Functional heterogeneity of polyphosphoinositides in human erythrocytes

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After labelling of erythrocytes with [^{32}P]P_i for 23 h, the specific radioactivities of the phosphomonoester groups of PtdIns4P and of PtdIns(4,5)P₂ approached equilibrium values which were close to that of the γ -phosphate of ATP (78–85 %), showing that almost all of these phosphate groups were metabolically active. Phosphoinositidase C (PIC) activation, using Ca²⁺ and the ionophore A23187, of ^{32}P -prelabelled erythrocytes was used to investigate a possible functional heterogeneity of the phosphoinositides. Hydrolysis of PtdIns(4,5)P₂, measured from its radioactivity, decreased as function of the time of prelabelling up to a constant value equal to that measured from its content. In contrast, hydrolysis of PtdIns4P, determined both from radioactivity and from content, was always the same. These data suggest that newly labelled molecules of PtdIns(4,5)P₂, initially accessible to PIC, then moved towards a PIC-resistant pool. This was further confirmed by measuring the fraction of labelled PtdIns(4,5)P₂ molecules accessible to PIC after a prelabelling period of 5 min and different times of reincubation. Hydrolysis by PIC was also measured in erythrocytes in which the phosphoinositide content had been modified by activation (Mg²⁺-enriched cells) or inhibition (ATP-depleted cells) of the phosphoinositide kinases. The sizes of the PIC-resistant pools of polyphosphoinositides were not affected by these treatments, indicating that the kinases (and the phosphatases) act on the PIC-sensitive pools. This was also shown by the decrease in the production of Ins(1,4,5)P₃ upon PIC activation in ATP-depleted erythrocytes. A model is presented in which the PIC-sensitive pools of polyphosphoinositides are those which are accessible to the kinases and the phosphatases and are rapidly turned over.

INTRODUCTION

Polyphosphoinositides constitute a minor fraction of total cell phospholipids. Nonetheless, they are involved in important functions such as receptor-mediated transmembrane signalling (for a review, see Berridge, 1984) facilitation of exocytic processes (Whitaker & Aitchison, 1985), the anchorage of membrane proteins (Low & Kincade, 1985; Lassing & Lindberg, 1985; Anderson & Marchesi, 1985) and the regulation of ion transport (Simmons *et al.*, 1986). Polyphosphoinositides undergo rapid phosphorylation and dephosphorylation, which affects only the phosphomonoester groups in erythrocytes. This turnover is catalysed by two kinases acting on PtdIns and PtdIns4P and two phosphatases acting on PtdIns(4,5)P₂ and PtdIns4P (for a review, see Allan, 1982). Evidence for metabolic compartmentation of these phospholipids in erythrocytes was provided recently by two independent groups (Müller *et al.*, 1986; King *et al.*, 1987). Müller *et al.* (1986) performed a computer analysis of the data obtained in the very early phases of the ^{32}P labelling of polyphosphoinositides, and found that acceptable fits were possible only by introducing compartmentation factors for both PtdIns4P and PtdIns(4,5)P₂ (i.e. assuming non-homogeneous pools). King *et al.* (1987), using a different strategy allowing rapid P_i exchange across the membrane (by using a chloride-free medium) and thus rapid labelling of the γ -phosphate of ATP, have observed that the fraction of the polyphosphoinositides which became labelled at isotopic equilibrium (5–7 h) represented only about 30 % of the

total pool of each phospholipid. The presence of a metabolically inert pool of phosphoinositides in erythrocytes is in contrast with previous data obtained in platelets (Verhoeven *et al.*, 1987).

In the present study, we have investigated the metabolic compartmentation of phosphoinositides in erythrocytes incubated with [^{32}P]P_i. In chloride medium, phosphomonoester groups of both PtdIns(4,5)P₂ and PtdIns4P were found to approach an equilibrium value close to that of the γ -phosphate of ATP after about 23 h. Similar data were also observed after 23 h in the Hepes medium used by King *et al.* (1987). However, a metabolic heterogeneity of the polyphosphoinositides was demonstrated by measuring their accessibility to endogenous phosphoinositidase C (PIC) either after different periods of labelling or after manipulation of the pool sizes by inhibiting or activating the kinases or the phosphatases. A model describing the compartmentation of PtdIns4P and PtdIns(4,5)P₂ is presented.

