Endogenous calcium in sickle cells does not activate polyphosphoinositide phospholipase C

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Sickle-cell-anaemia erythrocytes (SS cells) are known to have a high Ca^{2+} content (particularly the dense cell fraction) and to take up Ca^{2+} on deoxygenation. It has been reported that this high Ca^{2+} was responsible for the activation of the Ca^{2+} -dependent K^+ loss, and of the Ca^{2+} -sensitive polyphosphoinositide phospholipase C (PIC) in dense SS cells. We found that, either in the total population of SS cells or in the light or dense fractions, the content of phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$] was not changed, whereas that of phosphatidylinositol 4-phosphate was increased and that of phosphatidic acid (PtdOH) was decreased compared with normal (AA) erythrocytes. Deoxygenation-induced Ca^{2+} entry into SS cells did not change the concentration or, in ^{32}P -prelabelled cells, the radioactivity of polyphosphoinositides and PtdOH. It also failed to induce the formation of inositol 1,4,5-trisphosphate, the product of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis by PIC, which was measured by an original method using ion-pair reverse-phase h.p.l.c. Thus there was no evidence of an endogenous Ca^{2+} effect on the PIC activity in SS cells, in agreement with the demonstration that the excess Ca^{2+} in SS cells is compartmentalized into internal vesicles and unavailable as free Ca^{2+} . The ^{32}P incorporation in polyphosphoinositides and PtdOH was markedly higher in SS than in AA cells, but this increase was the same in both dense and light SS cells. The increase in the turnover of these phospholipids in SS cells is consistent either with an activation of the lipid kinases and phosphatases or with perturbation in the metabolic compartmentation of these lipids.

INTRODUCTION

In human erythrocytes, the only phospholipids to be labelled on incubation with $^{32}\text{P}\text{P}_i$ are polyphosphoinositides and PtdOH (Allan, 1982). This is because in these simple cells there is no synthesis of PtdIns, but only a turnover of the 4-phosphate of $\text{PtdIns}4\text{P}$ and of the 4- and 5-phosphate groups of $\text{PtdIns}(4,5)\text{P}_2$ by specific kinases and phosphatases (Hokin & Hokin, 1964) and synthesis of PtdOH from diacylglycerol by a specific kinase (Hokin & Hokin, 1963). The erythrocyte membrane also possesses a Ca^{2+} -sensitive polyphosphoinositide phospholipase C (PIC) (Allan & Michell, 1978; Downes & Michell, 1981), which leads to the formation of $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,4)\text{P}_2$ and diacylglycerol, but is thought to be inactive at physiological intracellular Ca^{2+} concentrations (Downes & Michell, 1982). However, Ca^{2+} loading in the presence of the ionophore A23187, by activating PIC, induces a decrease in the polyphosphoinositide content and an increase in that of PtdOH (by conversion of the diacylglycerol generated by the action of PIC) (Allan & Thomas, 1981).

Sickle-cell-anaemia (SS) erythrocytes are known to have an elevated Ca^{2+} content, particularly the dense cell fraction rich in 'irreversibly sickled cells' (ISC) (Eaton

et al., 1973; Palek, 1977; Bookchin *et al.*, 1981), and to accumulate Ca^{2+} on deoxygenation (Palek, 1977; Bookchin *et al.*, 1984). Ponnappa *et al.* (1980), observing a decrease in $^{32}\text{P}\text{PtdIns}4\text{P}$ and an increase in $^{32}\text{P}\text{PtdOH}$ when ^{32}P -labelled SS cells were deoxygenated in the presence of Ca^{2+} , suggested a role of Ca^{2+} in this process. More recently, Raval & Allan (1986) reported that SS erythrocytes (particularly the denser fractions) contain less polyphosphoinositides and more PtdOH than do normal erythrocytes. They attributed these changes to an activation of PIC by Ca^{2+} accumulated in the denser SS cells. Many other abnormalities, such as K^+ loss, dehydration, altered deformability (Glader & Nathan, 1978; Eaton *et al.*, 1978; Clark *et al.*, 1980) and Na^+ -pump inhibition (Clark *et al.*, 1978), have been also often attributed to the high Ca^{2+} content of SS cells. However, the demonstrations that in SS cells (i) the excess of Ca^{2+} is sequestered into endocytic inside-out vesicles (Lew *et al.*, 1985; Rubin *et al.*, 1986), (ii) the free cytoplasmic Ca^{2+} concentration is not higher than in normal erythrocytes (Bookchin *et al.*, 1984; Rhoda *et al.*, 1985; Murphy *et al.*, 1987) and (iii) the Ca^{2+} -sensitive K^+ -selective channel is not activated (Ortiz *et al.*, 1986) have led to questions about the putative role of Ca^{2+} in these alterations.

