Biochem. J. (1987) 244, 209–217 (Printed in Great Britain)

Multiple metabolic pools of phosphoinositides and phosphatidate in human erythrocytes incubated in a medium that permits rapid transmembrane exchange of phosphate

Christopher E. KING, Leonard R. STEPHENS, Phillip T. HAWKINS, Graeme R. GUY and Robert H. MICHELL
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT, U.K.

1. A Hepes-based medium has been devised which allows rapid P_i exchange across the plasma membrane of the human erythrocyte. This allows the metabolically labile phosphate pools of human erythrocytes to come to equilibrium with [^{32}P]P_i in the medium after only 5 h *in vitro*. 2. After 5–7 h incubation with [^{32}P]P_i in this medium, only three phospholipids, phosphatidic acid (PtdOH), phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) are radioactively labelled. The concentrations of PtdIns4*P* and PtdIns4,5P_2 remain constant throughout the incubation, so this labelling process is a reflection of the steady-state turnover of their monoester phosphate groups. 3. During such incubations, the specific radioactivities of the monoesterified phosphates of PtdIns4*P*, PtdIns4,5P_2 and PtdOH come to a steady value after 5 h that is only 25–30% of the specific radioactivity of the γ-phosphate of ATP at that time. We suggest that this is a consequence of metabolic heterogeneity. This heterogeneity is not a result of the heterogeneous age distribution of the erythrocytes in human blood. Thus it appears that there is metabolic compartmentation of these lipids within cells, such that within a time-scale of a few hours only 25–30% of these three lipids are actively metabolized. 4. The phosphoinositidase C of intact human erythrocytes, when activated by Ca^{2+}-ionophore treatment, only hydrolyses 50% of the total PtdIns4,5P_2 and 50% of ^{32}P-labelled PtdIns4,5P_2 present in the cells: this enzyme does not discriminate between the metabolically active and inactive compartments of lipids in the erythrocyte membrane. Hence at least four metabolic pools of PtdIns4*P* and PtdIns4,5P_2 are distinguishable in the human erythrocyte plasma membrane. 5. The mechanisms by which multiple non-mixing metabolic pools of PtdOH, PtdIns4*P* and PtdIns4,5P_2 are sustained over many hours in the plasma membranes of intact erythrocytes are unknown, although some possible explanations are considered.

INTRODUCTION

Incubation of mammalian erythrocytes with [^{32}P]P_i in physiological media leads to the incorporation of ^{32}P into three phospholipids: PtdOH, PtdIns4*P* and PtdIns4,5P_2 (Hokin *et al.*, 1963; Hokin & Hokin, 1964*a*; Peterson & Kirschner, 1970; Buckley & Hawthorne, 1972). This ^{32}P is derived directly from the γ-phosphate of ATP by the action of lipid kinases (diacylglycerol kinase, PtdIns kinase and PtdIns4*P* kinase) and is only found in the monoester phosphate groups of these lipids (Hokin & Hokin, 1964*a*; Schneider & Kirschner, 1970). These reactions progress in intact erythrocytes while the cellular concentrations of PtdOH, PtdIns4*P* and PtdIns4,5P_2 remain constant, hence the monoester phosphate groups must be turning over (Peterson & Kirschner, 1970; Müller *et al.*, 1986). The balancing degradative reactions which complete the turnover cycles of the monoester phosphate groups are catalysed by PtdOH phosphatase, PtdIns4*P* phosphomonoesterase and PtdIns4,5P_2 phosphomonoesterase (Hokin & Hokin, 1961; Brockerhoff & Ballou, 1962; Roach & Palmer, 1981; Mack & Palmer, 1984).

Studies using human erythrocytes have shown that the specific radioactivity of the γ-phosphate of ATP remains very much greater than the specific radioactivities of PtdOH, PtdIns4*P* and PtdIns4,5P_2, even in cells that have been incubated for several hours in media containing [^{32}P]P_i (Dale, 1985; Giraud *et al.*, 1984; Maretzki *et al.*, 1983), but these observations were neither emphasized by the authors of these papers nor effectively explained. Under the 'physiological' ionic conditions in which these experiments were carried out, the specific radioactivities of the γ-phosphate of ATP and the polyphosphoinositides were still rising after 3–5 h of incubation, probably because of the slow exchange of P_i on the erythrocyte membrane anion transporter, which it shares with Cl^- and other anions (Whittam, 1964). Thus the difference in the specific radioactivities of ATP and the lipids could be explained in either of two ways: (*a*) the turnover of the lipids is sufficiently slow to cause their labelling to lag well behind that of the γ-phosphate of ATP, or (*b*) there is metabolic compartmentation, with only a fraction of the total lipids undergoing metabolic turnover.

We have investigated possible metabolic pooling of these membrane phospholipids that bear monoester phosphate groups by using an incubation medium free of competing anions that therefore permits rapid P_i exchange across the plasma membrane of human erythrocytes, thus allowing [^{32}P]P_i to attain an equilibrium distribution more rapidly.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidate; GroPIns4,5P_2, glycerophosphoinositol 4,5-bisphosphate; PIC, phosphoinositidase C; 2,3-DPG, 2,3-diphosphoglycerate.

MATERIALS AND METHODS

Isolation of erythrocytes and incubation of cells with [^{32}P]P_i

Blood was either from the local Blood Transfusion Centre (maximum 7 days after donation), or was taken from a volunteer into a sterile donation bag containing CPD-adenine (115 mM-citrate/NaOH, pH 7.3, 160 mM-glucose, 21 mM-NaH_2PO_4, 2 mM-adenine) (Travenol Laboratories, Thetford, Norfolk, U.K.). The erythrocytes were washed four times in ice-cold 154 mM-NaCl/1.5 mM-Hepes, pH 7.2, the buffy coat and suernatant being discarded each time. Residual leucocytes were removed by passing the washed cells through a sterile cotton wool column (Cellselect Leukocyte filter). Erythrocytes were then washed once and incubated in one of two alternative media. The first, a NaCl-based medium ('chloride medium') based on that of Shukla *et al.* (1978), contained 137.3 mM-NaCl, 5.0 mM-NaHepes buffer, pH 7.2, 5 mM-sodium pyruvate, 1.18 mM-magnesium gluconate, 1.8 mM-K_2HPO_4, 1 mM-inosine, 1 mM-adenine, 10 mM-glucose 0.07% (w/v) dialysed bovine serum albumin, 2 mM-calcium gluconate, 100 μg of streptomycin/ml, 100 i.u. of penicillin/ml and 2.5 μg of amphotericin B (Flow Laboratories)/ml. This medium has free Ca^{2+} concentration of approx. 1 mM as measured with a Ca^{2+}-sensitive electrode. The alternative medium ('Hepes medium') was identical with the above except that the NaCl and NaHepes buffer were relaced with 188 mM-Hepes/NaOH, pH 7.2, so yielding a medium that was essentially free of Cl^-. For incubation, erythrocytes were suspended at a haematocrit of 20%. Cell suspensions were preincubated at 37 °C in stoppered polypropylene bottles for 1 h before addition of carrier-free [^{32}P]P_i (1–10 μCi/ml; PBS 11, Amersham International), although in some cases [^{32}P]P_i was added at later times (see the text for details).

