Metabolism and possible compartmentalization of inositol lipids in isolated rat-liver nuclei

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(1) The removal of the nuclear envelope from isolated rat-liver nuclei by washing with Triton X-100 (TX-100) was assessed by electron microscopy. All the envelope was removed by 0.04 % (w/v) TX-100. (2) After this removal, phosphorylation of inositol lipids and diacylglycerol (DAG) from [γ -³²P]ATP still occurs, despite the near complete absence of detectable (by mass assay) DAG and PtdIns. This suggests that the majority of these two lipids in nuclei are present in the nuclear membrane, but the small amounts remaining after extraction, defined as intranuclear, are available for phosphorylation by lipid kinases (36 % for DAG and 24 % for PtdIns respectively, when expressed as a percentage of incorporation of intact nuclei). (3) PtdIns(4,5) P_2 did not follow the same pattern as PtdIns and DAG; after removal of the nuclear membrane, 40 % of the mass of this lipid was left in the nucleus. Moreover, a similar amount of PtdIns(4,5) P_2 was also resistant to extraction with even higher concentrations of detergent, suggesting that PtdIns(4,5) P_2 has a discrete intranuclear location, probably bound to nuclear proteins. (4) Addition of exogenous substrates, PtdIns, PtdIns(4) P and DAG, to membrane-depleted nuclei resulted in reconstitution of the majority of lipid phosphorylations from [γ -³²P]ATP (70 %, 90 % and 94 % of intact nuclei respectively),

suggesting a predominantly intranuclear location for the respective kinases. (5) Nuclei also showed phosphomonoesterase and phosphatidic acid hydrolase activity; dephosphorylation of pre-radiolabelled PtdIns(4) P , PtdIns(4,5) P_2 and phosphatidic acid was observed when [γ -³²P]ATP was removed. However, some of the radioactivity was apparently resistant to these enzymes, suggesting the existence of multiple pools of these lipids. (6) Addition of excess non-radiolabelled ATP to nuclei pre-labelled with [γ -³²P]ATP resulted in an initial increase in the label in PtdIns(4,5) P_2 , implying a precursor–product relationship between the radiolabelled pools of PtdIns(4) P and PtdIns(4,5) P_2 . This was confirmed by analysis of the incorporation of ³²P into the 4'-phosphate group of PtdIns(4) P and the individual 4'- and 5'-phosphate groups of PtdIns(4,5) P_2 . The data from these experiments also indicated that PtdIns(4,5) P_2 can be produced from a pre-existing pool of PtdIns(4) P , as well as *de novo* from PtdIns. (7) Taken together our data suggest that isolated rat-liver nuclei have an intranuclear inositol lipid metabolism mechanism utilizing enzymes and substrates equivalent to those found in cytosol and plasma membrane, and that there may be some, but not complete, compartmentalization of the components of the nuclear inositol cycle.

INTRODUCTION

Receptor-agonist initiation of the plasma-membrane inositol cycle, leading to the production of the second messengers Ins(1,4,5) P_3 and 1,2-diacylglycerol (DAG), is well established [1–3]. There is now considerable evidence that nuclear inositol lipids, discrete from those in the plasma membrane, are likely to be involved in the regulation of some as-yet-unknown aspect of nuclear function [4]. The precise location, compartmentalization and inter-relationships between the inositol lipid substrates and enzymes in the nucleus has yet to be explored in detail. In particular, the relative contribution of the nuclear envelope to nuclear inositol metabolism remains to be elucidated. Most studies of inositol lipids involving nuclei have required removal of the nuclear envelope before lipid analysis [5–7]. However, when Jarpe et al. [8] investigated the source of DAG generated from isolated, entirely intact nuclei, they concluded that phosphatidylcholine, rather than inositides, was the primary source. It is therefore possible that there exists more than one signalling system in nuclei for which DAG is a second messenger, and overall it is relevant to investigate the location of the inositol lipids and DAG and their associated kinases, as well as phos-

phatidylcholine, by investigating how much of these remain in nuclei after removal of the nuclear membrane by detergent.

Only two laboratories [9,10] have provided any topographical insight into nuclear inositides. Payrastré et al. [9] showed that membrane-depleted nuclei could be further sub-fractionated into peripheral and internal matrices, the former fraction being rich in PtdIns 4-kinase, and the latter in PtdIns(4) P 5-kinase and phosphoinositidase C. In this study we have sought to clarify whether inositol lipid metabolism, determined by the incorporation of γ -³²P into PtdIns(4) P , PtdIns(4,5) P_2 and PtdOH (through the phosphorylation of their precursors PtdIns, PtdIns(4) P and DAG respectively), occurs within the nuclear matrix, the nuclear envelope or both. We have determined the mass levels of phospholipids and DAG in nuclei, both intact and with membranes removed. Furthermore, we have investigated any possible links between the pools of lipids, and the respective kinases and phosphatases metabolizing them.

MATERIALS AND METHODS

Radioisotopes were supplied from Amersham, all other reagents were of analytical grade.

Abbreviations used: DAG, diacylglycerol; TX-100, Triton X-100; PtdOH, phosphatidic acid.