In the present study, we have measured the

Abbreviations used: AA cells, normal erythrocytes; SS cells, sickle-cell-anaemia erythrocytes; ISC, 'irreversibly sickled cells'; PtdIns, phosphatidylinositol; $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-phosphate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid; PIC, polyphosphoinositide phospholipase C; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; 2,3-DPG, 2,3-diphosphoglycerate; $[\text{Ca}^{2+}]_i$, free cytosolic Ca^{2+} concentration.

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concentrations of polyphosphoinositides and PtdOH, the ^{32}P incorporation into these phospholipids and tried to detect the possible production of $\text{Ins}(1,4,5)\text{P}_3$ in normal (AA) and SS erythrocytes, under different conditions designed to reveal PIC activity. $[\text{P}]_i\text{-Ins}(1,4,5)\text{P}_3$ was measured by an original procedure, initially developed with Ca^{2+} -ionophore-treated AA cells and using ion-pair reverse-phase h.p.l.c. The results show that PIC is not activated in SS cells, even in the denser fractions or even when deoxygenated, and thus are consistent with the compartmentalization of SS-cell Ca^{2+} into endocytic vesicles.

The apparent discrepancies with the data of Ponnappa *et al.* (1980) are likely to be due to the greater number of samples analysed in the present work, and those with those of Raval & Allan (1986) to differences in the ionic conditions of the density-gradient solutions used for cell separations.

METHODS

Materials

$[\text{P}]_i\text{P}_i$ (sodium salt) was obtained from International CIS (Saclay, France), and $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ from Amersham (Paris, France). 2,3-DPG phosphatase and standards for t.l.c. and h.p.l.c. were purchased from Sigma (St Louis, MO, U.S.A.). Silica-gel t.l.c. plates with luminescer were obtained from Schleicher and Schuell (Dassel, Germany), and films for autoradiography (X-Omat AR) were from Kodak (Paris, France). Prepacked $0.46\text{ cm} \times 25\text{ cm}$ columns for h.p.l.c. were obtained from Waters Associates ($\mu\text{Bondapak C18}$), Beckman (Ultrasphere IP) and Whatman (Partisil 10 SAX).

Patients and controls

Blood samples from normal donors and from patients homozygous for haemoglobin S were obtained with informed consent. All patients had received their last blood transfusion at least 2 months before the study and had 8–27% ISC. After centrifugation (1000 g, 10 min), the plasma and buffy coat were removed by aspiration and the erythrocytes were washed three times in buffer A (140 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1 mM-CaCl₂, 10 mM-glucose, 1 mM-NaH₂PO₄/Na₂HPO₄, 10 mM-Hepes, pH 7.4). The cells were resuspended in buffer A, and, unless otherwise mentioned, the suspension was kept overnight at 4 °C.

Density-gradient separation of erythrocytes

A discontinuous gradient of stractan (arabinogalactan polysaccharide; Sigma) was used to fractionate the cells as described by Corash *et al.* (1974) with minor modifications. The cells were washed and resuspended (haematocrit 30%) in buffered saline (BSKG) containing 5 mM-KCl, 10 mM-glucose, 10 mM-sodium phosphate buffer (pH 7.4) and sufficient NaCl to increase the osmolarity to 291 mosm. Stractan was dissolved in water and deionized by successive equilibrations and filtrations at 4 °C with AG501-X8 (D)20–50-mesh mixed-bed resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) until the osmolarity was less than 100 mosm. A stock solution, about 40% (w/w) stractan (density 1.178 g/ml), pH adjusted to 7.4 with NaOH, was stored frozen. A discontinuous gradient was made by diluting the stock solution with BSKG to obtain six additional densities (1.068, 1.078, 1.082, 1.096, 1.105 and 1.115 g/ml) and

layering 1 ml or 2 ml of these density solutions and 3 ml of the erythrocyte suspension in 12 ml plastic centrifuge tubes. Centrifugation was done at 30000 rev./min for 60 min at 4 °C in a SW41 rotor of a Beckman L8-55 centrifuge. The erythrocyte bands obtained after centrifugation were harvested with a Pasteur pipette and washed twice in BSKG and once in buffer A.