Isolation of PtdIns4*P* and PtdIns4,5P_2

Samples of an erythrocyte suspension (5 ml, containing 1 ml of packed cells) were lysed in ice-cold 20 mM-Tris/HCl/2 mM-EDTA, pH 7.2 (70 ml; 'lysis buffer'). This procedure preserves the concentrations of polyphosphoinositides *in vivo* (Müller *et al.*, 1986). The membranes were sedimented (15000 *g* for 20 min) and washed in lysis buffer until white (usually four to six washes). Lipids were extracted from the membranes (final vol. 2 ml) by the addition of chloroform/methanol/HCl (20:40:1, by vol.; 7.5 ml). After 20 min at room temperature, this solvent mixture was partitioned by addition of 0.1 M-HCl (2.5 ml) and chloroform (2.5 ml). The resulting lower phase was taken, dried *in vacuo*, and the lipids were dissolved in chloroform. Samples were applied to t.l.c. plates [10 cm × 10 cm, cut from Polygram 20 cm × 20 cm SIL H-HR plastic-backed plates (Camlab)]. Two separation methods were employed. A one-dimensional technique (Jolles *et al.*, 1981) yielded chemically pure PtdIns4,5P_2 in a single step. Although this separation could produce radiochemically pure PtdIns4*P*, a phosphate-containing compound(s) co-chromatographed sufficiently closely to make assay of total PtdIns4*P* impossible. Consequently, the samples were chromatographed twice to obtain uncontaminated PtdIns4*P*. Firstly, they were resolved in a two-dimensional system (Ferrell & Huestis, 1984), leaving the polyphosphoinositides on the origin. PtdOH analyses were carried out on material from these plates. Secondly, the origin was scraped from the plate and the polyphosphoinositides were eluted with chloroform/methanol/HCl (20:40:1, by vol.) containing 5% (v/v) water, applied to a new plate, and PtdIns4*P* was separated by the one-dimensional separation described above. Recoveries through this procedure exceeded 85%.

Radioactive lipids were detected with Fuji X-ray film, and non-radioactive lipids with iodine vapour. Spots were scraped from the plate into acid-washed digestion tubes and digested with 72% (w/w) $HClO_4$. P_i in this hydrolysate was assayed by the method of Bartlett (1959) as modified by Galliard *et al.* (1965). Radioactivity was measured by Čerenkov counting in a liquid-scintillation counter.

Some ^{32}P-labelled PtdIns4,5P_2 spots were scraped off plates and the silica gel was placed in the deacylating mixture of Clarke & Dawson (1981). The resulting glycerophosphoinositol 4,5-bisphosphate was purified by their procedure.

Acid extraction of erythrocytes

A sample of cell suspension (0.5 ml) was withdrawn and immediately added to ice-cold 0.154 M-NaCl (0.5 ml)/1.5 mM-NaHepes, pH 7.2, in a Microfuge tube. The cells were sedimented at full speed in an MSE Micro Centaur centrifuge for 5 s, and a sample of the supernatant was removed (0.8 ml), to be stored frozen if P_i (Baginski *et al.*, 1967) or total radioactivity in the medium was to be determined. Ice-cold water (0.4 ml) was rapidly added to lyse the erythrocyte pellet. After vigorous mixing, 20% $HClO_4$ (0.1 ml) was added and, after 5 min on ice, the precipitated protein was sedimented at 15000 rev./min in an MSE Micro Centaur microcentrifuge for 1 min. A 0.4 ml sample of the supernatant was withdrawn and added to 0.5 ml of ice-cold fluorotrichloromethane/tri-n-octylamine (1:1, v/v) (Sharpes & McCarl, 1982). After vigorous mixing the samples were centrifuged at 15000 rev./min for 1 min, and a sample of the water-soluble supernatant (0.3 ml; the $HClO_4$-freed 'acid extract', now at pH 6.5) was stored frozen for assay of the specific radioactivity of the γ-phosphate of ATP and the radioactivity in, and the concentrations of, ATP and 2,3-DPG (described below). A sample of each acid extract was also analysed for its total radioactivity and total phosphorus content (Galliard *et al.*, 1965) to determine the specific radioactivity of the total water-soluble intracellular contents.

ATP

The specific radioactivity of the γ-phosphate of ATP in an 'acid extract' was assayed by the method of Hawkins *et al.* (1983). The concentration of ATP was assayed by the method of Lamprecht & Trautschold (1974). The total radioactivity in ATP was measured by applying a sample of the acid extract to a h.p.l.c. system (LDC Milton Roy) with an anion-exchange column (25 cm × 0.46 cm; Partisil 10 SAX) and a gradient formed at 1.25 ml/min from water (A) and 1.7 M-ammonium formate adjusted to pH 3.7 with H_3PO_4 (B) (0 min, 0% B; 5 min, 0% B; 10 min, 44.1% B; 12 min, 44.1% B; 18 min, 58.8% B; 23 min, 58.8% B, 33 min, 99.9% B, 39 min, 99.9% B). The A_{254} was continuously monitored, and the position of the peak corresponding to ATP was confirmed for each sample. Fractions were collected every 0.3 min and their ^{32}P content was