MATERIALS AND METHODS

Preparation of erythrocytes

Human blood was withdrawn from healthy volunteers, heparinized and centrifuged (1300 g, 10 min, 4 °C). Plasma and buffy coat were removed by aspiration and the packed erythrocytes were washed three times in a solution containing 154 mM-NaCl/1.5 mM-Hepes/10 mM-glucose (pH 7.4) and once either in 'chloride medium' or in 'Hepes medium'. Chloride medium (A)

Abbreviation used: PIC, phosphoinositidase C.

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contained 154 mM-NaCl, 1.5 mM-Hepes, 10 mM-glucose, 1 mM-adenine, 5 mM-inosine, 100 μ g of streptomycin/ml and 100 units of penicillin/ml (pH 7.4). Hepes medium (B) was the same as that used by King *et al.* (1987) but lacked bovine serum albumin and amphotericin B. With the exception of the incubations, all steps were performed at 4 °C.

Incubation of erythrocytes with [32 P]P_i

Cell suspensions in medium A or B (haematocrit 40 %) were prewarmed at 37 °C for 15 min before the addition of [32 P]P_i (0.2–4.4 MBq/ml of cells; International CIS, France). In phosphorylation time-course experiments, the reaction was stopped by the addition of ice-cold 154 mM-NaCl/1.5 mM-Hepes/1 mM-EGTA (pH 7.4). The cells were washed twice in this medium and haemolysed (see below). In PIC activation experiments, phosphorylation (in medium A) was stopped by the addition of ice-cold 154 mM-NaCl/1.5 mM-Hepes (pH 7.4).

PIC activation

32 P-prelabelled erythrocytes were washed twice in 154 mM-NaCl/1.5 mM-Hepes (pH 7.4) (step 1) and twice more in 75 mM-NaCl/75 mM-KCl/0.15 mM-MgCl₂/10 mM-Hepes (pH 7.4), and resuspended in this medium at a haematocrit of 10 % (step 2). Aliquots of the cell suspension were preincubated for 10 min at 37 °C in the presence of either EGTA (1 mM final concn.) or CaCl₂ (30 μ M–1 mM final concn.) before the addition of the Ca²⁺ ionophore A23187 (Sigma; final concn. 5 μ M from a 2 mM stock solution in dimethyl sulphoxide). After 20 min, the reaction was stopped by the addition of ice-cold 154 mM-NaCl/1.5 mM-Hepes/1 mM-EGTA (pH 7.4). The cells were washed twice in this medium and haemolysed. In some experiments, between step 1 and step 2, 32 P-labelled erythrocytes were either reincubated at 4 or 37 °C (with 10 mM-glucose) for different periods, or ATP-depleted by incubation (60 min, 37 °C) in a medium containing 154 mM-NaCl/10 mM-Hepes/6 mM-iodoacetamide/10 mM-inosine (pH 7.4).

Membrane isolation, lipid extraction and t.l.c. of lipids

Packed erythrocytes were haemolysed in 30 vol. of 10 mM-Tris/HCl/1 mM-EDTA (pH 7.4), centrifuged (25000 g, 10 min) and washed twice in the same buffer. Acidic lipid extraction of the resuspended membranes, t.l.c. separation and quantification of phospholipid content and radioactivity were carried out essentially as previously described (Giraud *et al.*, 1984). After t.l.c. on silica gel H plates (Schleicher and Schüell, Dassel, Germany) pretreated in 2 % EDTA, using chloroform/methanol/4.3 M-NH₄OH (9:7:2, by vol.) as solvent, phospholipids were detected by autoradiography. Although this separation produced radiochemically pure PtdIns4P, a phosphate-containing compound(s), revealed by I₂ vapour, co-chromatographed closely to it. This contaminant was carefully eliminated before PtdIns4P was scraped off the plates. Under these conditions, PtdIns4P content was lower than that measured in our previous work (Giraud *et al.*, 1984) and close to that reported by others (Müller *et al.*, 1986; King *et al.*, 1987). Spots of silica gel containing the phospholipids were digested with HClO₄ and assayed for phosphorus content. Radioactivity was measured in the same samples by Cerenkov counting.