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Purification of rat-liver nuclei

The procedure used was an adaptation of a previous method [11]. The livers from two male Wistar rats were collected on ice, washed twice in solution B [10 mM Hepes (pH 7.5)/5 mM MgCl_2 /25 mM KCl] and 14 g (wet wt.) was homogenized in 28 ml of the same solution using a power-driven pestle. To this was added 4.9 ml of solution C [10 mM Hepes (pH 7.5)/2 mM MgCl_2 /2.4 M sucrose], to give a final concentration of sucrose of 0.25 M. This was then mixed by inversion with solution D [10 mM Hepes (pH 7.5)/2 mM MgCl_2 /2.3 M sucrose; 90 ml]; this gave a final sucrose concentration of 1.62 M.

A 7.5 ml cushion of solution D was then layered carefully below 23 ml of the final (1.6 M sucrose) liver homogenate, in polypropylene centrifuge tubes. The samples were then spun at 106000 *g* for 30 min at 4 °C in a Beckman SW27 rotor. The pellet at the bottom of the cushion was considered to be the nuclear fraction and was vigorously resuspended in 5 ml of solution A [10 mM Hepes (pH 7.5)/2 mM MgCl_2 /0.25 M sucrose]. The fractions were then pooled and washed twice with 25 ml of solution A before being pelleted at 165 *g* for 6 min at 4 °C and finally being resuspended to 4 ml in this solution.

Preparation of membrane-depleted nuclei

A quantitative removal of the nuclear envelope was performed by titration with the non-ionic detergent Triton X-100 (TX-100). Briefly, a 0.5 ml aliquot of nuclei in solution A was mixed with 20 ml of ice-cold final resuspension buffer [5 mM Tris (pH 7.4)/5 mM MgCl_2 /1.5 mM KCl/290 mM sucrose/1 mM EGTA], and left for 20 min. TX-100 was added to this buffer where required before addition of the nuclei to yield final concentrations of between 0.01 and 1.0 % (w/v).

The nuclei were pelleted at 165 *g* for 6 min (4 °C) and the supernatant removed. The pellet was carefully resuspended in solution A (5 ml), and a cushion carefully laid beneath it [10 mM Hepes (pH 7.5)/2 mM MgCl_2 /0.5 M sucrose; 10 ml], and centrifuged at 165 *g* for 6 min (4 °C). This was assumed to remove any residual TX-100. The supernatant was removed and the final pellet resuspended in 0.5 ml of solution A. Protein estimations were performed using the Bio-Rad protein assay. From two rat livers approx. 20 mg of nuclear protein was recovered before depletion of the nuclear envelope.

Preparation of nuclei for electron microscopy

The method was adapted from that of Wooding et al. [12]. Nuclei (total protein 200 μg) were pelleted in a bench-top microfuge for 1 min. The pellet of nuclei was resuspended in an equal volume of fixative buffer [50 mM Tris/140 mM NaCl with 4 % (w/v) glutaraldehyde and 2 % (w/v) sucrose] and left for 20 min. The sample was once again spun as above. The supernatant was removed and the pellet surface washed gently with 50 mM Tris (pH 7.5)/140 mM NaCl. After 5 min the supernatant was removed, 1 ml of osmium ferrocyanide was added and it was then left for 1 h. Samples were passed through water, 50 % (v/v) ethanol, 75 % ethanol and eosin, and washed twice with 100 % ethanol. In all of these steps samples were left for 10 min.

After these dehydration steps the samples were left overnight in Araldite/propylene oxide (2:1, w/v). Next day the samples were transferred into 100 % Araldite (10 ml of CY212 Resin/10 ml of DDSA Hardener/0.4 ml of DMP 30 Accelerator) and left for 2 h. The pellets were then transferred to embedding moulds with fresh Araldite and again left overnight. The resin was finally cured at 60 °C for 6–8 h.

Sections for electron microscopy were taken using a Reichert-

Jung Ultracut E microtome and picked up on naked 300-mesh nickel grids. The grids were washed with a jet of distilled water, then stained with saturated uranyl acetate in 50 % (v/v) ethanol and lead citrate for 10 min. The grids were washed again in a water jet and left to dry. The sections were examined by using a JEOL 100 C microscope operated at 80 kV.

In vitro labelling of poly(PtdIns) lipids with [γ - ^{32}P]ATP

Endogenous lipid

Nuclei (total protein 100 μg) were resuspended in 90 μl of final resuspension (above) on ice. The samples were preincubated for 2 min at 30 °C to hydrolyse any remaining endogenous ATP. Then 10 μl of phosphorylation mixture (20 μCi of [γ - ^{32}P]ATP, 2 μl of 5 mM non-radiolabelled ATP, made up to 10 μl with final resuspension buffer) was added. The incorporation of ATP was stopped by the addition of 1 ml of chloroform/methanol (1:1, v/v) containing internal lipid standards (approx. 2 μg of phosphorus/ml).

To remove ATP from the incubations (and so to reveal phosphatase activities), hexokinase (20 units; Sigma) and glucose (final concentration 10 mM) were added to the incubations. The addition of 10 μl of 50 mM ATP to incubations was used to reduce the specific activity of the radiolabelled ATP during the chase experiments.