Labelling of phospholipids

Erythrocyte suspensions (haematocrit 20%) were preincubated for 45 min at 37 °C in buffer A supplemented with 2 mM-adenine and 10 mM-inosine. After three washes in buffer A, erythrocytes were suspended in the same buffer (haematocrit 40%) and incubated with $[\text{P}]_i\text{P}_i$ (1.1 MBq/ml of cells) at 37 °C. After different times, these suspensions were diluted in ice-cold buffer A, centrifuged (1000 g, 10 min) and washed twice more with buffer A.

Deoxygenation experiments

Erythrocytes were labelled for 90 min with $[\text{P}]_i\text{P}_i$ in buffer A as described above. Samples of AA and SS cells were then incubated under humidified N₂, either in buffer A (1 mM-CaCl₂) or in buffer A supplemented with Tris/EGTA (1.2 mM) to lower external free Ca^{2+} concentration below 2 μM . Other samples of AA and SS cells were incubated under air either in the Ca^{2+} -containing medium or in the presence of Tris/EGTA.

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

Packed erythrocytes were haemolysed in 30 vol. of 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA, centrifuged (25000 g, 10 min) and washed twice with the same buffer. Acidic lipid extraction, t.l.c. separations and quantification of phospholipid contents and radioactivities were carried out as previously described (Giraud *et al.*, 1984; Wallace *et al.*, 1983).

ATP content and specific radioactivity

Cells were extracted with ice-cold HClO₄ (final concn. 5%, w/v), neutralized with KOH and centrifuged (9000 g, 2 min) for removal of KClO₄. The extract was applied to a $\mu\text{Bondapak C18}$ h.p.l.c. column, and the nucleotides were eluted with 0.025 M-tetrabutylammonium hydrogen sulphate/0.1 M-KH₂PO₄ (pH 7.2)/10% (v/v) methanol, at a flow rate of 1 ml/min. This method achieved excellent separation of AMP, ADP and ATP, which were detected by absorption at 254 nm. The ATP mass was estimated from a calibration curve which was linear up to 30 nmol. The ^{32}P radioactivity of the ATP peak was measured in the effluent by Čerenkov radiation with a radioactivity detector (Flo-one HP; Radiomatic Instrument and Consommables), interfaced with the h.p.l.c. system.

Measurement of $[\text{P}]_i\text{Ins}(1,4,5)\text{P}_3$ in intact cells

The method was initially developed in Ca^{2+} -loaded AA cells in which the production of $[\text{P}]_i\text{Ins}(1,4,5)\text{P}_3$ could be correlated with the Ca^{2+} concentration of the medium and with the breakdown of $[\text{P}]_i\text{PtdIns}(4,5)\text{P}_2$. Washed erythrocytes were incubated for 2.5 h at 37 °C with $[\text{P}]_i\text{P}_i$ (1.85 MBq/ml of cells) in 150 mM-NaCl/10 mM-glucose/1.5 mM-Hepes (pH 7.4) at 40% haematocrit. Cells were washed three times in 150 mM-KCl/0.15 mM-MgCl₂/10 mM-glucose/1.5 mM-Hepes (pH 7.4) and

Table 1. Phosphoinositide and PtdOH contents of AA and SS cells

Fresh AA and SS cells were washed and haemolysed, and the resulting membranes were extracted and processed for lipid analysis (see the Methods section). Results are expressed as molar percentages of total phospholipids and represent means $\pm$ S.E.M. from duplicate or triplicate analyses of four to nine samples (number in parentheses). X refers to an unidentified phospholipid (see the text).

Cells	Content (mol %)				
	PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	PtdIns	PtdOH	X
AA	1.03 $\pm$ 0.07 (5)	0.45 $\pm$ 0.07 (5)	0.50 $\pm$ 0.05 (4)	1.78 $\pm$ 0.09 (4)	0 (5)
SS	1.05 $\pm$ 0.09 (6)	0.96 $\pm$ 0.13 (6)	0.50 $\pm$ 0.10 (4)	1.03 $\pm$ 0.10 (9)	0.46 $\pm$ 0.08 (5)

Table 2. Polyphosphoinositide and ATP contents of unfractionated and density-fractionated AA and SS cells

AA and SS cells were processed for ATP and lipid extraction either before or after fractionation on stractan gradients (see the Methods section). Results are expressed as molar percentages of total phospholipids, and represent the means $\pm$ S.E.M. of duplicate determinations in three independent experiments for the phospholipids and means of duplicate determinations in a single experiment for ATP. Values in parentheses show the approximate distribution of the cells in each fraction (expressed as the percentage of the total cell population).