ATP content and specific radioactivity

ATP content and specific radioactivity were measured as previously described (Giraud *et al.*, 1988; Rhoda *et al.*, 1988) except that methanol was replaced by acetonitrile (10 % v/v in the elution buffer), and that the radioactivity of the ATP peak was measured by Cerenkov counting of the effluent fractions.

Measurement of [32 P]Ins(1,4,5)P₃ in intact cells

The method used was the same as that described by Rhoda *et al.* (1988). Briefly, HClO₄ extracts of erythrocytes, neutralized with KHCO₃, were injected together with standard [3 H]Ins(1,4,5)P₃ on a C18 reverse-phase h.p.l.c. column (Ultrasphere IP). Ion-pair chromatography was performed by a two-step elution: with solvent A [0.025 M-tetrabutylammonium hydrogen sulphate/0.1 M-KH₂PO₄ (adjusted to pH 5.5)] for 40 min and then with solvent A/acetonitrile (4:1, v/v). Fractions were collected every 1 min and their 32 P and 3 H contents were measured by liquid-scintillation counting. Identification of the [32 P]Ins(1,4,5)P₃ peak was confirmed on an anion-exchange h.p.l.c. column (Partisil 10 SAX) (see Rhoda *et al.*, 1988).

Quenching corrections

Radioactivity measured by Cerenkov counting (coloured samples of digested phospholipids and fractions of the ATP peak collected from the h.p.l.c. column) was corrected for quenching (in d.p.m.) using an efficiency curve established by counting samples of 32 PO₄ in water alone or after addition of coloured samples of digested unlabelled phospholipids or portions of elution solvent used in the h.p.l.c. system.

RESULTS AND DISCUSSION

Kinetics of 32 P incorporation into PtdIns(4,5)P₂ and PtdIns4P

Erythrocytes were incubated with [32 P]P_i either in a chloride medium or in the Hepes medium used by King *et al.* (1987). Both media contained glycolytic substrates. However, long incubation periods led to a diminution in the concentration of ATP, particularly in the Hepes medium (Fig. 1). This ATP depletion was accompanied by a decrease in the concentration of PtdIns(4,5)P₂ and by an increase in that of PtdIns4P. These changes were more marked in the Hepes than in the chloride medium (Fig. 1), and were thus probably due to the ATP depletion. This effect of ATP depletion induced by iodoacetamide and inosine on the content of polyphosphoinositides was confirmed in other experiments as described below. This has been also reported previously by Ferrell & Huestis (1984), both after slow metabolic depletion (incubation in the absence of metabolic substrates) and after rapid depletion (induced by iodoacetamide and inosine). However, these authors did not observe any change in the content of PtdIns4P, although they reported a decrease in PtdIns(4,5)P₂ content (and an increase in PtdIns content). This lack of change of PtdIns4P content may be due to the fact that this phospholipid comigrated with (unlabelled) phosphate-containing compounds in the chromatographic system used by Ferrell & Huestis (1984) as shown by the high content of PtdIns4P reported in their publication

Fig. 1. Concentrations of ATP and polyphosphoinositides in erythrocytes incubated in chloride medium or in Hepes medium

Erythrocytes were incubated at 37 °C either in Hepes (□, ○, △) or chloride medium (■, ●, ▲) as described in the Materials and methods section. Portions of the incubation were removed at various times and assayed for ATP (□, ■), PtdIns(4,5) P_2 (○, ●) and PtdIns4P (△, ▲) concentrations (see the Materials and methods section). Data are means $\pm$ S.E.M. of duplicate determinations in three or four experiments.

(0.8 mol % instead of 0.3–0.4 mol % in this report) (see the Materials and methods section).

The specific radioactivity of ATP remained constant between 3 and 23 h of incubation in both media (Figs 2a and 2b). The specific radioactivity of both PtdIns(4,5) P_2 (mean value of 4- P + 5- P) and PtdIns4P (4- P) increased more rapidly in the Hepes medium (Fig. 2b). In that medium, the specific radioactivity of PtdIns4P increased more rapidly than that of PtdIns(4,5) P_2 during the first 6 h of incubation. In the chloride medium, the specific radioactivity of both phosphoinositides increased progressively, that of PtdIns4P always being greater than that of PtdIns(4,5) P_2 . In both media, after 23 h, the curves describing the variations of the specific radioactivity of the phosphate monoester groups of the phospholipids were still increasing. However the values of the phospholipid specific radioactivity were close to that of the γ -phosphate of ATP (78–85 %).

