supernatant applied on the gradient was above 95% compared to the homogenate, whereas more than 80% of mitochondrial succinate dehydrogenase sedimented. The specific activities of enzymes applied on the gradient were: galactosyltransferase, 62.5 pmol/min per mg; alkaline phosphodiesterase I, 54.1 nmol/min per mg; arylesterase, 1.20 μmol/min per mg; DAG kinase, 52.3 pmol/min per mg; phosphoinositidase C, 1.11 nmol/min per mg; CTP:PA cytidylyltransferase, 275 pmol/min per mg; PI kinase, 137 pmol/min per mg; PIP kinase, 10.3 pmol/min per mg; PI synthase, 43.7 pmol/min per mg. The figure shows a representative separation of 3 in which all components were analyzed. All enzymes, except cytidylyltransferase, have been monitored in 5 or more separations with essentially the same results. For further details, see section 2.

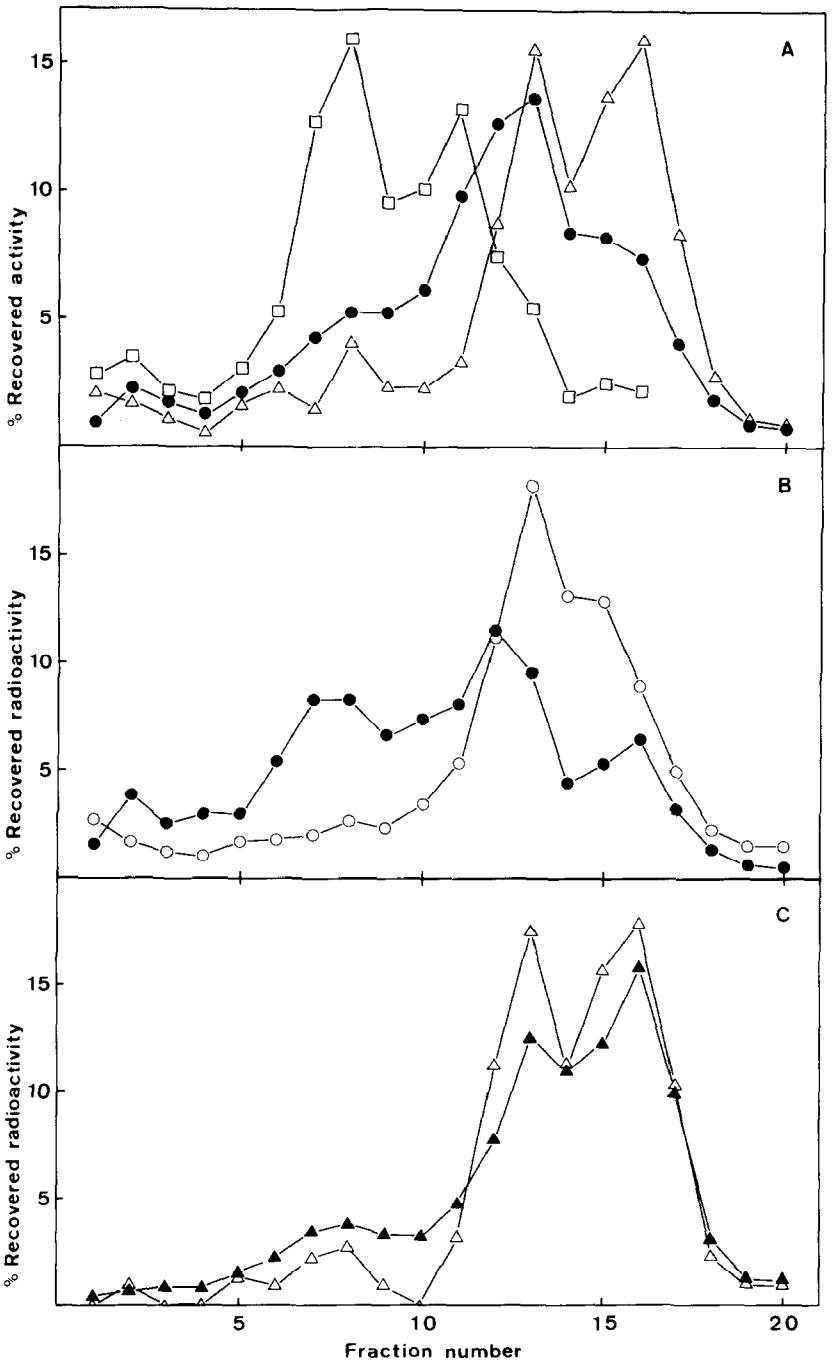

Fig.2. Subcellular distribution of phosphorylated intermediates of the phosphoinositide turnover cycle. Hepatocytes were prepared, incubated with [^{32}P]P_i for 10 min and processed as described under section 2. Distribution of marker enzymes: (A) □, galactosyltransferase, ●, alkaline phosphodiesterase I and △, NADPH-cytochrome *c*-reductase; (B) ●, PIP and ○, PIP_2; (C) ▲, PA and △, PI. The amount of label of intermediates applied on the gradient was: PIP, 3820 cpm; PIP_2, 5610 cpm; PA, 5890 cpm; for PI 950 cpm. Recoveries in the fractions were above 75% of the radioactivity applied, except for PI which was 55%. The figure shows a representative separation of 4.

with the plasma membrane marker, whereas soluble enzyme was recovered in the top fractions.