Exogenous lipid

For exogenous lipid, radiolabelling was carried out as above, except nuclei were resuspended in 40 μl of final resuspension buffer and made up to 90 μl with lipid vesicles. These consisted of PtdIns, PtdIns(4)*P*, DAG and phosphatidylserine, dried under a stream of N_2 , then resuspended in 50 μl of final resuspension buffer (final concentrations of lipids are 50 mM for all lipids except phosphatidylserine which is 100 mM). The vesicles were formed by sonication of the lipid mixture 3 times for 10 s on ice with a Heatsystems W-225 sonicator probe [9].

Identification of inositol lipids

The lipids were extracted as in ref. [13] and acidified as in ref. [5]. Phosphoinositides and phosphatidic acid (PtdOH) were separated by TLC on silica-gel-coated plates, pre-coated in 1 % (w/v) oxalate, then dried at 110 °C for 1 h. Resolution of the inositol lipids was achieved with the solvents chloroform/methanol/water/ammonia (45:35:8:2, by vol.). The plates were exposed to autoradiography, and then iodine to detect internal lipid standards. The radiolabelled lipids were scraped from the plate and counted by scintillation counting.

Determination of ATP hydrolysis during incubation with nuclei

Reactions were stopped with 750 μl of chloroform/methanol (1:2, v/v). Aliquots (2 μl) were taken and spotted on to a poly(ethyleneimine)-cellulose plate (Sigma), and run in 1 M phosphoric acid/KOH (pH 3.8) to separate ATP, ADP and P_i .

Mass determination of nuclear phospholipids and DAG

PtdIns(4,5) P_2

This lipid was quantified as described in ref. [5], by deacylating and deglycerating the lipid extract so that PtdIns(4,5) P_2 was converted into Ins(1,4,5) P_3 and then the latter was measured by

a specific binding assay using bovine adrenal $\text{Ins}(1,4,5)\text{P}_3$ receptors [14].

DAG mass assay

The method was adapted from that used previously [5]. Briefly, 200 μg of nuclear protein was mixed thoroughly with 0.75 ml of methanol/chloroform (2:1, v/v) and final resuspension buffer to a volume of 0.95 ml. The tubes used for this stage were thin-walled autoclaved glass, approx. 10 ml in volume.

The tubes were capped and stored at -20°C overnight, or for 1 h at room temperature, and the DAG was extracted by addition of 0.25 ml of chloroform and 0.25 ml of autoclaved water. The samples were vortex-mixed and centrifuged at 165 g for 5 min (20°C). This excluded the DAG and phospholipids to the lower (chloroform) phase, which was then carefully removed to fresh autoclaved tubes. The sample was then dried in a vacuum-assisted centrifuge at 30°C (Gyrovap GL; Philip Harris Scientific, Lichfield, Staffs.).

Approx. 200 μl of neomycin-linked glass beads (mesh size 200–400; Sigma), suspended in chloroform/methanol (1:1, v/v), were used to remove the phospholipids from the sample (phospholipids inhibit DAG kinase under these conditions). The lipid samples (resuspended in 0.5 ml of chloroform) and a further 0.5 ml chloroform rinse of the tubes, were combined with the beads in 2 ml autoclaved microfuge tubes. The tubes were vortex-mixed and left for 15 min, then centrifuged at 15000 g for 3 min. Then 900 μl of the supernatant was removed into fresh autoclaved microfuge tubes and dried under vacuum. The samples had 20 μl of 9.2 mg/ml CHAPS in DAG kinase buffer (50 mM Tris/acetate/80 mM KCl/10 mM magnesium acetate/2 mM EGTA) added to them and they were left for 5 min. Then a further 150 μl of DAG kinase buffer was added to the samples and the tubes were briefly probe-sonicated on ice, vortex-mixed and centrifuged at high speed for 10 s.

The reaction to convert DAG into PtdOH, the basis of the mass determination, was initiated by the addition of 20 μl of DAG kinase from rat brain [15], 1 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 0.8 μl of 5 mM ATP (final concentration 20 μM) and made up to a final volume of 200 μl with DAG kinase buffer. The reaction was allowed to proceed for 90 min and was stopped by the addition of 1 ml of chloroform/methanol (1:1, v/v) that also included PtdOH as a carrier (2 μg of phosphorus/sample). The PtdOH was extracted by means of an acid Bligh and Dyer phase split [13]. The lower phase was dried and resolution achieved by TLC on silica-gel-coated plates [sprayed with 1 mM EDTA (pH 5.5)/methanol (1:1, v/v), then dried for 1 h at 110°C], using chloroform/methanol/water/acetic acid (65:50:4:1, by vol.) as the developing solvent. The plates were autoradiographed before exposure to iodine to detect internal lipid standards. The radiolabelled PtdOH was scraped from the plate and counted by scintillation counting.

Phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine mass assay

The procedure was to separate these from each other by TLC, and then to quantify them by perchloric acid digestion to P_i , followed by phosphate measurement, exactly as described in ref. [16].

