Biochem. J. (1989) 259, 893–896 (Printed in Great Britain)

Determination of the steady-state turnover rates of the metabolically active pools of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in human erythrocytes

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When intact human erythrocytes are incubated at metabolic steady state in a chloride-free medium containing [^{32}P]P_i, there is rapid labelling of the γ-phosphate of ATP, followed by a slower labelling of the monoester phosphate groups of phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] [King, Stephens, Hawkins, Guy & Michell (1987) Biochem. J. **244**, 209–217]. We have analysed the early kinetics of the labelling of these phosphate groups, in order to determine: (a) the steady-state rates of the interconversions of phosphatidylinositol, PtdIns4*P* and PtdIns(4,5)P_2; and (b) the fractions of the total cellular complement of PtdIns4*P* and PtdIns(4,5)P_2 that participate in this steady-state turnover. The experimental data most closely fit a pattern of PtdIns4*P* and PtdIns(4,5)P_2 turnover in which one-quarter of the total cellular complement of each lipid is in the metabolic pool that participates in rapid metabolic turnover, with rate constants of 0.028 min^{-1} for the interconversion of PtdIns and PtdIns4*P*, and of 0.010 min^{-1} for the PtdIns 4*P*/PtdIns(4,5)P_2 cycle. These rate constants represent metabolic fluxes of approx. 2.1 nmol of lipid/h per ml of packed erythrocytes between PtdIns and PtdIns4*P* and of approx. 5.7 nmol/h per ml of cells between PtdIns4*P* and PtdIns(4,5)P_2.

INTRODUCTION

PtdIns4*P*, PtdIns(4,5)P_2 and phosphatidate are the only membrane lipids into which the human erythrocyte incorporates added [^{32}P]P_i, and this cell has therefore served as a valuable system in which to define the basic aspects of the metabolism of the monoester phosphate groups of these lipids (see Allan, 1982; King *et al.*, 1987). Up to the present, studies of lipid metabolism in intact erythrocytes have usually employed 'physiological' incubation media containing a high concentration of Cl^-, which potently inhibits the entry of added [^{32}P]P_i by competing with it for the membrane anion transporter (Whittam, 1964; King *et al.*, 1987). As a result, these studies have shown relatively slow uptake of label into ATP and the polyphosphoinositides, so limiting somewhat the precision of kinetic analyses based on such measurements (see, for example, Müller *et al.* 1986).

We recently reported studies in which the ^{32}P-labelling of ATP and lipids in human erythrocytes was accelerated by incubation in a Cl^--free but otherwise physiological medium, allowing the relatively rapid attainment of isotopic equilibrium (King *et al.*, 1987). In the present paper, we report the use of this medium to determine the rates of interconversion of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2. This kinetic analysis also confirms the existence of metabolic compartmentation of these lipids (Müller *et al.*, 1986; King *et al.*, 1987).

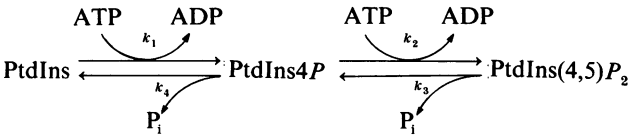
MATERIALS AND METHODS

Labelling of cells and analysis of ATP and lipids

The methods used for the isolation of erythrocytes, incubation of cells with [^{32}P]P_i under steady-state conditions in the Cl^--free medium ('Hepes medium'), assay of ATP-γ-phosphate specific radioactivity, extraction and analysis of PtdIns4*P* and PtdIns(4,5)P_2 (4- and 5-phosphate analysis by Method 2) were exactly as in King *et al.* (1987).