Kinetic analysis of the data shown in Figs. 2(a) and 2(b) was not possible because (i) the specific radioactivity of ATP between 0 and 3 h was not measured; nor was that of each of the two phosphate groups (4- P and 5- P) of PtdIns(4,5) P_2 , and (ii) the pool sizes of ATP and polyphosphoinositides were not constant during the period of the experiments, especially in the Hepes medium. However, from the data of the specific radioactivity of ATP (γ - P) at equilibrium and of the polyphosphoinositides (4- P or 4- P + 5- P) after 23 h of incubation, it may be concluded that the phosphomonoester groups of at least 78–85 % of these phospholipids are being turned over by exchange with the γ -phosphate of ATP. This finding is in agreement with similar data obtained in platelets (Verhoeven *et al.*, 1987) but contrasts with the conclusions of King *et al.* (1987) of

Fig. 2. Specific radioactivities of ATP and polyphosphoinositides in erythrocytes incubated in chloride medium (a) or in Hepes medium (b)

Erythrocytes were incubated with [32 P] P_i (370 kBq/ml of cells) in either Hepes or chloride medium as described in the Materials and methods section. At various times, portions of the incubation were removed and assayed for ATP and polyphosphoinositide content and radioactivity (see the Materials and methods section). The specific radioactivity of the γ -phosphate of ATP (■) was calculated by dividing the specific radioactivity of ATP by 2 (see Müller *et al.*, 1986). The specific radioactivity of 4- P of PtdIns4P (▲) and of 4- P + 5- P of PtdIns(4,5) P_2 (●) was calculated by multiplying specific radioactivities of total phosphorus by 2 and 1.5 respectively. Data are means $\pm$ S.E.M. of duplicate determinations in three or four experiments.

the occurrence of metabolically inert pools representing 65–70 % of phosphoinositides in erythrocytes. In fact, it is possible that under the conditions used by these authors

(Hepes medium and only 5–7 h of incubation), the specific radioactivity of PtdIns4P and of PtdIns(4,5) P_2 has reached only an apparent equilibrium value. We have indeed observed that the specific radioactivity of PtdIns4P, after a rapid augmentation, continued to increase more slowly up to 13 h (see Fig. 2b). This may be explained by the biphasic kinetics of the specific radioactivity of ATP in this medium as shown by King *et al.* (1987) (see Fig. 5 in their publication), which rendered difficult the prediction of the kinetics of labelling of polyphosphoinositides. The interpretation of the data obtained in the chloride medium was more straightforward since, under these conditions, the specific radioactivity of ATP exhibited a more simple time course (see Müller *et al.*, 1986; King *et al.*, 1987; Rhoda *et al.*, 1988). However, long-term experiments (more than 23 h) were required to reach isotopic equilibrium for the phosphoinositides. Although a small loss of ATP (20%) and changes in the contents of PtdIns4P and PtdIns(4,5) P_2 were observed, these variations occurred before 15 h so that during the final 8 h of observation, the pool sizes of all metabolites could be considered as stationary. Thus the data obtained with the chloride medium were taken as a demonstration that at least 85% of the phosphomonoester groups of the polyphosphoinositides were being turned over in erythrocytes.

Evidence for compartmentation of PtdIns(4,5) P_2 and PtdIns4P

Although the majority of the phosphoinositides were metabolically active, this does not necessarily mean that the polyphosphoinositide molecules behave as homogeneous pools. Endogenous PIC activity (stimulated by Ca^{2+} in the presence of the ionophore A23187) was used to probe the possible sub-compartmentation of these phospholipids.