Determination of specific radioactivities of the individual phosphate groups in the 4'- and 5'-positions of the inositol ring of $\text{PtdIns}(4,5)\text{P}_2$

Lipids extracted from nuclei that were radiolabelled by in-

cubation with 80 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (as above) were deacylated and deglycerated [5]. The radiolabelled $\text{Ins}(1,4,5)\text{P}_3$ in the resulting extracts was purified by HPLC [17], desalted by passing through Dowex beads and the $\text{Ins}(1,4,5)\text{P}_3$ eluted with formate/formic acid as described previously [18], and freeze-dried. It was then analysed essentially as described by Hawkins et al. [19], who employed the InsP_3 5-phosphatase in human erythrocyte ghosts to analyse the 4- and 5-phosphate moieties individually. $\text{Ins}(1,4,5)\text{P}_3$ was incubated for 30 min with erythrocyte ghosts [19] to hydrolyse the 5'-phosphate from the inositol ring, it was spiked with approx. 500 d.p.m. of $[\text{14C}]\text{Ins}(1,4)\text{P}_2$ as an internal marker, and the $[\text{32P}]\text{Ins}(1,4)\text{P}_2$ and $[\text{32P}]\text{P}_i$ were resolved by HPLC [17].

RESULTS

Characterization of the *in vitro* labelling of isolated nuclei

For many of the *in vitro* labelling assays, it was desirable that the experimental variables operated with first-order kinetics. Initially, radiolabelled ATP was incubated with varying amounts of nuclear protein, using intact nuclei (incubation for 5 min for all concentrations). Incorporation of the radiolabel was linear with up to 100 μg of protein for PtdIns and DAG phosphorylation (results not shown), and up to 200 μg for PtdIns(4,5) P_2 (results not shown). We therefore used 100 μg of nuclear protein for the incubations.

Incubation of 100 μg of nuclear protein with the radiolabel over a range of different times revealed that a 5 min incubation was close to first-order conditions for all three lipids, but declined subsequently (results not shown). Analysis of the nucleotides showed that at 5 min the level of ATP had fallen to 42% of its initial concentration. Moreover, the addition of a second dose of radiolabelled ATP after 5 min restored phosphorylation of the inositol lipids and DAG to a rate approaching first-order kinetics. Thus a decline in rate after 5 min was due to ATP consumption, not inactivation of kinases or depletion of lipid substrates.

Many procedures required the isolated nuclei to be viable for up to 20 min, so their incorporation was assessed after preincubation (without ATP) for a range of times. It was found that nuclei preincubated for 15 min had the same potential to incorporate radiolabelled ATP into PtdIns(4) P , PtdIns(4,5) P_2 and PtdOH as nuclei that had been subject to only 2 min of preincubation (results not shown). This indicated that nuclei maintain a similar biochemical activity with regard to incorporation of radioactivity into phospholipids over the time course of these experiments.

Electron-microscopic analysis of nuclei

Central to this study was the quantitative removal of the nuclear envelope with low concentrations of TX-100, followed by quantification of lipid phosphorylation. The removal was assessed by visualizing the nuclei, before and after treatment with detergent, by electron microscopy. The isolated control nuclei (those not washed with TX-100) could be clearly seen to possess an intact nuclear envelope consisting of the characteristic double membrane (Figure 1a); examination at a lower-power magnification revealed no evidence of contamination by non-nuclear membranes such as endoplasmic reticulum (results not shown). A high proportion of the double membrane was solubilized when isolated nuclei were washed with 0.02% TX-100 (Figure 1b), with only small fragments of the inner and outer membranes remaining. Treatment with 0.04% of the detergent completely removed the nuclear envelope to leave the naked laminar layer and nuclear pore remnants (Figure 1c). Three separate nuclear

Figure 1 Electron micrographs of nuclear preparations

(a) Control rat-liver nuclei. The double membrane (DM) surrounds the nucleus, with no visible contamination with either cytoplasmic or endoplasmic reticulum membranes. (b) Treatment of the nuclei with 0.02% TX-100 results in solubilization of the majority of the nuclear envelope, with only remnants of the inner membrane (IM) remaining. (c) An increase in the TX-100 concentration to 0.04% results in the complete removal of both leaflets of the double membrane, revealing the lamellar layer (X) and nuclear pore remnants (N).

Table 1 Phospholipid mass measurements from isolated rat-liver nuclei

A comparison between control, intact nuclei and those washed with the detergent TX-100 at three different concentrations is shown. Measurements are taken from at least three independent experiments and expressed as a percentage of the intact control nuclei $\pm$ S.E.M. Typical absolute values for control (washed with buffer containing no TX-100) nuclei are: total phospholipid, 66 nmol/mg of protein; PtdIns, 3.2 nmol/mg of protein; DAG, 139 pmol/mg of protein; phosphatidylcholine (PtdCho), 21 nmol/mg of protein; PtdIns(4,5) P_2 , 80 pmol/mg of protein. N.D., not detectable to a significant level by the method used.

TX-100 (% w/v) ...		Lipid remaining (%)			
		0	0.02	0.04	0.08
Total phospholipid	100	32 $\pm$ 10	2 $\pm$ 2	N.D.	
DAG	100	7 $\pm$ 3	7 $\pm$ 3	5 $\pm$ 2	
PtdCho	100	50 $\pm$ 17	18 $\pm$ 10	6 $\pm$ 9	
PtdIns	100	64 $\pm$ 23	N.D.	N.D.	
PtdIns(4,5) P_2	100	79 $\pm$ 23	40 $\pm$ 9	35 $\pm$ 9	

preparations treated with 0.04% TX-100 were examined, and more than 100 randomly selected nuclei were examined in each case. None showed any recognizable unit membrane structure,

even when the potential visibility of this was enhanced by tannic acid pre-treatment before the osmium fixation.