Cells	Fraction	Content (mol %)		ATP (mmol/l of cells)
		PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	
AA	Unfractionated	1.03 $\pm$ 0.03	0.36 $\pm$ 0.05	0.84
	1.068 < <i>d</i> < 1.078 (40)	0.92 $\pm$ 0.03	0.34 $\pm$ 0.08	0.89
	1.078 < <i>d</i> < 1.096 (40)	0.88 $\pm$ 0.04	0.32 $\pm$ 0.04	0.81
SS	Unfractionated	0.92 $\pm$ 0.05	0.81 $\pm$ 0.04	1.00
	1.068 < <i>d</i> < 1.082 (30–40)	0.90 $\pm$ 0.03	0.74 $\pm$ 0.01	1.07
	<i>d</i> > 1.096 (10–20)	0.88 $\pm$ 0.04	0.88 $\pm$ 0.04	0.98

resuspended at 10% haematocrit. After 10 min pre-incubation at 37 °C in the presence of different Ca²⁺ concentrations (15–1000 μ M), 5 μ M-A23187 (from a 2 mM stock solution in dimethyl sulphoxide) was added to the suspensions. After 20 min, cells were washed twice in 150 mM-NaCl (pH adjusted to 7 with NaOH). Then 1 vol. of ice-cold HClO₄ (10%) was added to 2 vol. of cell suspension. HClO₄ extracts were neutralized with KHCO₃, centrifuged (9000 *g*, 2 min) and analysed with an ion-pair chromatographic system on C18 reverse-phase Ultrasphere IP h.p.l.c. column. Samples were injected together with [³H]Ins(1,4,5)*P*₃. A two-step elution was performed at a flow rate of 1 ml/min, with solvent A [0.025 M-tetrabutylammonium hydrogen sulphate/0.1 M-KH₂PO₄ (adjusted to pH 5.5 with KOH)] for 40 min and then for 15 min with solvent A/acetonitrile (4:1, v/v). Before another run, the column was reconditioned with solvent A for 10 min. Fractions were collected every 1 min, and their ³²P and ³H contents were measured by liquid-scintillation counting after addition of 5 ml of Aqualuma Plus (Lumac, Shaesberg, The Netherlands). This system permitted elution of P_i, glucose and fructose 1,6-bisphosphate together, 2,3-DPG, AMP and

Ins(1,4,5)*P*₃ respectively at 3, 6, 15, 20 and 26 min and ADP and ATP at 49 and 53 min. Peaks were identified by using authentic standards {P_i, glucose and fructose 1,6-bisphosphate, AMP, [³H]Ins(1,4,5)*P*₃, ADP and ATP} and by using 2,3-DPG phosphatase for 2,3-DPG (King *et al.*, 1987). Identification of [³²P]Ins(1,4,5)*P*₃ was further confirmed as follows: the fractions co-eluting with [³H]Ins(1,4,5)*P*₃ were collected, desalted, freeze-dried and re-injected on an anion-exchange h.p.l.c. column (Partisil 10 SAX) and eluted with a gradient of ammonium formate as previously described (Irvine *et al.*, 1985). In this second system ³²P radioactivity again co-eluted with [³H]Ins(1,4,5)*P*₃ (see Fig. 3*d*).

In the experiments involving sickle cells, AA and SS erythrocytes were prelabelled for 2 h at 37 °C with [³²P]P_i under standard conditions (see under 'Labelling of phospholipids'), but with 1.85 MBq/ml of cells. Samples of AA and SS cells were then deoxygenated in the same medium for 15–60 min, and other samples were incubated for the same period under oxygenated conditions. The cells were washed in 150 mM-NaCl (adjusted to pH 7 with NaOH) and treated with HClO₄ and KHCO₃ for h.p.l.c. analysis as described above.

Fig. 1. Kinetics of ^{32}P incorporation into [γ - ^{32}P]ATP (a), PtdIns(4,5)P₂ (b), PtdIns4P (c) and PtdOH (d)

AA (●) and SS (○) cells were incubated with [γ - ^{32}P]P_i for different times and processed for ATP and lipid extraction, followed by separation by h.p.l.c. or t.l.c. and measurement of their amount and of their radioactivity (see the Methods section). Results are means of duplicate determinations in a single experiment. Specific radioactivity of [γ - ^{32}P]ATP was calculated by dividing the specific radioactivity of ATP, which is labelled on β - and γ -P, by 2. Specific radioactivities of 4-P of PtdIns4P and of 4-P + 5-P of PtdIns(4,5)P₂ were calculated by multiplying the specific radioactivities of total phosphorus by 2 and 1.5 respectively (Müller *et al.*, 1986).