In the first group of experiments, erythrocytes were labelled with [^{32}P]P_i for different periods (from 5 min to 15 h), washed and immediately assayed for PIC activation in the presence of the ionophore and either 1 mM-EGTA (control) or 1 mM- $CaCl_2$. The percentage of Ca^{2+} -dependent hydrolysis of each phosphoinositide was determined from the diminution of its radioactivity and of its mass (Fig. 3). The percentage hydrolysis of radioactive PtdIns(4,5) P_2 , which amounted to $80.0 \pm 1.6\%$ after 5 min of labelling, decreased to $61.3 \pm 2.8\%$ after 15 h of labelling. However, the percentage hydrolysis of PtdIns(4,5) P_2 as measured from its mass was constant, regardless of the time of labelling ($60.3 \pm 2.9\%$ after 5 min and $57.5 \pm 2.9\%$ after 15 h). In contrast, hydrolysis of PtdIns4P, measured from its radioactivity, remained nearly constant throughout the experiment ($78.4 \pm 2.2\%$ after 5 min of labelling and $77.8 \pm 2.3\%$ after 15 h) and was not significantly different from that determined from its mass ($72.3 \pm 3.5\%$ after 5 min and $74.5 \pm 2.0\%$ after 15 h). These results indicate that after a short period of incubation with [^{32}P]P_i, during which only a small fraction of the PtdIns(4,5) P_2 pool became labelled, PIC was able to hydrolyse almost all the radioactive molecules, but only 60% of the total pool of PtdIns(4,5) P_2 . After longer periods of labelling, less and less radioactive molecules were accessible to PIC until a constant state was reached at which about 60% of both labelled and total molecules were susceptible to hydrolysis. The situation was different for PtdIns4P, since the fraction of accessible radioactive molecules did not change with

Fig. 3. Hydrolysis of phosphoinositides by PIC in erythrocytes prelabelled with ^{32}P for different periods

Erythrocytes were preincubated with [^{32}P]P_i for the periods indicated on the abscissa, washed twice and assayed for PIC activation by treatment with A23187 ionophore and either 1 mM- $CaCl_2$ or 1 mM-EGTA (control). Polyphosphoinositide content and radioactivity were measured (see the Materials and methods section). Ca^{2+} -induced hydrolysis of the content (○, △) or the radioactivity (●, ▲) of PtdIns(4,5) P_2 (○, ●) and PtdIns4P (△, ▲) is shown. Data are means $\pm$ S.E.M. of duplicate determinations from four experiments.

time and was not different from that of the total pool of PtdIns4P. Therefore it seems that the newly synthesized molecules of PtdIns(4,5) P_2 were exchanging between a PIC-accessible and a PIC-resistant pool during the experiment. In the case of PtdIns4P, it may be assumed that a similar process occurred, but equilibrium had already been achieved at the beginning of the experiment. Alternatively PIC, but not PtdIns kinase and PtdIns4P phosphatase, could be fatty-acid selective. However, the fact that the new diacylglycerol formed in erythrocyte membranes upon PIC activation has the same fatty acid composition as that of PtdIns4P and PtdIns(4,5) P_2 (Allan & Cockcroft, 1983) argues against this second hypothesis.

In the second group of experiments, erythrocytes were labelled for 5 min, washed and assayed for PIC activation after different periods of reincubation at 37 °C (5–90 min) (Fig. 4a). Results were the same as those observed with continuous labelling. Hydrolysis of [^{32}P]PtdIns(4,5) P_2 decreased progressively as a function of the time of reincubation, whereas hydrolysis of [^{32}P]PtdIns4P stayed constant. In addition, when after 5 min of labelling reincubation was carried out at 4 °C, the percentage of hydrolysis of [^{32}P]PtdIns(4,5) P_2 (and also of [^{32}P]PtdIns4P) did not decrease during the 3 h of reincubation (Fig. 4b). The exchange of the labelled molecules of PtdIns(4,5) P_2 from their site of synthesis (PIC-accessible) towards a PIC-resistant pool was thus inhibited at 4 °C.