Kinetic calculations

In these experiments, the only reactions involved were the following two kinase/phosphatase cycles, and the steady-state conditions dictated that for each cycle the forward rate of the kinase reaction was exactly balanced by the opposing phosphatase (i.e. that $k_1 = k_4$ and $k_2 = k_3$):



We collected our data following the addition of a tracer quantity of [^{32}P]P_i to cells that had previously been brought to metabolic steady state in unlabelled medium. This information therefore describes the incorporation of ^{32}P into the γ-phosphate of ATP and the monoester phosphate groups of PtdIns4*P* and PtdIns(4,5)P_2 as each of these components progresses asymptotically towards isotopic equilibrium with its immediate metabolic precursor. The data were analysed by nonlinear regression techniques to fit an appropriate curve through each set of specific radioactivities (see Fig. 1).

One complication in the quantitative interpretation of

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this information is that only a limited subcompartment of the PtdIns4*P* and PtdIns(4,5)P_2 in human erythrocytes participates in rapid metabolic turnover (Müller *et al.*, 1986; King *et al.*, 1987), so calculations to derive turnover rates require the inclusion of 'compartmentation factors' for PtdIns4*P* and PtdIns(4,5)P_2 (represented below as $\phi_{\text{PtdIns4}P}$ and $\phi_{\text{PtdIns(4,5)}P_2}$ respectively). In principle, each compartmentation factor can take any value between 0.0 (no lipid subject to turnover) and 1.0 (all of the lipid subject to turnover). From our previous studies in which the γ-phosphate of ATP and the monoester phosphates of PtdIns4*P* and PtdIns(4,5)P_2 were labelled to isotopic equilibrium with [^{32}P]P_i in Hepes medium, the compartmentation factors for both lipids were expected to be approx. 0.25–0.30 (King *et al.*, 1987), so in our kinetic modelling we independently varied these two factors over the rather wider range 0.1–0.6.

The rate of labelling of the 5-phosphate of PtdIns(4,5)P_2 at any time depends upon the specific radioactivity of the precursor (ATP-γ-*P*), the value of $\phi_{\text{PtdIns(4,5)}P_2}$, and how close the system is to isotopic equilibrium. At any time after the addition of label, the instantaneous rate of labelling is:

$$\left(\frac{\delta S_{\text{PtdIns(4,5)}P_2\text{(5-}P\text{)}}}{\delta t}\right) = k_2\left[S_{\text{ATP-}\gamma\text{-}P} - \left(\frac{S_{\text{PtdIns(4,5)}P_2\text{(5-}P\text{)}}}{\phi_{\text{PtdIns(4,5)}P_2}}\right)\right] \quad (1)$$

where S_X is the specific radioactivity of component *X*.

^{32}P is incorporated into the 4-phosphate of PtdIns(4,5)P_2 when [^{32}P]PtdIns4*P* is phosphorylated by PtdIns4*P* kinase. Eqn. 2, which describes the labelling of the 4-phosphate of PtdIns(4,5)P_2, is similar to Eqn. 1, except that PtdIns4*P* is the precursor:

$$\left(\frac{\delta S_{\text{PtdIns(4,5)}P_2\text{(4-}P\text{)}}}{\delta t}\right) = k_2\left[\left(\frac{S_{\text{PtdIns4}P}}{\phi_{\text{PtdIns4}P}}\right) - \left(\frac{S_{\text{PtdIns(4,5)}P_2\text{(4-}P\text{)}}}{\phi_{\text{PtdIns(4,5)}P_2}}\right)\right] \quad (2)$$

Since the labelling of the 4-phosphate and the 5-phosphate are both consequences of the same reaction, the rate of PtdIns(4,5)P_2 labelling derived from eqn. 2 must be the same as that derived from eqn. 1.