Changes in mass levels of phospholipids in membrane-depleted nuclei

The mass of total phospholipid fell to less than 3% of the control value, in nuclei washed with 0.04% TX-100 (Table 1). Under the same conditions, the mass of PtdIns followed a similar profile, but as it fell to a level close to the limit of detection of the assay used, we could not quantify how much remained at 0.04% TX-100. DAG (Table 1) was clearly different from these two phospholipids in that it fell to 7% of the control value when the nuclei were treated with only 0.02% of the detergent; this residual mass of DAG then remained almost constant even when the nuclei were treated with much higher concentrations of TX-100. The mass of phosphatidylcholine followed a profile similar to total phospholipids, and approx. 18% of the control level of this lipid remained once the double membrane had been removed. The only lipid whose mass did not fall dramatically after removal of the nuclear envelope was PtdIns(4,5) P_2 ; 40% of the control value remained after treatment with 0.04% TX-100 (Table 1), and further increases in the concentration of TX-100 applied to

Figure 2 An autoradiograph of a TLC plate of a typical experiment showing the incorporation of radiolabel from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into phospholipids of nuclei pre-washed with different concentrations of TX-100

The odd-numbered lanes are from nuclei incubated with no added substrate, and the even-numbered lanes are from nuclei that had an exogenous mixture of phospholipids (see the Materials and methods section) added to them along with the radiolabelled ATP. Lane 1, control nuclei; lane 2, control nuclei with exogenous substrate; lane 3, 0.02% TX-100; lane 4, 0.02% TX-100 and exogenous substrate; lane 5, 0.04% TX-100; lane 6, 0.04% TX-100 and exogenous substrate; lane 7, 0.08% TX-100; lane 8, 0.08% TX-100 and exogenous substrate; lane 9, 0.1% TX-100; lane 10, 0.1% TX-100 and exogenous substrate; lane 11, 1.0% TX-100; lane 12, 1.0% TX-100 and exogenous substrate.

the nuclei resulted in little further loss in the mass of this lipid [mass of $\text{PtdIns}(4,5)\text{P}_2$ in nuclei treated with 0.08% TX-100 was 35% of control].

Biochemical changes in inositol lipid turnover in membrane-depleted nuclei

Isolated nuclei are able to incorporate phosphate from ATP into nuclear inositol lipids and PtdOH [7]. It is not clear whether the lipid precursors for this phosphorylation are provided by the nuclear envelope or by the internal matrix of the nucleus. To investigate this we have used the strategy of removing the nuclear envelope, measuring the change in the masses of the various lipid precursors (see also Table 1), and comparing these numbers with the ability of the nuclei to phosphorylate the remaining precursors to their respective products $\text{PtdIns}(4)\text{P}$, $\text{PtdIns}(4,5)\text{P}_2$ and PtdOH (see odd-numbered lanes of Figure 2, and Table 2).

Intact nuclei (control) incorporated radiolabelled phosphate into $\text{PtdIns}(4)\text{P}$, $\text{PtdIns}(4,5)\text{P}_2$ and PtdOH , as revealed by autoradiography of resolved TLC plates (Figure 2, lane 1). However, the level of incorporation fell as the nuclei were washed with an increasing concentration of TX-100. The crucial observation is that when nuclei were washed with 0.04% TX-100 (Figure 2, lane 5), the concentration at which the nuclear envelope was

Figure 3 Reconstitution of lipid phosphorylation by endogenous lipid kinases in TX-100-washed nuclei when supplied with exogenous lipid substrates, compared with the incorporation of radiolabel from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ occurring in TX-100-washed nuclei not supplied with exogenous substrate

An exemplar of raw data from such an experiment is shown qualitatively in Figure 2, and the data are the means of duplicate samples from a typical experiment, quantified by scraping the radioactive spots from the TLC plate followed by scintillation counting. PtdOH produced from exogenous substrate ($\square$); $\text{PtdIns}(4)\text{P}$ produced from exogenous substrate ($\triangle$); $\text{PtdIns}(4,5)\text{P}_2$ produced from exogenous substrate ($\circ$). PtdOH produced from endogenous substrate ($\blacksquare$); $\text{PtdIns}(4)\text{P}$ produced from endogenous substrate ($\blacktriangle$); $\text{PtdIns}(4,5)\text{P}_2$ produced from endogenous substrate ($\bullet$).

removed, significant incorporation of the radiolabel into $\text{PtdIns}(4)\text{P}$, $\text{PtdIns}(4,5)\text{P}_2$ and PtdOH still occurred, at 24%, 18% and 36% of control values respectively (Table 2). We interpret these data to suggest that there exists, within the intranuclear region, PtdOH , $\text{PtdIns}(4)\text{P}$ and DAG, available for phosphorylation.