A third group of experiments investigated whether PIC, polyphosphoinositide phosphatases and PtdIns4P kinase attack the same pool of polyphosphoinositides. The kinase activities were modulated by changing the cellular concentrations of ATP and Mg^{2+} as follows. Erythrocytes were ATP-depleted by incubation with inosine and iodoacetamide and treated with the ionophore and either EGTA or $CaCl_2$ in the presence of two

Fig. 4. Hydrolysis of phosphoinositides by PIC in erythrocytes prelabelled with ^{32}P for 5 min and reincubated for different periods either at 37 °C (a) or at 4 °C (b)

Erythrocytes were preincubated with $^{32}\text{P}\text{P}_i$ for 5 min, washed twice, reincubated for the periods indicated on the abscissa and directly assayed for PIC activation by treatment with A23187 ionophore and either 1 mM- CaCl_2 or 1 mM-EGTA (control). Polyphosphoinositide radioactivity was measured (see the Materials and methods section). Ca^{2+} -induced hydrolysis of the radioactivity of PtdIns(4,5) P_2 (●) and PtdIns4P (▲) is shown. Data are means $\pm$ S.E.M. of duplicate determinations from four experiments.

different MgCl_2 concentrations. At an external MgCl_2 concentration of 0.15 mM, intracellular free $[\text{Mg}^{2+}]$ is about 0.4 mM, corresponding to the physiological level; and at 1.2 mM external MgCl_2 , intracellular free $[\text{Mg}^{2+}]$ is about 2 mM (Flatman & Lew, 1977). Control erythrocytes

were treated similarly. The ATP content was decreased by more than 95 % in iodoacetamide-treated cells (results not shown). At physiological Mg^{2+} concentration, ATP depletion led to a decrease in the concentration of PtdIns(4,5) P_2 and to an increase in PtdIns4P and PtdIns resulting from the inhibition of both PtdIns4P and PtdIns kinase activities (Table 1). Mg^{2+} enrichment, in control cells, caused an increase in the concentration of PtdIns4P and a decrease in that of PtdIns, resulting from an activation of PtdIns kinase. This effect of Mg^{2+} enrichment was not seen in ATP-depleted cells (Table 1). Interestingly, under all these conditions, i.e. the presence or absence of ATP and/or Mg^{2+} , the PIC-resistant pools of PtdIns(4,5) P_2 and of PtdIns4P (that remaining after PIC activation by Ca^{2+}) were the same (0.31–0.41 mol % for PtdIns(4,5) P_2 and 0.10–0.12 mol % for PtdIns4P) (Table 1 and Fig. 5). PIC activation by ionophore and various external Ca^{2+} concentrations (30 μM , 100 μM and 1 mM) was also assayed in erythrocytes prelabelled for 90 min, washed and either ATP-depleted for 1 h as above or kept at 4 °C for the same period (Fig. 5). In both control and ATP-depleted cells, hydrolysis of PtdIns(4,5) P_2 by PIC resulted in a decrease in its specific radioactivity (Figs 5c and 5d). In contrast, the specific radioactivity of PtdIns4P was not significantly changed after PIC activation (Figs 5a and 5b). The decrease in the specific radioactivity of PtdIns(4,5) P_2 after PIC activation and the lack of change in that of PtdIns4P under the same conditions are both in agreement with the data of Figs. 3 and 4. They indicate again that PIC preferentially attacks the most heavily labelled pool of PtdIns(4,5) P_2 . In addition, Ins(1,4,5) P_3 produced in intact erythrocytes upon PIC activation by ionophore treatment in the presence of various external Ca^{2+} concentrations was measured both in control and in ATP-depleted cells. Fig. 6 shows that after ATP depletion, when the PtdIns(4,5) P_2 pool has been reduced by the action of PtdIns(4,5) P_2 phosphatase, Ins(1,4,5) P_3 production was less than in control cells. This indicates that PIC and phosphatase act on the same pool of PtdIns(4,5) P_2 .

These data support the concept that both PtdIns(4,5) P_2 and PtdIns4P molecules exist within metabolically distinct pools (Fig. 7). The effect of the activation of PtdIns kinase (an increase in the content of PtdIns4P) caused an

Table 1. Effects of ATP depletion, Mg^{2+} and Ca^{2+} on the concentrations of phosphoinositides

Control (A) or ATP-depleted (B) erythrocytes were treated with A23187 ionophore in the presence of 1 mM-EGTA/0.15 mM- MgCl_2 (EGTA), 1 mM-EGTA/1.2 mM- MgCl_2 (EGTA + Mg^{2+}), 1 mM- CaCl_2 /0.15 mM- MgCl_2 (Ca^{2+}), or 1 mM- CaCl_2 /1.2 mM- MgCl_2 (Ca^{2+} + Mg^{2+}). Cells were washed, haemolysed and processed for lipid extraction, phospholipid separation and assay (see the Materials and methods section). Data are means $\pm$ S.E.M. of duplicate determinations in three or four experiments.