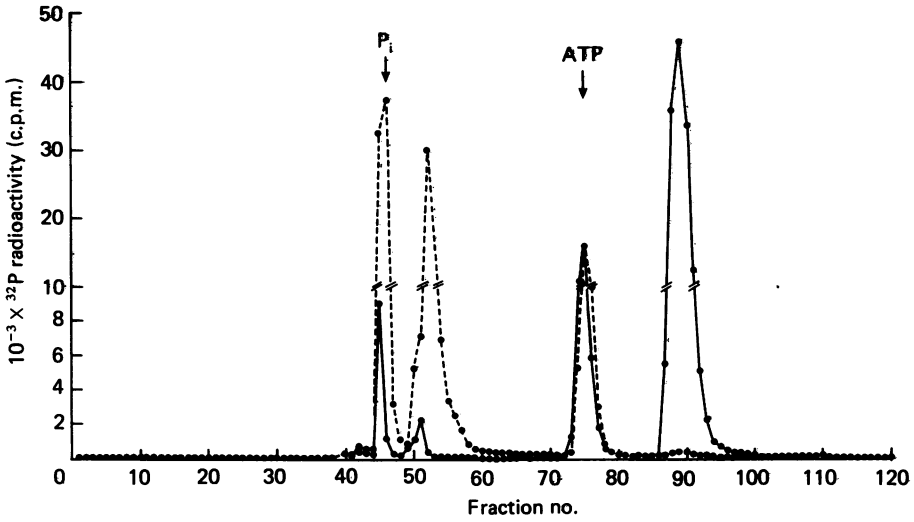

Fig. 1. Separation of [^{32}P]P_i-labelled compounds from erythrocytes

Erythrocytes were incubated with [^{32}P]P_i in Hepes medium for 5 h, acid-extracted (see the Materials and methods section), and a sample of the neutralized acid extract was applied to an anion-exchange h.p.l.c. column (see the Materials and methods section). Radioactivity in fractions of the eluate was determined by Čerenkov counting (continuous line). Another sample of the same extract was incubated with 2,3-DPG phosphatase for 30 min at 37 °C, then separated by h.p.l.c. (broken line). The enzyme treatment resulted in the quantitative transfer of radioactivity from a peak eluted after ATP (i.e. 2,3-DPG) into two earlier-eluted peaks (P_i and 3-phosphoglycerate). The peak labelled 'ATP' coincided with a peak of u.v.-absorbing material, which was eluted in the same fractions as authentic ATP [Boehringer Corp. (London)].

measured by Čerenkov counting. This revealed that the total specific radioactivity of ATP was approximately twice that of its γ-phosphate for the first 3 h of incubation, confirming the observation by Müller *et al.* (1986) that the β- and γ-phosphate groups of ATP are rapidly equilibrated by adenylate kinase. After longer periods, radioactivity began to appear in the α-phosphate (6% of total ^{32}P in α-phosphate after 2 h, 18% after 7 h).

Assay of 2,3-DPG

2,3-DPG concentrations in the acid extracts were assayed with a diagnostic kit (Sigma). This is a linked enzyme assay involving the hydrolysis of 2,3-DPG and the stoichiometric oxidation of NADH via a series of linked enzyme reactions. The quantity of [^{32}P]P_i in the 2,3-DPG of an acid extract was determined by Čerenkov counting of the appropriate fractions of eluent from an h.p.l.c. column (see Fig. 1 and its legend for details).

Distribution of radioactivity between 4- and 5-phosphate groups of PtdIns4,5P_2

Ghosts from erythrocytes incubated for 7 h in [^{32}P]P_i-containing HEPES medium (prepared as described above) were analysed by one of two methods.

Method 1. Ghosts were extracted with chloroform/methanol/HCl (as described above) to yield a mixture of lipids which were deacylated by the method of Clarke & Dawson (1981). The resulting ^{32}P-labelled glycerophosphoinositol phosphates were separated on small Bio-Rad anion-exchange resin columns (AG 1X8, 200–400 mesh; formate form). The fraction containing radiochemically pure [^{32}P]Gro*P*Ins4,5P_2 was desalted by freeze-drying, resuspended in 50 mM-Hepes/5 mM-MgCl2, pH 7.2, and then split into two portions. One was incubated for 90 min at 37 °C with human erythrocyte ghosts to determine the amount of radioactivity in the 4- and 5-phosphate groups, by the method of Hawkins *et al.* (1984). In membranes from cells labelled with [^{32}P]P_i for 7 h in Hepes medium, 50±2% of the ^{32}P was in the 5-phosphate. The remainder of the sample was treated with periodate under conditions which restricted oxidation to the glycerol moiety (Brown & Stewart, 1964). It should be noted that similar treatment of Gro*P*Ins4*P* results in significant oxidation of the inositol ring, resulting in the production of an unidentified ^{32}P-labelled compound. The reaction was terminated by the addition of excess ethylene glycol and the mixture processed exactly as described by Brown & Stewart (1964) to yield radiochemically pure [^{32}P]Ins1,4,5P_3. The radioactivity in the 4- and 5-phosphate groups of this compound was determined as described by Hawkins *et al.* (1984). In the sample mentioned above, 49.5±3% was found in the 5-phosphate, as in Gro*P*Ins4,5P_2 obtained from that sample.

Method 2. The ^{32}P-labelled ghosts were incubated for 20 min at 37 °C in the presence of 1 mM-Ca^{2+}. The [^{32}P]Ins1,4,5P_3 appearing in the supernatant after sedimentation of the membranes (15000 *g* for 20 min at 4 °C) was separated on Bio-Rad Ag1 × 10 anion-exchange resin, desalted, and the radioactivity in its 4- and 5-phosphate groups determined as described above. The [^{32}P]PtdIns4,5P_2 remaining in the membranes (40% of the original) was extracted, deacylated, radiochemically purified, and the distribution of radioactivity between its 4- and 5-phosphate groups assayed as described above. With the same sample as mentioned above, there was equal radioactivity in the 4- and 5-phosphate groups both of the [^{32}P]Ins1,4,5P_3 produced by the PIC (Downes & Michell, 1981) and of the PtdIns4,5P_2 remaining in the membranes after PIC activation.

In most experiments in which knowledge of the

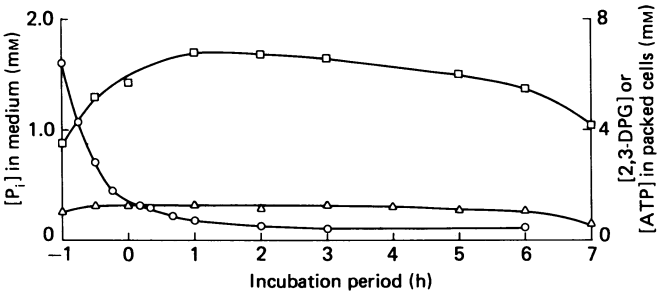

Fig. 2. Cellular concentrations of ATP, 2,3-DPG and uptake of P_i in erythrocytes incubated in Hepes medium

Erythrocytes were incubated in Hepes medium as described in the Materials and methods section. Portions of the incubation were removed at various times and assayed for extracellular P_i (○), ATP (△) and 2,3-DPG (□) concentrations (see the Materials and methods section). Times of −1 and 0 h represent the start and end of the preincubation period respectively. Data are pooled from several experiments, plotted as means (P_i, $n = 4$; ATP, $n = 6$; 2,3-DPG, $n = 3$).