Enzyme and substrate availability in membrane-depleted nuclei

The above data did not indicate to what degree the reduction in radiolabelling of the lipids was due to loss of lipid substrate (from the nuclear membrane or the nuclear matrix) or to loss of the enzymes that phosphorylate them, PtdIns 4-kinase, $\text{PtdIns}(4)\text{P}$ 5-kinase and DAG kinase.

To address this question exogenous lipid substrates, in the form of vesicles [9], were supplied to both intact and membrane-depleted nuclei (even-numbered lanes in Figure 2). As previously demonstrated, nuclei depleted of their membranes by 0.04%

Table 2 A comparison of the mass levels of precursor lipids DAG and PtdIns in control and detergent-washed nuclei

Data are from experiments exemplified by those in Table 1. The concomitant availability of these lipids for phosphorylation to their respective products PtdOH and $\text{PtdIns}(4)\text{P}$ is shown. Data for $\text{PtdIns}(4,5)\text{P}_2$ mass levels are also shown. Results are taken from four experiments exemplified by those in Figure 2 (odd-numbered lanes) and in Figure 3 (solid symbols). N.D., not detectable to a significant level by the method used; N.A., not assayed.

Concn. of TX-100 (%, w/v)	DAG mass (% of control)	$[\text{}^{32}\text{P}]\text{PtdOH}$ (% of control)	PtdIns mass (% of control)	$[\text{}^{32}\text{P}]\text{PtdInsP}$ (% of control)	$[\text{}^{32}\text{P}]\text{PtdInsP}_2$ (% of control)
0	100	100	100	100	100
0.02	7 $\pm$ 3	65 $\pm$ 23	54 $\pm$ 15	74 $\pm$ 4	46 $\pm$ 5
0.04	7 $\pm$ 3	36 $\pm$ 9	N.D.	24 $\pm$ 7	18 $\pm$ 1
0.08	5 $\pm$ 0	8 $\pm$ 1	N.D.	6 $\pm$ 3	13 $\pm$ 7
0.1	N.A.	9 $\pm$ 3	N.D.	4 $\pm$ 2	10 $\pm$ 6

Figure 4 Radiolabel in (a) PtdOH, (b) PtdIns(4)P and (c) PtdIns(4,5)P₂ in nuclei prelabelled for 5 min with [γ -³²P]ATP

Prelabelling was followed by: no additions (●), addition of non-radiolabelled 5 mM ATP (■), or hexokinase and glucose (▲). Data are expressed as a percentage of the label at the end of the 5 min prelabelling period; $n = 2$.

TX-100 (Figure 2, lane 5) showed a concomitant decrease in incorporation of the radiolabel into the lipids. However, addition of exogenous lipid substrate to these nuclei (Figure 2, lane 6) resulted in the reconstitution of incorporation into PtdIns(4)P, PtdIns(4,5)P₂ and PtdOH to the levels of 70%, 90% and 94% respectively, when compared with intact nuclei also incubated with exogenous substrates (Figure 2, lane 2). Indeed, even in those nuclei washed with 0.1% TX-100 and then incubated with exogenous lipid, the activity was reconstituted to 64%, 76%, 83% of the control values for these lipids (Figure 2, lane 10). The contrast in radiolabelling of the lipids in nuclei with and without exogenous substrates is clearly seen in the quantification shown in Figure 3. Even nuclei washed with as much as 1.0% detergent (Figure 2, lane 12) exhibited little change in their ability to phosphorylate exogenous PtdIns(4)P and DAG. However, a significant reduction in PtdIns phosphorylation was seen in nuclei treated with this concentration of TX-100, with only 13% of the control value remaining (see also Figure 2, lane 12). This

may be significant in view of data from Payraastre et al. [9], who demonstrated that PtdIns 4-kinase is prevalent in the periphery of the nuclear matrix, and therefore is perhaps more susceptible to detergent extraction than PtdIns(4)P 5-kinase and DAG kinase, which are suggested by Payraastre et al. [9] to be located in the internal matrix of the nucleus.

Product-precursor relationship between PtdIns(4)P and PtdIns(4,5)P₂

Synthesis of PtdIns(4,5)P₂ from a pre-existing pool of PtdIns(4)P

Resolution of the incorporated radiolabelled phosphate into the 4'- and 5'-positions of the inositol ring of PtdIns(4,5)P₂ (see the Materials and methods section) revealed that when intact nuclei were incubated with [γ -³²P]ATP for 2 min, radiolabel was not distributed equally between the two positions. Indeed, in a typical experiment (one of three) a ratio of 1.8:1 in favour of

counts incorporated into the 5'-position was observed at this time point. This ratio decreased with time, so that after 10 min of incubation, the ratio had fallen to 1.6:1 in favour of the 5'-position, and by 30 min to 1.2:1. These data suggest that some of the PtdIns(4)*P* substrate for PtdIns(4)*P* 5-kinase was unlabelled and therefore already present as a pool in the isolated nuclei (see also [7]). However, increasingly with time, radiolabelled PtdIns(4)*P* (synthesized during the incubation from PtdIns) was apparently used as a substrate by the PtdIns(4)*P* 5-kinase, probably as the pre-existing pool of PtdIns(4)*P* was depleted.