RESULTS

Lipid content

Membranes from fresh AA and SS erythrocytes were used for these experiments. In Table 1, the concentration of each phospholipid is expressed as a molar percentage of the total phospholipids. The concentrations of PtdIns and PtdIns(4,5)P₂ were the same in AA and SS erythrocytes. In contrast, major differences were found in the content of PtdIns4P, which approximately doubled, and in that of PtdOH, which decreased about 2-fold, in SS cells. In addition, in the lipid extracts from SS cells, a compound migrating just below PtdOH in system B (see Giraud *et al.*, 1984) and marked X in Table 1 was revealed by iodine staining. It was not seen in the extract from AA cells. It contained phosphate, was not lysoPtdOH (as indicated by comparison of its R_f with that of an authentic standard) and it was not labelled with ^{32}P (as shown by autoradiography).

The concentration of polyphosphoinositides was also measured in AA and SS cells before and after fractionation on a stractan density gradient (Table 2). There was no effect of cell density on the contents of PtdIns4P and PtdIns(4,5)P₂ in either AA or SS cells. ATP content, measured in one experiment, was not affected by cell density.

^{32}P incorporation into ATP, polyphosphoinositides and PtdOH

AA and SS erythrocytes were incubated with [γ - ^{32}P]P_i under oxygenated conditions. At different times, samples were processed for measurements of the content and radioactivity of ATP and of phospholipids. The results were expressed as c.p.m./pmol of labelled phosphate. ATP specific radioactivity reached an equilibrium value after 3 h in AA cells (Fig. 1a). It was slightly lower in SS cells than in AA cells for the period preceding this equilibration.

Fig. 2. Distribution of ³²P incorporation into PtdIns(4,5)P₂ (a), PtdIns4P (b) and PtdOH (c)

AA and SS cells were incubated with [³²P]P_i for 3 h and processed for lipid extraction and separation (see the Methods section). The columns (■, AA; □, SS) represent the mean values of ³²P incorporation into each phospholipid. Individual values from each independent experiment (means of duplicate determinations) are also shown (AA, ●; SS, ○).

The specific radioactivities of PtdIns(4,5)P₂, PtdIns4P and PtdOH were always higher in SS cells than in AA cells (Figs. 1b–1d). The specific radioactivity of PtdIns(4,5)P₂ was nearly equal to that of PtdIns4P, in each type of cells, at the latest time of observation (4.5 h). At that time, PtdIns4P specific radioactivity was about 40% of that of ATP in SS cells, whereas it was only 17% in AA cells. For PtdOH, these values were 22% and 6%.

Fig. 2 shows the variability of ³²P incorporation, after incubation for 3 h, into the phospholipids of erythrocytes from eight controls and nine individual patients, expressed in c.p.m./nmol of total phospholipids. Clearly, the values from SS cells showed a larger scatter than those from AA cells. To investigate whether this was due to different proportions of ISC (which ranged between 8 and 27% in the different patients), similar experiments were conducted with density-fractionated AA and SS cells (Table 3). The same pattern of incorporation was found in total, light and dense cell fractions of AA cells. This was also the case in SS cell fractions with no preferential increase in the dense cells enriched in ISC and which have the highest Ca²⁺ content. In SS cells, the

ATP specific radioactivity was slightly lower than that in AA cells, as mentioned above, in both the light and the dense cell fractions.

Lack of effect of deoxygenation on the concentration and on the radioactivity of phospholipids

These experiments were designed to investigate whether a stimulation of the Ca²⁺ uptake in SS cells, as induced by deoxygenation in a Ca²⁺-containing medium, could have any effect on the activity of PIC. AA and SS cells, preincubated with [³²P]P_i, were deoxygenated either in the normal Ca²⁺-containing medium or in this medium supplemented with EGTA (external free [Ca²⁺] < 2 μM). Two other samples were incubated for the same period under air with or without EGTA. Phospholipid contents and radioactivities were measured. The results in Table 4 show that none of these treatments had any significant effect on the radioactivity incorporated into each phospholipid. In particular, there was no special difference for deoxygenated SS cells incubated with or without Ca²⁺. In addition, the concentrations of PtdIns(4,5)P₂ and PtdIns4P were not modified in SS or in AA cells after the deoxygenation (results not shown). Variations in the experimental conditions (deoxygenation period shortened to 30 min or extended to 1 h, Ca²⁺ concentration in the medium increased to 2 mM) also failed to reveal any effect of deoxygenation on the content or on the radioactivities of the phospholipids. ⁴⁵Ca uptake, measured under the same conditions, was clearly stimulated in deoxygenated SS cells incubated in the presence of Ca²⁺ (results not shown), in agreement with previous reports (Palek, 1977; Bookchin *et al.*, 1984).