PtdIns4*P* is synthesized by phosphorylation of PtdIns with the γ-phosphate group of ATP in a manner analogous to the phosphorylation of PtdIns4*P* to PtdIns(4,5)P_2:

$$\left(\frac{\delta S_{\text{PtdIns4}P}}{\delta t}\right) = k_1\left[S_{\text{ATP-}\gamma\text{-}P} - \left(\frac{S_{\text{PtdIns4}P}}{\phi_{\text{PtdIns4}P}}\right)\right] \quad (3)$$

However, this equation does not fully describe the labelling of PtdIns4*P*, as PtdIns4*P* is also formed by the dephosphorylation of PtdIns(4,5)P_2 and removed by the action of PtdIns4*P* kinase. This cycle causes the net transfer of label from PtdIns4*P* into PtdIns(4,5)P_2 and thus the rate of labelling of PtdIns4*P* is the sum of these two processes, one of which incorporates label whilst the other removes it:

$$\left(\frac{\delta S_{\text{PtdIns4}P}}{\delta t}\right) = k_1\left[S_{\text{ATP-}\gamma\text{-}P} - \left(\frac{S_{\text{PtdIns4}P}}{\phi_{\text{PtdIns4}P}}\right)\right] + k_2\left[\left(\frac{S_{\text{PtdIns(4,5)}P_2\text{(4-}P\text{)}}}{\phi_{\text{PtdIns(4,5)}P_2}}\right) - \left(\frac{S_{\text{PtdIns4}P}}{\phi_{\text{PtdIns4}P}}\right)\right] \quad (4)$$

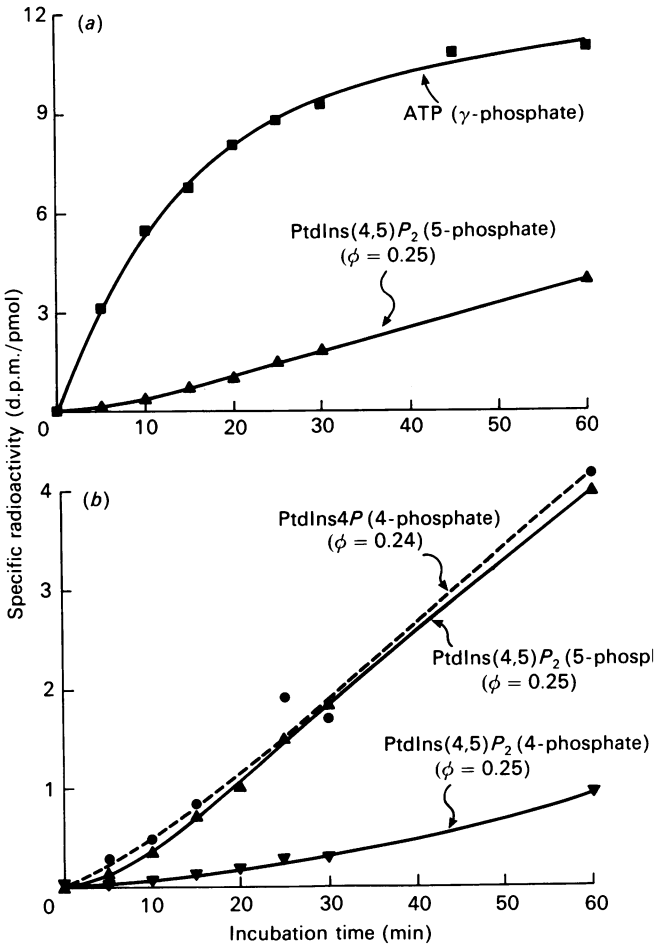

Fig. 1. Labelling of the γ-phosphate of ATP and of the monoester phosphates of PtdIns4*P* and PtdIns(4,5)P_2 following the addition of [^{32}P]P_i to human erythrocytes incubated at metabolic steady state in a Hepes-based medium

The plotted specific radioactivities of the γ-phosphate of ATP were determined directly (see the Materials and methods section). The specific radioactivities of the individual lipid monoester phosphate groups were determined directly on the extracted lipids of erythrocyte membranes, and the resulting values were used to model lipid turnover, assuming various values of $\phi_{\text{PtdIns4}P}$ and $\phi_{\text{PtdIns(4,5)}P_2}$. The values plotted in the Figure represent the specific radioactivities of the metabolically active pools of PtdIns4*P* and PtdIns(4,5)P_2, assuming $\phi_{\text{PtdIns4}P} = 0.24$ and $\phi_{\text{PtdIns(4,5)}P_2} = 0.25$, and the plotted curves are the best-fit curves calculated using these values for the compartmentation factors.