Evidence for phosphoinositide monoesterases and a PtdOH hydrolase in the nucleus

Addition of non-radiolabelled ATP (which reduced the specific activity of the radiolabelled ATP), or hexokinase and glucose (which removed any remaining ATP from the incubation) to pre-radiolabelled nuclei resulted in the displacement of ³²P from the pre-radiolabelled lipids.

Addition of hexokinase and glucose to pre-radiolabelled nuclei resulted in all free ATP being consumed in under 15 s (results not shown). The result of this was a subsequent reduction in the counts in PtdIns(4)*P*, PtdIns(4,5)*P*₂ and PtdOH as they were dephosphorylated to their respective products PtdIns, PtdIns(4)*P* and DAG by PtdIns(4)*P* monoesterase, PtdIns(4,5)*P*₂ monoesterase and PtdOH hydrolase respectively (Figure 4, triangles). However, after 5 min, counts were no longer removed from the first two of these lipids and at a much reduced rate from PtdOH.

Addition of excess non-radiolabelled ATP ensured that ATP was still available to the PtdIns lipid kinases. However, the specific activity of the radiolabelled ATP was significantly reduced, and little more radiolabelled ATP could be incorporated into the lipids. As with the addition of hexokinase, counts in PtdIns(4)*P* and PtdOH were reduced because the radiolabel was removed from these lipids as they were dephosphorylated to their respective precursors (Figures 4a and 4b, squares). However, when the radiolabel in PtdIns(4,5)*P*₂ was analysed after addition of the excess non-radiolabelled ATP, there was an increase in the counts detected in this lipid for up to 10 min, after which they returned to control levels (Figure 4c, squares). We interpret this as a mass action effect, whereby the high ATP concentration drives PtdIns(4)*P* to be phosphorylated to PtdIns(4,5)*P*₂.

DISCUSSION

The study of inositol lipids in the nucleus has been significantly hampered by the fact that we are not sure of their distribution within the organelle, and in particular what proportion exists in the nuclear envelope.

Most of the data collected to date by ourselves and others [5–7,9] have utilized nuclei that have been stripped of their envelopes by detergents. Moreover, we have so far assumed that nuclear inositides are the major source of the DAG generated in this location, which in turn probably serves to recruit protein kinase C to the nucleus [5] and activate it. However, Jarpe et al. [8] found by analysis of DAG species, that DAG produced by nuclei in thrombin-stimulated cells was predominantly derived from phosphatidylcholine. The nuclei they prepared still had their envelopes intact, and our data (Table 1) clearly suggest that the great majority of DAG and phosphatidylcholine may be located in the envelope. This leads us to suggest that Jarpe et al. [8] may be studying an entirely different signalling system, possibly located in the endoplasmic reticulum as well as in the nuclear membrane. It is relevant to note, however, that intra-

nuclear phosphatidylcholine was still at 18 % of the control value when the nuclear membrane was removed, and this raises the possibility that non-membranous intranuclear phosphatidylcholine contributes to intranuclear DAG production. The demonstration that there is an intranuclear phosphatidylcholine pool is also of some interest in the context of the demonstration that phospholipase A₂ exists in the nucleus [20]. The detergent-resistant pools of DAG and inositides that were demonstrated and inferred in these experiments, and which we have presumably been measuring in our previous experiments (e.g. [5–7]), are most likely to be truly intranuclear, probably attached to the nuclear matrix or laminar layer in a manner similar to the attachment of inositides to the cytoskeleton [21].

We have also studied here some aspects of inositol lipid synthesis and turnover by radiolabelling intact nuclei with [γ -³²P]ATP (in the presence of EGTA so that phosphoinositidase C activity does not complicate the interpretation of the data). The incorporation of radiolabel reveals that the PtdIns(4)*P* [used as a substrate by PtdIns(4)*P* 5-kinase] is a mixture of some that is already present in the nucleus and (increasingly with time) some that is synthesized during the incubation (see the Results section). Payraastre et al. [9] concluded that PtdIns(4)*P* kinase is in a different location in the nucleus from PtdIns(4)*P* 5-kinase (peripheral and internal matrices respectively), and if this is so, then our data suggest that at least some of the PtdIns(4)*P* synthesized by PtdIns(4)*P* kinase can rapidly transfer between the locations. Payraastre et al. [9] also found DAG kinase and phospholipase C activity in the internal matrix of the nucleus.

Our experiments cannot address the contribution, if any, of inositol lipids from the nuclear envelope to the turnover of this system within the nucleus, as we cannot quantify how much intranuclear lipid (i.e. that not in the envelope) is also removed by 0.04 % (w/v) TX-100. Nevertheless, the incorporation of radiolabel into the PtdIns lipids that persists after such treatment does indicate that significant phosphorylation of the lipids occurs in the absence of the nuclear membrane. We can say that for membrane-depleted nuclei, there is a minimum contribution of approx. 36 %, 24 % and 18 %, for DAG, PtdIns and PtdIns(4)*P* phosphorylation respectively, when compared with intact nuclei, and as nuclei stripped of their nuclear membranes have also lost > 90 % of the mass levels of PtdIns and DAG, a preferential usage of non-envelope (and presumably non-membranous) lipids by the kinases is implied.