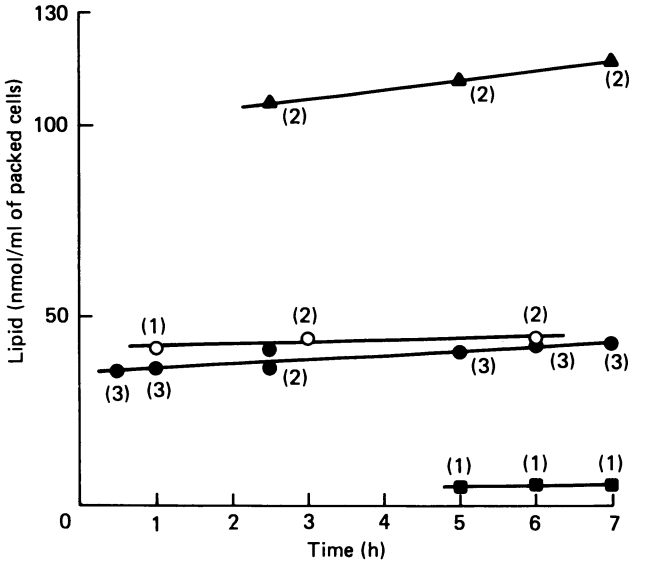

Fig. 3. Cellular concentrations of PtdOH, PtdIns4*P* and PtdIns4,5P_2

Erythrocytes were incubated with [^{32}P]P_i in either Hepes or chloride medium (see the Materials and methods section). At various times, portions of each incubation were removed into ice-cold 'lysis buffer' (see the Materials and methods section), membranes were prepared, and the lipids extracted. Lipid extracts were separated on t.l.c. plates, and the concentration of each lipid determined as described in the Materials and methods section. The black symbols show the concentrations of PtdOH (▲), PtdIns4*P* (■) and PtdIns4,5P_2 (●) in cells incubated in Hepes; the ○ symbol shows the concentration of PtdIns4,5P_2 in cells incubated in chloride medium.

distribution of radioactivity between the 4- and 5-phosphate groups of [^{32}P]PtdIns4,5P_2 was required, the protocol employing Ca^{2+} activation of erythrocyte ghost PIC, followed by analysis of the released [^{32}P]Ins1,4,5P_3, was used because of its greater simplicity.

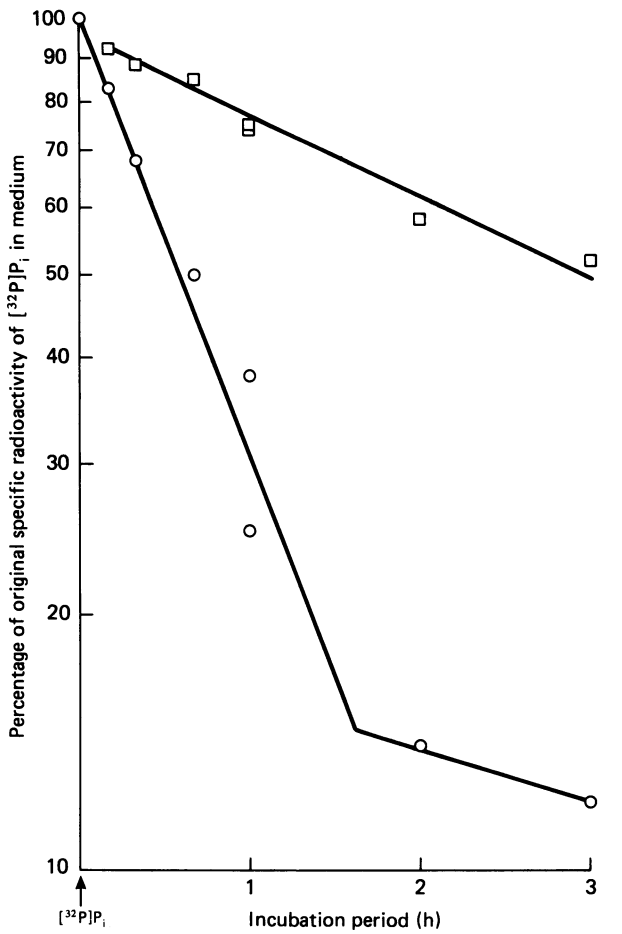

Fig. 4. Exchange of [^{32}P]P_i across the human erythrocyte membrane

Erythrocytes were incubated with [^{32}P]P_i in either Hepes (○) or chloride (□) medium (see the Materials and methods section). At various times, samples of each incubation were taken, and the specific radioactivity of P_i in the extracellular medium was assayed (calculated from P_i concentration and radioactivity; see the Materials and methods section). Data shown are means of triplicate determinations; data were pooled from two experiments.

Separation of erythrocytes on the basis of density

The method used was that of Murphy (1973). Basically, 50 ml of washed packed erythrocytes (approx. 95% haematocrit) were centrifuged for 70 min at 32000 *g* in an angle rotor at 30 °C. After cooling to 4 °C, 2 ml fractions were taken sequentially from the top of the erythrocyte column, yielding a series of fractions containing erythrocytes of increasing density. Samples of each fraction were assayed for haemoglobin (Sigma diagnostic assay kit) and acetylcholinesterase activity (Beutler, 1984). Erythrocyte acetylcholinesterase activity and the acetylcholinesterase/haemoglobin ratio both decrease as the cells age (Cohen *et al.*, 1976), so providing a means of verification of the age-separation procedure. Changes in the acetylcholinesterase/haemoglobin ratio between the top (least dense) and bottom (most dense) fractions agreed well with values reported by Cohen *et al.* (1976) (see the Results section). Pooled fractions from different portions of the cell column were

washed once in Hepes medium (to remove Cl^- ions), and incubated with $[^{32}P]P_i$ as described above.

RESULTS

Metabolic state of erythrocytes incubated in Hepes medium

When human erythrocytes were incubated in Hepes medium (described in the Materials and methods section), the concentration of P_i in the extracellular medium fell during the first 60 min, during which time there was an essentially stoichiometric rise in the intracellular concentration of 2,3-DPG (see Fig. 2). After this first 1 h of incubation, and for at least 7 h thereafter, the cells were healthy and close to metabolic steady state, as confirmed by almost constant cellular concentrations of ATP (Fig. 2), 2,3-DPG (Fig. 2), PtdOH (Fig. 3) and polyphosphoinositides (Fig. 3) and minimal haemoglobin leakage (results not shown). The cells showed normal biconcave disc morphology throughout, as assessed by scanning electron miroscopy after fixation with glutaraldehyde and OsO_4 and coating with gold (results not shown). In addition, the lipid contents were the same as in cells incubated in the 'more physiological' chloride medium (Fig 3). In experiments designed to study the steady-state metabolism of the polyphosphoinositides, tracer quantities of $[^{32}P]P_i$ were added to the cell suspensions after 1 h of incubation (see the Materials and methods section).