		Concentration (mol %)		
		PtdIns(4,5) P_2	PtdIns4P	PtdIns
(A)	EGTA	0.89 $\pm$ 0.11	0.29 $\pm$ 0.01	0.70 $\pm$ 0.05
	EGTA + Mg^{2+}	0.82 $\pm$ 0.03	0.42 $\pm$ 0.02	0.55 $\pm$ 0.05
	Ca^{2+}	0.34 $\pm$ 0.06	0.11 $\pm$ 0.03	0.79 $\pm$ 0.03
	Ca^{2+} + Mg^{2+}	0.41 $\pm$ 0.03	0.12 $\pm$ 0.03	0.56 $\pm$ 0.04
(B)	EGTA	0.61 $\pm$ 0.05	0.41 $\pm$ 0.05	0.86 $\pm$ 0.07
	EGTA + Mg^{2+}	0.60 $\pm$ 0.07	0.44 $\pm$ 0.05	0.55 $\pm$ 0.03
	Ca^{2+}	0.33 $\pm$ 0.06	0.11 $\pm$ 0.02	0.68 $\pm$ 0.05
	Ca^{2+} + Mg^{2+}	0.31 $\pm$ 0.05	0.10 $\pm$ 0.03	0.52 $\pm$ 0.09

Fig. 5. Effect of the concentration of external CaCl_2 on polyphosphoinositide content and specific radioactivity in ionophore-treated control (a, c) and ATP-depleted (b, d) erythrocytes

Erythrocytes were preincubated with $[^{32}\text{P}]\text{P}_i$ (555 kBq/ml of cells) for 90 min, washed twice and either maintained at 4 °C for 60 min or ATP-depleted for the same period at 37 °C. Control and ATP-depleted cells were washed twice and treated with 5 μM -A23187 in the presence of 0.15 mM- MgCl_2 and either 1 mM-EGTA or different CaCl_2 concentrations (30, 100 or 1000 μM). Under the conditions of unbuffered $[\text{Ca}^{2+}]$ in the medium used in these experiments, $[\text{Ca}^{2+}]_i$, the free cytosolic Ca^{2+} concentration, was respectively 37, 122 and 1220 μM in control erythrocytes and 31, 102 and 1020 μM in ATP-depleted erythrocytes (Ferreira & Lew, 1976; Escobales & Canessa, 1985). The content and the radioactivity of polyphosphoinositides was measured (see the Materials and methods section). (a) and (b) PtdIns4P content (▲) and specific radioactivity (Δ); (c) and (d), PtdIns(4,5) P_2 content (●) and specific radioactivity (○). Data are means $\pm$ S.E.M. of duplicate determinations from three experiments or means of duplicate determinations in two experiments.

increase in the size of the PIC-sensitive PtdIns4P pool, showing that the newly synthesized PtdIns4P molecules are PIC-sensitive. The effect of the inhibition of PtdIns4P kinase (an increase in the content of PtdIns4P) also caused an increase in the size of the PIC-sensitive PtdIns4P pool, indicating that PIC and PtdIns4P kinase act on the same pool of PtdIns4P. Thus PtdIns4P molecules behave as two distinct pools: (i) a PIC-sensitive one (pool 1), the product of PtdIns kinase and the substrate for PtdIns4P kinase and (ii) a PIC-insensitive one (pool 2) linked neither to PtdIns nor to PtdIns(4,5) P_2 .

Fig. 6. Effect of the concentration of external CaCl_2 on Ins(1,4,5) P_3 content in ionophore-treated control (●) and ATP-depleted erythrocytes (○)

Experimental conditions were the same as those described in the legend to Fig. 5, except that cell prelabelling was for 2.5 h. Ins(1,4,5) P_3 radioactivity was measured in neutralized HClO_4 extracts of erythrocytes by h.p.l.c. (see the Materials and methods section). Ins(1,4,5) P_3 content was calculated from the specific radioactivity of the PIC-sensitive pool of PtdIns(4,5) P_2 . This latter was itself calculated from the specific radioactivity of the total and of the PIC-resistant pools of PtdIns(4,5) P_2 and from the size of these pools. Results are means of duplicate determinations in a single experiment; three others gave similar values.