If the value of *k* is known for any reaction, the flux of lipid through that reaction (e.g. in nmol/min per ml of cells) can be calculated. For the reaction described by eqn. 1, for example:

$$\text{flux} = k_2(\phi_{\text{PtdIns(4,5)}P_2} \cdot C_{\text{PtdIns(4,5)}P_2})$$

where $C_{\text{PtdIns(4,5)}P_2}$ (nmol/ml of cells) is the total cellular content of PtdIns(4,5)P_2.

Numerical integration algorithms were used to determine the slopes at a large number of points on the best-fit curves, and thus the rates at which the specific radioactivities of individual phosphate groups progressed

Table 1. Rate constants (min^{-1}) for the interconversion of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, calculated using different compartmentation factors for PtdIns4*P* and PtdIns(4,5)P_2

Compartmentation factor	PtdIns $\rightleftharpoons$ PtdIns4*P*	PtdIns4*P* $\rightleftharpoons$ PtdIns(4,5)P_2 (based on 5-phosphate)	PtdIns4*P* $\rightleftharpoons$ PtdIns(4,5)P_2 (based on 4-phosphate)
(*a*) With $\phi_{PtdIns4P} = \phi_{PtdIns(4,5)P_2}$			
$\phi = 0.1$	0.35±0.28	0.054±0.024	0.011±0.003
0.2	0.044±0.013	0.014±0.001	0.011±0.003
0.25	0.028±0.007	0.010±0.001	0.011±0.003
0.3	0.020±0.004	0.008±0.001	0.011±0.003
0.6	0.006±0.001	0.004±0.001	0.011±0.003
(*b*) With $\phi_{PtdIns(4,5)P_2}$ held constant at 0.25			
$\phi_{PtdIns4P} = 0.15$	0.053±0.015	0.010±0.001	0.006±0.002
0.20	0.037±0.010	0.010±0.001	0.008±0.002
0.24	0.029±0.007	0.010±0.001	0.010±0.003
0.28	0.024±0.006	0.010±0.001	0.012±0.003
0.35	0.018±0.004	0.010±0.001	0.016±0.004
(*c*) With $\phi_{PtdIns4P}$ held constant at 0.24			
$\phi_{PtdIns(4,5)P_2} = 0.20$	0.035±0.010	0.014±0.001	0.013±0.003
0.25	0.029±0.007	0.010±0.001	0.010±0.003
0.30	0.026±0.006	0.008±0.001	0.008±0.002

from particular intermediate values towards their final values. From these we derived 'instantaneous' rate constants for at least 500 points along each curve which could be averaged to yield an overall rate constant for each reaction.

RESULTS AND DISCUSSION

ATP and polyphosphoinositide labelling at early times

The detailed analysis of the kinetics of PtdIns4*P* and PtdIns(4,5)P_2 turnover that is presented below is derived from data obtained using a single preparation of erythrocytes. However, in three separate experiments in which the labelling of the monoester phosphates of these lipids was allowed to achieve a close to constant value during prolonged incubation under the conditions used in this experiment (an example is shown in Fig. 7 of King *et al.*, 1987), the period taken for the specific radioactivity of PtdIns(4,5)P_2 to reach one-half of its final value was 60–75 min, indicating that the same temporal pattern of lipid turnover is seen in erythrocytes from different individuals.