Labelling of the acid-extractable components of erythrocytes with $[^{32}P]P_i$

Fig. 4 shows the difference in the rate of exchange of $[^{32}P]P_i$ between the extracellular medium and cells, when incubated in chloride medium and in Hepes medium. The

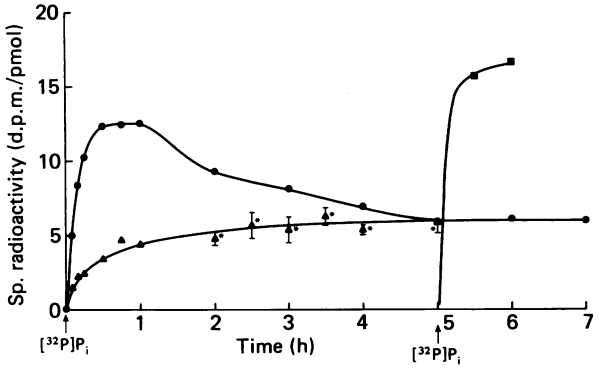

Fig. 5. Incorporation of $[^{32}P]P_i$ into γ-phosphate of ATP in Hepes and chloride media

Erythrocytes were incubated with $[^{32}P]P_i$ in either Hepes or chloride medium (see the Materials and methods section). At various times, samples of each incubation were taken, acid-extracted, and the extract was assayed for ATP γ-phosphate specific radioactivity (see the Materials and methods section). In a separate incubation, $[^{32}P]P_i$ was also added to a suspension of cells in Hepes medium 5 h after the end of the preincubation period. Specific radioactivities are shown of the γ-phosphate of ATP from cells in: ●, Hepes medium, $[^{32}P]P_i$ added at 0 h; ■, Hepes medium, $[^{32}P]P_i$ added after 5 h; ▲, chloride medium, $[^{32}P]P_i$ added at 0 h. All points are means of duplicates from a single experiment, except for chloride data (*), which are pooled from two experiments, and are means ± S.D. ($n = 3$).

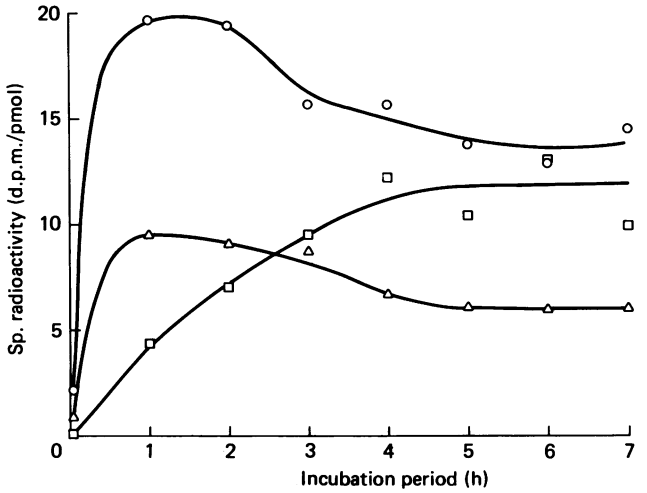

Fig. 6. Specific radioactivities of ATP and 2,3-DPG in erythrocytes incubated in Hepes medium

Erythrocytes were incubated in Hepes medium as described in the Materials and methods section. At various times, portions of the incubation were removed, acid-extracted (see the Materials and methods section), and assayed for 2,3-DPG concentration and radioactivity, ATP concentration and radioactivity, and ATP γ-phosphate specific radioactivity (see the Materials and methods section). ATP total and 2,3-DPG specific radioactivities were calculated from the concentration and radioactivity of each compound. △, ATP γ-phosphate specific radioactivity; ○, ATP total specific radioactivity; □, 2,3-DPG specific radioactivity. All points are means of duplicate or triplicate determinations from a single experiment.

$t_{\frac{1}{2}}$ of $[^{32}P]P_i$ exchange in cells incubated in Hepes medium was ≃ 45 min, compared with ≃ 180 min in chloride medium. The increased rate of exchange of $[^{32}P]P_i$ in Hepes medium was reflected in an increased rate of ATP labelling (see Fig. 5). The $[^{32}P]P_i$ entered the β- and γ-phosphate pools of ATP (see Fig. 6) at similar rates, presumably as a consequence of the large adenylate kinase activity in erythrocytes (Müller *et al.*, 1986). During this phase, in which the $[^{32}P]P_i$ in ATP rose rapidly, the ATP concentration remained constant (see above and Fig. 2). Consequently, this labelling of ATP can be attributed to metabolic turnover rather than net synthesis. Taken together, these data suggest that in Cl^--based media the rate of appearance of $[^{32}P]P_i$ in ATP is heavily restrained at the level of phosphate entry (Reich, 1968), and that in the Hepes medium this restraint has been substantially decreased.

During the incubation of erythrocytes with $[^{32}P]P_i$ in Hepes medium, the specific radioactivity of ATP (both γ-phosphate and total) rapidly attained a peak value, followed by a decline to a steady state that was maintained for at least 2–3 h (see Figs. 5 and 6). $[^{32}P]P_i$ added during the late plateau phase was rapidly incorporated into ATP (see Fig. 5), demonstrating that the steady specific radioactivity during this period represents a dynamic equilibrium owing to ATP turnover, rather than a static value maintained because ATP synthesis had ceased. The decline in the specific radioactivity of ATP was due to metabolic equilibration of $[^{32}P]$ATP with unlabelled 2,3-DPG, which contains the