Since PtdIns kinase only had access to pool 1, the labelling of pool 2 was achieved through exchange from pool 1. The second effect of the inhibition of PtdIns4P kinase [a decrease in the content of PtdIns(4,5) P_2 due to the activity of the PtdIns(4,5) P_2 phosphatase] caused (i) a decrease in the size of the PIC-sensitive PtdIns(4,5) P_2 pool, indicating, as already shown by Ins(1,4,5) P_3 measurements, that PIC and PtdIns(4,5) P_2 phosphatase act on the same pool of PtdIns(4,5) P_2 ; and (ii) a decrease in the specific radioactivity of PtdIns(4,5) P_2 , indicating that the PIC-sensitive pool of PtdIns(4,5) P_2 left after the phosphatase action had a higher specific radioactivity than the PIC-resistant pool and thus was its metabolic precursor. PtdIns(4,5) P_2 molecules behave as three distinct pools: (i) a phosphatase and PIC-sensitive one (pool 3), a substrate for PtdIns(4,5) P_2 phosphatase and a product of PtdIns4P kinase, (ii) a PIC-sensitive one (pool 4) insensitive to PtdIns(4,5) P_2 phosphatase, and (iii) a PIC-insensitive one (pool 5) linked only with pool 4, its

Fig. 7. Schematic model of the metabolic compartmentation of polyphosphoinositides in human erythrocytes

PtdIns4P (pools 1 and 2) and PtdIns(4,5)P₂ (pools 3, 4 and 5) are divided into rapidly labelled pools (1 and 3), PIC-sensitive pools (1, 3 and 4) and PIC-resistant pools (2 and 5) (see text). The rapidly turned-over pools amount to about 70% of PtdIns4P and 20% of PtdIns(4,5)P₂.

precursor. An exchange between the molecules of the three pools also occurs as in the case of PtdIns4P, but the rate of exchange is slower, so that if the labelling time is short enough, differences in the specific radioactivities of each pool could be observed. In this model, the sizes of the 'rapid pools' (pool 1 and pool 3) can be estimated from the data of Table 1 and Figs 3 and 5. Pool 1 amounted to 62–74% of total PtdIns4P, and pool 3 to 16–31% of total PtdIns(4,5)P₂. Müller *et al.* (1986), using the very early dynamic phase of ³²P labelling of polyphosphoinositides, have calculated that the rapidly turned over pools amount to 25–60% of PtdIns4P and to 6–10% of PtdIns(4,5)P₂ molecules. Presumably pools 1 and 3 in our model contain the sum of several rapid subcompartments including those detected by the methodology used by Müller *et al.* (1986).

Conclusion

The data presented here demonstrate that after 23 h of labelling with ³²P, the specific radioactivity of the monoester phosphate groups of polyphosphoinositides in erythrocytes approached an equilibrium value close to that of the γ -phosphate of ATP (78–85%). However, in addition to this apparent metabolic homogeneity, ac-

tivation of PIC and inhibition of kinases have revealed a functional heterogeneity of these phospholipids, those which are rapidly turned over being the PIC-sensitive ones. This concept of compartmentalization of phosphoinositides, first suggested by Fain & Berridge (1979) in blowfly salivary glands, has been further demonstrated in cells such as WRK-1 (Monaco, 1982), platelets (Billah & Lapetina, 1982; Vickers & Mustard, 1986) and pancreatic islets (Rana *et al.*, 1985) in terms of agonist-sensitive and -insensitive pools. Functional heterogeneity of phosphoinositides with specialized pools involved in signal transduction, in protein–lipid interactions to anchor membrane-associated proteins (Low & Kincade, 1985) and in protein–protein interactions as in the case of glycophorin–protein 4.1 in erythrocytes (Anderson & Marchesi, 1985), may be a general feature in various cellular membranes.

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