The incorporation of ^{32}P into the γ-phosphate of ATP and the monoester phosphate groups of PtdIns4*P* and PtdIns(4,5)P_2 was measured during the first 60 min following the addition of [^{32}P]P_i to erythrocytes suspended in Hepes medium (Fig. 1). During this time, the specific radioactivity of the γ-phosphate of ATP reached its maximum value (Fig. 1*a*, see also Figs. 5 and 6 of King *et al.*, 1987). Fig. 1(*a*) shows the time-course of labelling of the γ-phosphate of ATP, together with the labelling curve for the 5-phosphate of PtdIns(4,5)P_2, assuming a value for $\phi_{PtdIns(4,5)P_2}$ of 0.25 (see the Materials and methods section for definition of the compartmentation factors $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$). Thus the lipid-specific radioactivity plotted is a measure of the labelling of the metabolically active pool of PtdIns(4,5)P_2, and would reach a value equal to that of the γ-phosphate of ATP at isotopic equilibrium (see King *et al.*, 1987). Fig. 1(*b*) shows the time-courses of incorporation of ^{32}P into the 4- and 5-phosphates of PtdIns(4,5)P_2 and the 4-phosphate of PtdIns4*P*, assuming that $\phi_{PtdIns4P} = 0.24$ and $\phi_{PtdIns(4,5)P_2} = 0.25$ (see below).

Kinetic analysis of the labelling curves

The rate constants k_1 and k_2 were calculated from the best-fit curves using a range of compartmentation factors (see Table 1*a*). Initially, $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ were assigned the same value, and this was varied between 0.1 and 0.6. k_2 was calculated from the labelling of both the 4- and the 5-phosphate of PtdIns(4,5)P_2. As discussed in the Materials and methods section, values from these two calculations must by definition be the same; and the most satisfactory value for $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ was judged to be that which best satisfied this condition. The best fit was found when $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ were set at 0.25 (Table 1*a*).

It is not necessary, however, for $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ to be set to the same value. With $\phi_{PtdIns(4,5)P_2} = 0.25$, the value of $\phi_{PtdIns4P}$ that gave the best fit between the two estimates for k_2 was 0.24. Other values for $\phi_{PtdIns4P}$ caused a rapid divergence of these two values (Table 1*b*). By contrast, with $\phi_{PtdIns4P} = 0.24$, variation of $\phi_{PtdIns(4,5)P_2}$ caused no divergence of the two estimates of k_2, but did substantially influence the absolute value of k_2 (Table 1*c*). Our best-fit criterion thus allows us to select a value for $\phi_{PtdIns4P}$ that uniquely fits the data, but does not allow this to be done for $\phi_{PtdIns(4,5)P_2}$.

In previous experiments (King *et al.*, 1987), $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ were estimated by the quite different procedure of labelling erythrocytes with [^{32}P]P_i for a time sufficient to achieve isotopic equilibrium between the γ-phosphate of ATP and the monoester phosphates of PtdIns4*P* and PtdIns(4,5)P_2, and then measuring the specific radioactivities of these phosphate groups. Both $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ were estimated to be in the range 0.25–0.30. The fact that an independent kinetic technique using cells labelled for only a short time has yielded a very similar value suggests that the compartmentation seen is not an artefact of long-term incubation of

erythrocytes in a non-standard medium, but is a real phenomenon.

By using the kinetic constants derived from the above analysis in combination with the best-fit values of $\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$ (see above) and the known concentrations of PtdIns4*P* and PtdIns(4,5)P_2 (King *et al.*, 1987), it can be calculated that the lipid labelling seen in these experiments represents steady-state bidirectional lipid fluxes of approx. 2.1 nmol/h per ml of erythrocytes between PtdIns and PtdIns4*P*, and of approx. 5.7 nmol/h per ml of erythrocytes between PtdIns4*P* and PtdIns(4,5)P_2. The normal maintenance of PtdIns4*P* and PtdIns(4,5)P_2 levels in cells incubated under these conditions therefore requires the expenditure of about 8 nmol of ATP/h per ml of cells, which represents about 0.4% of the approx. 2 μmol of ATP that is synthesized by 1 ml of healthy human erythrocytes in an hour (Momsen & Vestergarrd-Bogind, 1978; Maretzki *et al.*, 1981).