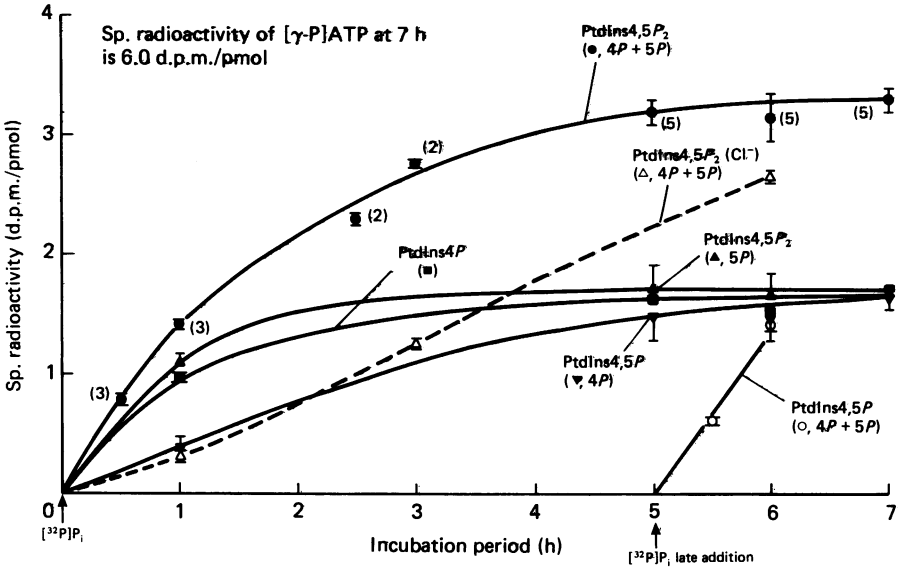

Fig. 7. Incorporation of $[^{32}P]P_i$ into the phospholipids of erythrocytes incubated in Hepes and chloride media

Erythrocytes were incubated with $[^{32}P]P_i$ in either Hepes or chloride medium (see the Materials and methods section). Concentrations of PtdOH, PtdIns4*P* and PtdIns4,5P_2 were determined as described in the legend to Fig. 3. The radioactivity in each lipid, and the distribution of $[^{32}P]P_i$ between the 4- and 5-phosphates of PtdIns4,5P_2, were determined as described in the Materials and methods section. Data from cells incubated in Hepes medium: ●, PtdIns4,5P_2 specific radioactivity; ▼, PtdIns4,5P_2 4-phosphate specific radioactivity; ▲, PtdIns4,5P_2 5-phosphate specific radioactivity; ■, PtdIns4*P* specific radioactivity. In a separate incubation, addition of $[^{32}P]P_i$ was delayed until 5 h after the end of the preincubation period (see the Materials and methods section). After this late addition of $[^{32}P]P_i$, the specific radioactivity of PtdIns4,5P_2 was determined (○). Data from cells incubated in chloride medium: PtdIns4,5P_2 specific radioactivity (△, broken line). The specific radioactivity of PtdOH attained a final steady-state value at 5 h of 1.61 ± 0.06 d.p.m./pmol (mean ± S.D., $n = 3$). Data are single point determinations pooled from three experiments, shown either as single points or as means ± S.D. for two to five determinations.

majority of the intracellular phosphate (see Figs. 1 and 2), and turns over relatively slowly (see Fig. 6).

Labelling of erythrocyte lipids with $[^{32}P]P_i$

As noted above, the concentrations of PtdOH, PtdIns4*P* and PtdIns4,5P_2 remained approximately constant throughout the 7 h incubation period. The rapid incorporation of $[^{32}P]P_i$ into these lipids in both incubation media must therefore be a consequence of the turnover of their monoester phosphate groups. In cells incubated in Hepes medium, the specific radioactivities of PtdOH, PtdIns4*P* and PtdIns4,5P_2 rose much more rapidly than in cells in chloride medium (Fig. 7, and results not shown), suggesting that in Cl^- medium the rate of labelling of the lipids was heavily constrained by the rate of P_i entry. Inspection of Fig. 7 makes it clear that incubations in the Cl^- medium of considerably more than 7 h would have been needed before the specific radioactivity of PtdIns4,5P_2 reached a constant value.

The rapid initial rise in specific radioactivity of all three lipids seen in cells incubated in Hepes medium reached a plateau after approx. 5 h at a value that was 25–30% of that of the γ-phosphate at ATP (see Fig. 7). This difference could be explained either by a cessation of lipid turnover in cells incubated for long periods, or because only a fraction of the lipids were being turned over. Addition of $[^{32}P]P_i$ after 5 h led to an initial rate of incorporation of $[^{32}P]P_i$ into PtdIns4,5P_2 identical with that seen with the earlier $[^{32}P]P_i$ addition (see Fig. 7). Thus metabolic turnover was still occurring at the end of our incubations, and the failure of the lipids to achieve isotopic equilibrium with the γ-phosphate of ATP must be due to metabolic compartmentation. Whether the 'unlabelled' compartment is simply slowly metabolized or is metabolically inactive over the entire lifespan of the erythrocytes cannot be established from this type of experiment.

There are two basic ways in which such metabolic compartmentation could arise: the two pools of lipids (those turned over, and those not) could either co-exist in each cell or be present in different cells. The only well-characterized heterogeneity in the circulating erythrocytes of any individual at a given time is that related to cell age (Cohen *et al.*, 1976). So one possible explanation of the observed compartmentation was that older cells incorporate $[^{32}P]P_i$ into ATP, but do not turn over the monoester phosphate groups of their lipids. To test this possibility, cells were separated by density into different age fractions (Table 1): older cells have a lower acetylcholinesterase/haemoglobin ratio than do newly formed cells (see the Materials and methods section). The cells of different ages were incubated with $[^{32}P]P_i$ in Hepes medium for 7 h. No difference either in PtdIns4,5P_2 concentration or in the size of the metabolically 'active' compartment of PtdIns4,5P_2 could be detected between old and young cells (see Table 1).

Phosphoinositidase C of the human erythrocyte

Human erythrocytes possess a membrane-bound phosphoinositidase C (PIC) that can be activated by high concentrations of Ca^{2+}, either by direct addition to a suspension of erythrocyte ghosts or by Ca^{2+}-ionophore treatment of intact cells (Allan & Michell, 1978; Allan &

Table 1. Metabolic heterogeneity of polyphosphoinositide metabolism in erythrocytes of different ages

The specific radioactivities of PtdIns4,5P_2 and the γ-phosphate of ATP from erythrocytes of different ages, incubated with [^{32}P]P_i in Hepes medium for 7 h, were determined as described in the Materials and methods section. Control and fractionated cells were from the same sample of blood. Control cells were subjected to the age-separation procedure, but were thoroughly mixed before being labelled with [^{32}P]P_i. Acetylcholinesterase activities (nmol/min per g of haemoglobin) for each fraction were as follows: 29.9, young; 27.6, medium; 22.8, old. [γ-^{32}P]ATP specific radioactivities are means ($n = 6$) of determinations from a single experiment. PtdIns4,5P_2 specific radioactivities are means ($n = 4$) of determinations from a single experiment. '% pool' indicates % of total cellular PtdIns4,5P_2 that is metabolically active.