Lack of effect of deoxygenation on the production of Ins(1,4,5)P₃

Because a small decrease in the concentrations or radioactivities of polyphosphoinositides, induced by Ca²⁺ uptake on deoxygenation of SS cells, could have been compensated by their immediate resynthesis, we have investigated whether [³²P]Ins(1,4,5)P₃, one of the products of the hydrolysis of PtdIns(4,5)P₂ by PIC, could be detected under the same conditions. The procedure used for the measurement of [³²P]Ins(1,4,5)P₃ in intact cells was initially developed with AA cells treated with ionophore A23187 in the presence of different Ca²⁺ concentrations. This permitted us to determine the threshold of free cytosolic Ca²⁺ concentration, [Ca²⁺]_i, at which [³²P]Ins(1,4,5)P₃ could be detected. The production of Ins(1,4,5)P₃, measured by h.p.l.c. analysis of a neutralized HClO₄ extract of the cells, increased with the Ca²⁺ concentration of the medium and was correlated with a decrease in the radioactivity of PtdIns(4,5)P₂ (results not shown). Under the conditions used in these experiments (extent and duration of the cell labelling, dilution of the cell extract and volume injected), the limit of detection of the [³²P]Ins(1,4,5)P₃ peak corresponded to a Ca²⁺ concentration in the medium of 15 μM. At the high ionophore concentration used (5 μM, haematocrit 10%, i.e. 50 μmol/l of cells), the Ca²⁺ pump became saturated and [Ca²⁺]_i could be determined by measurement of ⁴⁵Ca uptake (Ferreira & Lew, 1976). At 15 μM external Ca²⁺, [Ca²⁺]_i was 30 μmol/l of cell water or 20 μmol/l of cells. Deoxygenation of ³²P-labelled SS cells for 15–45 min failed to induce any [³²P]Ins(1,4,5)P₃ formation. A small ³²P peak co-eluted with [³H]-

Table 3. Distribution of ^{32}P incorporation into polyphosphoinositides, PtdOH and ATP in unfractionated and density-fractionated AA and SS cells

AA and SS cells were fractionated on stractan gradients, incubated with [^{32}P]P_i for 90 min, and processed for ATP and lipid extraction (see the Methods section). Unfractionated cells were treated similarly. ^{32}P incorporation into each phospholipid (measured as c.p.m./nmol of total phospholipids) is expressed as percentage of the incorporation into PtdIns(4,5)P₂ in unfractionated AA cells. ^{32}P incorporation into ATP (c.p.m./pmol) is expressed as percentage of the incorporation into unfractionated AA cells. Values are means $\pm$ S.E.M. of duplicate determinations in three independent experiments for the phospholipids, and means of duplicate determinations in a single experiment for ATP. The approximate distribution of the cells in each fraction was as indicated in Table 2.

Cells	Fraction	^{32}P incorporation (%)			
		PtdIns(4,5)P ₂	PtdIns4P	PtdOH	ATP
AA	Unfractionated	100	32 $\pm$ 9	31 $\pm$ 6	100
	1.068 < <i>d</i> < 1.078	112 $\pm$ 14	41 $\pm$ 13	48 $\pm$ 6	97
	1.078 < <i>d</i> < 1.096	81 $\pm$ 5	25 $\pm$ 5	44 $\pm$ 12	119
SS	Unfractionated	186 $\pm$ 5	102 $\pm$ 8	79 $\pm$ 6	81
	1.068 < <i>d</i> < 1.082	187 $\pm$ 15	106 $\pm$ 10	73 $\pm$ 16	69
	<i>d</i> > 1.096	144 $\pm$ 13	94 $\pm$ 14	99 $\pm$ 9	84

Table 4. Lack of effect of deoxygenation on the incorporation of ^{32}P into polyphosphoinositides and PtdOH

AA and SS cells were incubated for 90 min with [^{32}P]P_i in buffer A (1 mM-CaCl₂). One part was then deoxygenated for a further 45 min either in the same medium or in the presence of 1.2 mM-Tris/EGTA (free [Ca²⁺] < 2 μM). The other part was incubated under oxygenated conditions either in buffer A or with EGTA (see the Methods section). Results (measured as c.p.m./nmol of total phospholipids) were expressed as a percentage of the incorporation into PtdIns(4,5)P₂ in AA cells. Values are means $\pm$ S.E.M. of duplicate determinations in five independent experiments with AA cells and in seven independent experiments with SS cells. In some experiments the Ca²⁺ concentration of the medium was increased to 2 mM, and in others the deoxygenation period was either 30 or 60 min. Results were the same as those obtained in the standard conditions (1 mM-CaCl₂ and 45 min) and hence were included in the means presented in this Table.