The removal of radiolabelled ATP (Figure 4, triangles) demonstrated nuclear PtdOH, PtdIns(4)*P* and PtdIns(4,5)*P*₂ phosphatase activity, and this is in agreement with earlier findings [22]. The data again suggest some form of compartmentalization in pools, in so far as only a proportion of the radiolabelled PtdOH, and (in particular) PtdIns(4)*P* and PtdIns(4,5)*P*₂ are accessible to the phosphatases. This could be due to inactivation of the phosphatases during the incubation, but if not, then at least two pools of these lipids, with some slow transfer between them, is the most likely explanation. Interestingly, it was possible to chase the counts of radiolabel from PtdIns(4)*P* to PtdIns(4,5)*P*₂ by the addition of excess ATP (Figure 4c, squares). Reported *K_m* values for ATP of known PtdIns(4)*P* 5-kinases are 5–25 μ M (see e.g. [23,24]), so we can be reasonably confident that at 5 mM ATP a mass action effect could drive significant mass formation of PtdIns(4,5)*P*₂, but with comparatively little radioactive incorporation. If so, it indicates a link between pools of these two lipids [PtdIns(4)*P* and PtdIns(4,5)*P*₂]; a link that is supported by the data on the labelling of the 4'- and 5'-phosphates of PtdIns(4,5)*P*₂ (see the Results section).

In conclusion, although it is difficult to assess quantitatively how many pools of PtdIns lipids there are within the nucleus, and

how they interrelate, we believe that the present data do give us some insight into which enzymes and substrates are present and their location within the nucleus. Above all, our findings reinforce the suggestion that within the nuclei of eukaryotic cells are all the enzymes and substrates necessary to form an inositide signalling system that is entirely distinct from that located in the plasma membrane.

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REFERENCES

- Berridge, M. J. and Irvine, R. F. (1989) *Nature* (London) **341**, 197–205
- Hug, H. and Sarre, T. F. (1993) *Biochem. J.* **291**, 329–343
- Nishizuka, Y. (1988) *Nature* (London) **334**, 661–665
- Divecha, N., Banfic, H. and Irvine, R. F. (1993) *Cell* **74**, 405–407
- Divecha, N., Banfic, H. and Irvine, R. F. (1991) *EMBO J.* **10**, 3207–3214
- Banfic, B., Zizak, M., Divecha, N. and Irvine, R. F. (1993) *Biochem. J.* **290**, 633–636
- Cocco, L., Gilmour, R. S., Ognibene, A., Letcher, A. J., Manzoli, F. A. and Irvine, R. F. (1987) *Biochem. J.* **248**, 765–770
- Jarpe, M. B., Leach, K. L. and Raben, D. M. (1994) *J. Biol. Chem.* **269**, 526–534
- Payraastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J. and Van Bergen en Henegouwen, P. M. P. (1992) *J. Biol. Chem.* **267**, 5078–5084
- Capitani, S., Bertagonolo, V., Mazzoni, M., Santi, P., Previata, M., Antonucci, A. and Manzoli, F. A. (1989) *FEBS Lett.* **245**, 194–198
- Divecha, N., Rhee, S. G., Letcher, A. J. and Irvine, R. F. (1993) *Biochem. J.* **289**, 617–620
- Wooding, F. B. F., Chambers, S. G., Perry, J. S., George, M. and Heap, R. B. (1980) *Anat. Embryol.* **158**, 361–370
- Bligh, E. G. and Dyer, J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Palmer, S. and Wakelam, M. J. O. (1990) in *Methods in Inositide Research*, (Irvine, R. F., ed.), pp. 127–133, Raven Press Ltd, New York
- Divecha, N. and Irvine, R. F. (1990) in *Methods in Inositide Research*, (Irvine, R. F., ed.), pp. 179–185, Raven Press Ltd., New York
- Rouser, G., Fleischer, S. and Yamamoto, A. (1970) *Lipids* **5**, 494–496
- Stephens, L. R., Hawkins, P. T. and Downes, C. P. (1989) *Biochem. J.* **262**, 727–737
- Stephens, L. R., Hawkins, P. T., Barker, C. J. and Downes, C. P. (1988) *Biochem. J.* **253**, 721–733
- Hawkins, P. T., Kirk, C. J. and Michell, R. H. (1984) *Biochem. J.* **218**, 785–793
- Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., Ishihara, H. and Kojima, K. (1989) *Biochim. Biophys. Acta* **1002**, 182–188
- Mazzotti, G., Zini, N., Rizzi, E., Rizzola, R., Galanzi, A., Ognibene, A., Santi, S., Matteucci, A., Martelli, A. M. and Maraldi, N. M. (1995) *J. Histochem. Cytochem.* **43**, 181–191
- Santi, P., Martelli, A. M., Gilmour, R. S., Falcieri, E., Rana, E., Cataldi, A., Lattanzi, G., Bareggi, G. and Cocco, L. (1992) *Cell. Signalling* **4**, 385–391
- Bazenet, C. E., Ruano, A. R., Brockman, J. L. and Anderson, R. A. (1990) *J. Biol. Chem.* **265**, 18012–18022
- Divecha, N., Brooksbank, C. E. L. and Irvine, R. F. (1992) *Biochem. J.* **288**, 637–642