Age fraction	[γ-^{32}P]ATP sp. radioactivity (d.p.m./pmol)	PtdIns4,5P_2 sp. radioactivity (d.p.m./pmol)	% pool
Control	9.2	6.76	37%
Young	9.2	5.78	31%
Medium	10.4	6.33	31%
Old	10.3	7.48	36%

Table 2. Ca^{2+} activation of phosphoinositidase C in human erythrocyte membranes

^{32}P-labelled membranes were prepared from erythrocytes that had been incubated in Hepes medium for 7 h. The membranes were incubated with or without 1 mM-Ca^{2+} in 20 mM-Tris/HCl, pH 7.2, for 20 min at 37 °C. At the end of this period, the membranes were sedimented (15000 *g* for 20 min at 4 °C), and the lipids were extracted and analysed as described in the Materials and methods section. All data are means ($n = 4$) of determinations from a single experiment, one of two which gave essentially identical results: nd, not determined.

	Amount of lipid (nmol/ml of packed cells)	Radioactivity (d.p.m./ml of packed cells)	Sp. radioactivity (d.p.m./pmol)
PtdIns4*P*			
No Ca^{2+}	nd	41400	nd
1 mM-Ca^{2+}	nd	16200	nd
PtdIns4,5P_2			
No Ca^{2+}	34.3	281000	7.7
1 mM-Ca^{2+}	14.0	106000	7.5

Thomas, 1981; Downes & Michell, 1981; Allan, 1982). However, Downes & Michell (1981) observed that a maximum of 60% of ^{32}P-labelled PtdIns4*P* and PtdIns4,5P_2 is hydrolysed in Ca^{2+}-treated ghosts, and we wondered whether there was a relationship between the pools of PIC-susceptible and PIC-resistant lipid and the metabolic compartments that we have described above.

When a ^{32}P-labelled ghost preparation identical with that of Downes & Michell (1981) was incubated with Ca^{2+}, approx. 60% of the total and 60% of the radioactive PtdIns4,5P_2 was hydrolysed (Table 2). In this ghost preparation, therefore, PIC was unable to distinguish between the metabolically active and inactive pools of lipid. However, the isolation of the ghosts, a procedure which involves repeated washing of the membranes with low-ionic-strength buffers, might have destroyed any pre-existing compartmentation of the membrane lipids. We therefore took intact erythrocytes that had been labelled to isotopic equilibrium and briefly exposed them to Ca^{2+} ionophore to activate their PIC. Under these conditions, there was hydrolysis of half of the total PtdIns4,5P_2 and half of the [^{32}P]PtdIns4,5P_2 (Table 3), indicating that the ability of PIC to hydrolyse both pools of lipid is an authentic attribute of the enzyme in the intact cell rather than a consequence of membrane isolation.

Table 3. Effect of Ca^{2+}-ionophore treatment on human erythrocyte polyphosphoinositides

Human erythrocytes were incubated for 6½ h with [^{32}P]P_i in Hepes medium. Control cells were treated with 2 μg of ionomycin/ml (added in ethanol) and 1 mM extra Ca^{2+} for 10 min. Cells were lysed, membranes prepared, and lipids extracted and analysed as described in the Materials and methods section. Control cells were treated identically, except for the addition of ethanol instead of ionophore. Data are means ($n = 4$) from a single experiment.

	[ATP] in cells (mM)	PtdIns4,5P_2 (nmol/ml of packed cells)	PtdIns4,5P_2 Sp. radioactivity (d.p.m./pmol)
Control	1.22	14.4	2.55
Ionophore-treated	0.86	7.5	2.45

DISCUSSION

Several previous workers have seen a difference between the specific radioactivities of the γ-phosphate of ATP and the polyphosphoinositides in human erythrocytes incubated for several hours in [^{32}P]P_i-containing physiological media, but the origin of this difference has never been seriously considered (see the Introduction). The data in the present paper show that only 25–30% of the PtdOH, PtdIns4*P* and PtdIns4,5P_2 in human erythrocytes is rapidly turned over, even in cells incubated with [^{32}P]P_i for prolonged periods, and that this discrepancy is largely or solely a consequence of metabolic compartmentation. Furthermore, the distribution of radioactivity between the 4- and 5-phosphate groups of PtdIns4,5P_2 equilibrates after 7 h incubation at a ratio of 1:1 (see Fig. 7). We can therefore suggest a model (Fig. 8) in which (*a*) the metabolically active pool of PtdIns4*P* is the precursor of the metabolically active pool of PtdIns4,5P_2 and (*b*) the PtdIns4*P* produced by the dephosphorylation of PtdIns4,5P_2 re-enters the original active pool of PtdIns4*P*.

Müller *et al.* (1986) have independently concluded that there is metabolic pooling in the turnover of human erythrocyte polyphosphoinositides. They arrived at this conclusion through a process of fitting their kinetic data on the very early dynamic phase of [^{32}P]P_i flux into the polyphosphoinositides to a metabolic model similar to

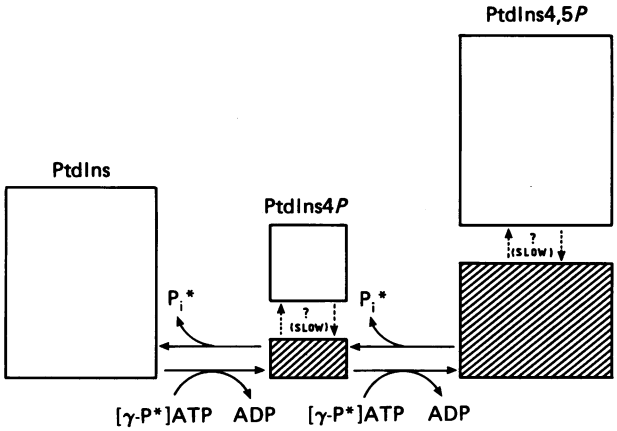

Fig. 8. Diagrammatic representation of the metabolic heterogeneity in human erythrocyte polyphosphoinositide metabolism

The relative pool sizes of the lipids PtdIns, PtdIns4*P* and PtdIns4,5P_2 are represented by the area of each box (shaded + unshaded for PtdIns4*P* and PtdIns4,5P_2). The shaded portions of the PtdIns4*P* and PtdIns4,5P_2 boxes represent the fraction of the total cellular pool of each lipid that is metabolically active and becomes labelled with ^{32}P (*). The arrows between the shaded and unshaded portions of the PtdIns4*P* and PtdIns4,5P_2 boxes represent mixing of the labelled and unlabelled pools of each lipid. Although not seen in the time-scale of the experiments reported here, this process may occur slowly *in vivo*. Pooling (or otherwise) of PtdIns cannot be determined in human erythrocytes, which cannot incorporate [^{32}P]P_i into phospholipid phosphodiester phosphate groups.

that of Fig. 8. They only obtained good coincidence between experimental data and the model by including a series of compartmentation factors in their analysis. Optimum fit between their data and the model was achieved by assuming that about one-half of PtdIns4*P* and one-tenth of PtdIns4,5P_2 were being turned over, but with large statistical variance in these estimates. The results that we present here confirm their claim that most of the human erythrocyte polyphosphoinositides are metabolically inactive, and give much more precise estimates of the metabolically active pools. In addition, we show that PtdOH metabolism is similarly compartmentalized.