Conditions		Cells...	³² P incorporation (%)					
			AA			SS		
			PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	PtdOH	PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	PtdOH
O ₂	Ca ²⁺		PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	PtdOH	PtdIns(4,5) <i>P</i> ₂	PtdIns4 <i>P</i>	PtdOH
+	+		100	25 ± 4	27 ± 3	189 ± 16	111 ± 16	84 ± 14
+	—		103 ± 7	26 ± 4	26 ± 4	178 ± 17	119 ± 19	77 ± 15
—	+		91 ± 4	25 ± 3	28 ± 3	190 ± 14	111 ± 16	93 ± 16
—	—		108 ± 11	28 ± 6	26 ± 5	189 ± 19	114 ± 19	79 ± 14

Ins(1,4,5)P₃ was always observed in these samples, especially in SS cells (Figs. 3*b* and 3*c*). However, it was not increased by deoxygenation and, when re-injected on the Partisil 10 SAX column, it was not co-eluted with [^3H]-Ins(1,4,5)P₃. Although it was not possible to identify this peak, it was checked that it was not glycerophosphoinositol 4,5-bisphosphate or 4-phosphate. It was markedly increased when the step of preincubation of the cells with adenine and inosine was omitted (results not shown). In comparison, ionophore treatment of AA cells in the presence of 15 μM -Ca²⁺ induces the appearance of a ^{32}P peak, co-eluted with [^3H]Ins(1,4,5)P₃ also when re-injected on the Partisil column (Fig. 3*d*).

DISCUSSION

The high Ca²⁺ content of SS erythrocytes has been often implicated in some of the alterations found in these cells, such as dehydration resulting from Ca²⁺ activation

of the K⁺ channel (Glader & Nathan, 1978; Eaton *et al.*, 1978). This interpretation presumed that most of the excess of Ca²⁺ in these cells was freely distributed in their cytoplasm. As a consequence, PIC should be activated in SS cells, as it is in ionophore-treated AA cells (Allan & Thomas, 1981). The SS erythrocytes should exhibit a decrease in the content of polyphosphoinositides and an increase in that of PtdOH, particularly the denser fractions rich in ISC and which have the higher Ca²⁺ content. This is what was reported by Raval & Allan (1986). In contrast, we have found that the concentration of PtdIns(4,5)P₂ (as well as that of PtdIns) was not changed in SS cells compared with AA cells, but that the concentration of PtdIns4P was increased and that of PtdOH was decreased. The same modifications were found in dense or light SS cells. Raval & Allan (1986) did not provide the phospholipid concentrations of the unfractionated AA and SS cells, so that it is not clear whether the stractan fractionation method in itself

Fig. 3. Separation of [³²P]P_i-labelled compounds from cytosolic extracts by ion-pair h.p.l.c.

Erythrocytes (AA or SS as indicated) were incubated with [³²P]P_i for 2 h 45 min under air (a, b), or for 2 h under air followed by 45 min under N₂ (c), or for 2.5 h under air, washed and reincubated for 20 min in a medium containing 15 μM-Ca²⁺ and 5 μM-A23187 (d) (see the Methods section). A sample of the neutralized acid extract was applied to the h.p.l.c. column (Ultrasphere IP). Radioactivity in fractions of the eluate (1 ml) was determined either by Čerenkov counting (for fractions 0–20 and 40–60) or by liquid-scintillation counting (for fractions 20–40) (note the change in the scale). The arrow indicates the change of the eluting solution. In (c) and (d), pooled fractions 26–29, collected from the ion-pair h.p.l.c. column, were desalted, freeze-dried and re-injected into an anion-exchange h.p.l.c. column (Partisil 10 SAX) (see the Methods section). Elution of the radioactivity from this second column is shown on the right (SAX). ●, ³²P; ▲, ³H.

introduced some modification in the concentration of the phospholipids. The procedure used by the above authors did not seem to involve a deionization step of the stractan. Hyperosmotic stractan medium causes dehydration and increases the cell density. The density limits at which both AA and SS cells were isolated were higher than those used in the present work or in other papers (Clark *et al.*, 1980; Ortiz *et al.*, 1986; Kuypers *et al.*, 1987; Jain, 1988). Whether this difference in cell density is the origin of the changes in the concentrations of the phospholipids remains to be determined. However other data presented here also argue against PIC activation in SS cells.