Comparison with previous studies

We have confirmed the conclusion, originally reported by Müller *et al.* (1986), that accurate kinetic modelling of the rapid metabolic turnover of PtdIns4*P* and PtdIns(4,5)P_2 in human erythrocytes can be achieved only if it is assumed that a limited fraction of the total erythrocyte membrane polyphosphoinositide participates in this metabolic turnover. Moreover, the compartmentation factors ($\phi_{PtdIns4P}$ and $\phi_{PtdIns(4,5)P_2}$) that we have obtained from these kinetic studies are similar to those that we previously determined by labelling erythrocyte lipids to equilibrium with the γ-phosphate of ATP in the same incubation medium (King *et al.*, 1987). As noted by King *et al.* (1987), our value of approx. 0.25 for $\phi_{PtdIns(4,5)P_2}$ is much larger than the value of 0.06–0.1 reported by Müller *et al.* (1986) from kinetic studies alone. The reason for this discrepancy is not clear. However, we believe that our estimate is correct because our steady-state labelling studies involved a direct demonstration that the specific radioactivity of the 5-phosphate of PtdIns(4,5)P_2 achieved a value that was one-quarter to one-third of that of the γ-phosphate of ATP within a few hours of the addition of labelled P_i to cells: this observation is incompatible with any value of $\phi_{PtdIns(4,5)P_2}$ that is less than 0.25–0.33. As discussed before (King *et al.*, 1987), the physical basis for this metabolic compartmentation of the PtdIns and PtdIns(4,5)P_2 of erythrocytes, which appears to occur within the inner leaflet of the plasma membrane, remains unknown.

Although some earlier studies suggested that polyphosphoinositide turnover accounts for 20–70% of the total ATP consumption of human erythrocytes (e.g. Maretzki *et al.*, 1983), Dale (1985) concluded that polyphosphoinositide metabolism can be sustained by less than 1% of the normal cellular ATP turnover. The figures he obtained by two independent experimental techniques were that this lipid turnover consumed about 14 nmol of ATP/h per ml of cells and about 0.5% of the phosphate that was lost from pulse-labelled ATP. Our estimate of a consumption of 8 nmol of ATP/h per ml of cells, equivalent to about 0.4% of total ATP turnover, agrees remarkably well with Dale's conclusion. By contrast, Müller *et al.* (1986) concluded that polyphosphoinositide turnover consumes 2–5% of erythrocyte ATP. This larger estimate arose primarily from their conclusion that the rate constant for the conversion of PtdIns4*P* to PtdIns(4,5)P_2 was remarkably high, at 0.18–0.60 min^{-1}. In their analysis, this conclusion was supported by the observation that the specific radioactivity of the 5-phosphate of PtdIns(4,5)P_2 was about 10% of that of the γ-phosphate of ATP after only 20 min of labelling (Table 3 of Müller *et al.*, 1986); given their assumed value of approx. 0.1 for $\phi_{PtdIns(4,5)P_2}$, it therefore appeared that the metabolically-active pool of PtdIns(4,5)P_2 had isotopically equilibrated with ATP by that time. However, under our incubation conditions, which lead to a very rapid labelling of ATP, we find that the specific radioactivity of the 5-phosphate of PtdIns(4,5)P_2 after 20 min is only about 3–4% of that of ATP (Fig. 1*a*). When this figure is combined with our much larger estimate of $\phi_{PtdIns(4,5)P_2}$, it suggests a much slower turnover of the 5-phosphate of PtdIns(4,5)P_2 than that estimated by Müller *et al.* (1986). This interpretation is confirmed by the approximate half-time for the turnover of the 5-phosphate of PtdIns(4,5)P_2 that can be directly estimated from inspection of the time-course of PtdIns(4,5)P_2 labelling: this is of the order of 30–60 min rather than a very few minutes.

We are grateful to the Medical Research Council for Programme Grant support and a research studentship (to C.E.K.).

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Received 18 October 1988/1 February 1989; accepted 9 February 1989