Müller *et al.* (1986) did not consider potential explanations of this pooling phenomenon, which fall into two groups. The first type of explanation would be that the active and inactive pools exist in separate cells. The simplest model of this type would involve a parallel decline in the turnover of all three lipids as cells age, but with the retention of the cells' ability to incorporate [^{32}P]P_i into their ATP. Erythrocytes can be separated into different age fractions on the basis of their density (Murphy, 1973; and Table 1), and young and old cells from such a separation show no significant difference in their ability to turn over PtdIns4,5P_2 (Table 1). This result is in accord with a report by Palmer (1985), in which he showed no correlation between the activities of the individual enzymes that catalyse turnover of the polyphosphoinositides in human erythrocytes and the age of the cells.

It thus seems that the different lipid pools must co-exist within individual cells. Essentially this means that the lipid kinases and phosphomonoesterases within a single erythrocyte must have access to only a limited portion of the potential total lipid substrate or, for the kinases, to a fraction of the cellular ATP that has an atypically low turnover rate. Our results are in accord with those of Beutler *et al.* (1978), who showed that erythrocyte ATP is metabolized in a homogenous manner within a time scale of only a few minutes, so it seems very unlikely that the observed failure of lipids and ATP to come to metabolic equilibrium is a consequence of heterogeneous ATP metabolism.

Two basic mechanisms can be imagined by which a group of enzymes acting on lipid substrates in a membrane could have access to only a proportion of their potential substrate molecules. Either: (*a*) some of the lipid molecules do not serve as substrates for the relevant enzymes because they have inappropriate fatty acids; or (*b*) the metabolically inert lipids are held within 'domains' of the membrane structure in which they are inaccessible to the enzymes in question. Nothing is known of the effects of the fatty acid substituents on the catalytic activities of the erythrocyte lipid kinases and phosphatases.

Possible structural bases for membrane domains in which some lipid molecules could be inaccessible to enzymes within the same membrane are of two types. Either the enzyme and the lipids with which they fail to react could be in different leaflets of the lipid bilayer, or there could be inhibitory and long-term (hours or more) molecular associations between the particular lipid molecules and other components within one leaflet of the bilayer.

It appears unlikely, in our experiments, that the metabolically active and inert pools of lipid were present in the inner and outer leaflets of the lipid bilayer, respectively. Although it is widely accepted that PtdIns4*P* and PtdIns4,5P_2 are localized in the inner leaflet of the plasma membrane, where there are active kinases (Garrett & Redman, 1975) and a ready supply of ATP (reviewed by Downes & Michell, 1982), none of the available evidence indicates either the biosynthetic origin or the intramembrane location of the 'metabolically inert' lipid molecules. In this context, it is notable that the membrane PIC of the erythrocyte does not distinguish between the metabolically active and inert pools of polyphosphoinositides. This enzyme is only activated by exposure to Ca^{2+} on the cytoplasmic surface of the membrane, so it would be remarkable if this enzyme showed no discrimination between lipid molecules in the two leaflets of the erythrocyte membrane. It thus seems probable that the metabolically active and inert pools of PtdIns4*P*, PtdIns4,5P_2 and PtdOH co-exist in the inner leaflet of the erythrocyte plasma membrane, with the lipid molecules in the 'inert' pools somehow being environmentally restrained from interaction with the three kinases and phosphomonoesterases that achieve metabolic turnover of their monoesterified phosphate groups.

Most past studies of membrane lipid dynamics at physiological temperatures have emphasized the free and rapid mixing of lipid molecules within one leaflet of any lipid bilayer, whether in biological membranes or in model lipid systems. This would suggest that lipid–lipid interactions are unlikely to account for the metabolic compartmentation of lipids within the erythrocyte membrane. However, studies by Giraud and co-workers

suggest that this possibility should not be ignored (Giraud *et al.*, 1984; M'Zali & Giraud, 1986). They showed that decreasing the cholesterol content of erythrocyte membranes decreases the expressed activities of diacylglycerol kinase, PtdIns kinase and PtdIns4*P* kinase, but that the total enzyme activities, assayed in the presence of a detergent, remain unchanged.

It is perhaps most likely that these three lipids all form relatively stable associations with protein components at the inner surface of the erythrocyte membrane, thus limiting turnover of their monoester phosphate groups. In the past, such interactions have been suggested both with glycophorin (Buckley, 1978; Shapiro & Marchesi, 1977; but see also Shukla *et al.*, 1979; Anderson & Marchesi, 1985) and with at least two components of the sub-membrane cytoskeletal network (Sheetz *et al.*, 1982; Lassing & Lindberg, 1985). Whatever the nature of these interactions, it appears that PtdOH, PtdIns4*P* and PtdIns4,5P_2 must participate with similar affinities and that the associations must be stable over several hours. Furthermore, either the membrane PIC has equal access to the bound and unbound forms of the polyphosphoinositides, or else the high concentration of Ca^{2+} that is needed to activate the PIC *in situ* causes their dissociation and hence mixing of the metabolically active and inert pools of these lipids.

Previous studies have shown that complex eukaryotic cells possess hormone-sensitive and hormone-insensitive pools of PtdIns (Hokin & Hokin, 1964*b*; Fain & Berridge, 1979; Monaco & Woods, 1983; reviewed in Downes & Michell, 1985) and that the hormone-responsive PtdIns pool is the precursor of the cellular pool of PtdIns4,5P_2 that is broken down in response to receptor activation (Koreh & Monaco, 1985). In all of these studies, the metabolism of the lipid pools was analysed isotopically, and hence they were all metabolically active over a time-scale of hours. None of these studies would have detected an additional and essentially metabolically inert pool, such as that demonstrated by our studies and by Müller *et al.* (1986).

P. T. H. and C. E. K. were funded by studentships and G. R. G. by a project grant from the M.R.C. We thank Deryk Mills for electron microscopy.

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Received 23 September 1986/23 January 1987; accepted 10 February 1987