It is known that deoxygenation of SS cells, but not of AA cells, induced a stimulation of Ca^{2+} uptake (Palek, 1977; Bookchin *et al.*, 1984). Deoxygenation of ^{32}P -labelled SS cells did not modify their polyphosphoinositide content or radioactivity. An increase in the Ca^{2+} concentration of the medium or decrease or increase in the duration of the deoxygenation period also failed to induce a decrease in polyphosphoinositides. Ponnappa *et al.* (1980), in apparently the same experiments, were able to detect a decrease in ^{32}P radioactivity of PtdInsP and a small increase in that of PtdOH in SS cells, but no change in ^{32}P $\text{PtdIns}(4,5)\text{P}_2$. $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ should be broken down to the same extent, since PIC attacks both phosphoinositides with equal affinity (Downes & Michell, 1981). In addition, the variations found in AA cells under the same conditions are indicative of a large scatter in the data of Ponnappa *et al.* (1980), presumably because they were obtained from a single experiment (not indicated in their publication; see Table IV of Ponnappa *et al.*, 1980).

Upon deoxygenation of SS cells, in agreement with the lack of degradation of $\text{PtdIns}(4,5)\text{P}_2$ there was no production of $\text{Ins}(1,4,5)\text{P}_3$. Taking into account that the smallest $[\text{Ca}^{2+}]_i$ at which $\text{Ins}(1,4,5)\text{P}_3$ could be detected in AA cells after 20 min treatment with Ca^{2+} ionophore was about 20 $\mu\text{mol/l}$ of cells (see the Results section), the lack of production of $\text{Ins}(1,4,5)\text{P}_3$ could indicate that $[\text{Ca}^{2+}]_i$ in deoxygenated SS cells never reaches that value. The increase in Ca^{2+} permeability, induced by deoxygenation, may result in only a transient increase in $[\text{Ca}^{2+}]_i$, because of a rapid extrusion by the Ca^{2+} pump both into intracellular endocytic vesicles and out of the cell (Bookchin & Lew, 1983; Lew *et al.*, 1985; Murphy *et al.*, 1987). These brief transients in $[\text{Ca}^{2+}]_i$ would not be able to produce a sustained activation of PIC.

An important feature of SS cells relative to AA cells was the acceleration of the ^{32}P turnover in $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}4\text{P}$ and PtdOH . A similar observation has been reported by Ponnappa *et al.* (1980). Interestingly, these increases were of the same order of magnitude in both the light and the dense SS cell fractions, indicating that the acceleration of ^{32}P turnover was not the consequence of the high Ca^{2+} content of SS cells. The increased ^{32}P turnover of polyphosphoinositides and PtdOH in SS cells could have been due to an increase in the ^{32}P specific radioactivity of their precursor ATP because of a possible greater metabolic activity, owing to the relative youth of these cells (Bertles & Milner, 1968). Instead, ATP specific radioactivity was the same or even slightly lower in SS than in AA cells, and was also the same in both the light and dense SS cell fractions, in agreement with previous results (Dzandu & Johnson, 1980). One possibility to explain the increased ^{32}P turnover of polyphospho-

inositides and PtdOH is that the activity of the enzymes involved in the metabolism of these phospholipids (kinases and phosphatases) was modified. The increase in the content of $\text{PtdIns}4\text{P}$ could indicate an increase in the activity of PtdIns kinase, as shown in isolated SS cell membranes (P. Boivin & C. Galand, personal communication). The evidence for the existence of multiple pools of polyphosphoinositides and PtdOH in AA-cell membranes, with only a fraction of these lipids being metabolically active (Müller *et al.*, 1986; King *et al.*, 1987), offers an alternative explanation. An apparent acceleration in the turnover of these lipids, in SS cells, can be solely the consequence of a metabolic compartmentation different from that found in AA cells, with more molecules within the metabolically active pools. The observations of perturbations in the asymmetric distribution of aminophospholipids in SS cells are consistent with this hypothesis (Chiu *et al.*, 1979; Lubin *et al.*, 1981; Zachowski *et al.*, 1985). More experiments are needed to determine whether the increased turnover of polyphosphoinositides and PtdOH in SS cells is due to a change in enzymic activities or to a different organization of these lipids in membrane domains where they are more accessible to the enzymes.

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