Membrane-bound DAG kinase coincided with PI kinase in a slightly lower density than the Golgi marker (fig.1B), with additional soluble activity in the top fractions. Both cytidylyltransferase and PI synthase (fig.1B,C) showed the same bimodal distribution as the endoplasmic reticulum marker.

3.3. *Subcellular distribution of inositol phospholipids*

The subcellular distribution of phosphorylated intermediates of the inositol phospholipid turnover pathway was examined after incubation of hepatocytes with [^{32}P]P_i (fig.2). PIP was observed together with PI kinase in the vicinity of the Golgi marker and in a less dense region of the plasma membrane peak not coinciding with PI kinase activity. Interestingly, PIP_2 was reproducibly found in a denser region of the plasma membrane peak than PIP, overlapping PIP kinase (cf. fig.1C). PI and PA were recovered in two peaks coincident with the endoplasmic reticulum marker NADPH-cytochrome *c*-reductase, which had the same distribution as arylesterase.

4. DISCUSSION

The use of Nycodenz gradient centrifugation has allowed us to examine the subcellular distribution of enzymes in the phosphoinositide turnover cycle in greater detail than has been done before. Both the distribution of these enzymes and phosphorylated intermediates support the conclusion that, in rat hepatocytes, the synthesis of PI takes place in the endoplasmic reticulum and that its phosphorylation to PIP occurs intracellularly before it has been translocated to the plasma membrane. There PIP is further phosphorylated to PIP_2 before hydrolysis by phosphoinositidase C in an event triggered by signal substances. An intracellular formation of PIP implies a vesicular transport of PIP to the plasma membrane as transport proteins for polyphosphoinositides have not been identified.

The PIP consistently recovered in the plasma membrane region (4 separations) did not exactly overlap PIP_2 or PIP kinase. A tentative explanation is the presence of two regions of the plasma membrane (assuming that alkaline phosphodiesterase I activity equates with plasma membrane), and that PIP enters one of them, then translocates to a second region, where it is rapidly phosphorylated to PIP_2.

From a functional point of view it is thought that PI kinase should, at least in part, reside in the plasma membrane [2]. Our results show clearly that this is not the case, at least in unstimulated hepatocytes. Instead, the enzyme sedimented with material of low density. Although we have earlier reported a Golgi location of PI kinase [5], this seems now less certain because of the consistent retrieval of the kinase at a lighter density than the trans-Golgi marker galactosyltransferase. In addition, these enzymes separated nearly completely in a modified Nycodenz gradient. Also, our results do not support the lysosomal location of PI kinase reported [20]; the lysosomal marker acid phosphatase sedimented in a region of higher density than the Golgi marker with little PI kinase activity (not shown). If the enzyme does not reside in a Golgi fraction other than trans-Golgi, it may be present in vesicles involved in membrane trafficking, earlier shown to have densities lighter than that of Golgi membranes [21,22]. The finding of PI kinase in chromaffin granules [23,24] and endocytic vesicles [25] also suggests that the enzyme is present in transport vesicles.

DAG kinase has been identified in cytosol and endoplasmic reticulum [26]. In addition, the enzyme is expected to be found in the plasma membrane regulating the second messenger function of DAG [27]. Our results, using exogenous substrate, show that the membrane-bound enzyme is located preferentially in light-density membranes rather than in the endoplasmic reticulum, and that practically no activity is associated with plasma membranes. Clearly, the subcellular localization of DAG kinase, and its physiological implications, merits further examination.

PI synthase sedimented with the endoplasmic reticulum marker supporting the contention that de novo synthesis of PI occurs there [2]. We did not distinguish distinct PI synthases in plasma membranes and endoplasmic reticulum, contrary to GH_3 cells [4]. Thus, the regeneration of polyphosphoinositides in hepatocytes is dependent on intracellular enzymes. As PI kinase is located in low-density membranes, it will be of interest to examine whether PI is phosphorylated to PIP prior to, or

during, transport to the plasma membrane and, if so, whether phosphorylation is coupled to the transport process.

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