Influence of Ca^{2+} and Mg^{2+} on the turnover of the phosphomonoester group of phosphatidylinositol 4-phosphate in human erythrocyte membranes

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In isolated erythrocyte membranes, increasing the free Mg^{2+} concentration from 0.5 to 10 mM progressively activates the membrane-bound phosphatidylinositol (PtdIns) kinase and leads to the establishment of a new equilibrium with higher phosphatidylinositol 4-phosphate (PtdIns4P) and lower PtdIns concentrations. The steady-state turnover of the phosphomonoester group of PtdIns4P also increases at high Mg^{2+} concentrations, indicating a simultaneous activation of PtdIns4P phosphomonoesterase by Mg^{2+} . Half-maximum inhibition of PtdIns kinase occurs at 10 μM free Ca^{2+} in the presence of physiological free Mg^{2+} concentrations. Increasing free Mg^{2+} concentrations overcome Ca^{2+} inhibition of PtdIns kinase. In the presence of Ca^{2+} , calmodulin activates Ca^{2+} -transporting ATPase 5-fold, but does not alter pool size and radiolabelling of PtdIns4P. In intact erythrocytes, adding EGTA or EGTA plus Mg^{2+} and the ionophore A23187 to the external medium does not exert significant effects on concentration and radiolabelling of polyphosphoinositides when compared with controls in the presence of 1.4 mM free Ca^{2+} .

INTRODUCTION

Ca^{2+} has been shown to stimulate the breakdown of polyphosphoinositides in erythrocytes and isolated erythrocyte membranes of different mammalian species. Primarily this is the result of a Ca^{2+} -sensitive phosphodiesterase, which specifically cleaves PtdIns4P and PtdIns(4,5) P_2 (Allan & Michell, 1978; Ponnappa *et al.*, 1980; Downes & Michell, 1981, 1982; Giraud *et al.*, 1984; Moore & Appel, 1984). In addition, several reports also invoke the participation of specific phosphomonoesterases in the Ca^{2+} -dependent breakdown of polyphosphoinositides (Peterson & Kirschner, 1970; Garrett *et al.*, 1976; Lang *et al.*, 1977; Quist, 1980, 1985), although direct evidence for the existence of such enzyme activities in erythrocyte membranes is lacking.

Mg^{2+} was found to increase synthesis and extent of radiolabelling of polyphosphoinositides by stimulating Mg^{2+} -dependent kinases (Marche *et al.*, 1982; Quist, 1982) and to reduce the apparent Ca^{2+} affinity of the enzymes involved in the Ca^{2+} -dependent breakdown of polyphosphoinositides (Buckley & Hawthorne, 1972; Quist, 1982; Ferrell & Huestis, 1984; Quist, 1985). Quantitative data on the Ca^{2+} - Mg^{2+} antagonism as well as data on possible enzyme pathways by which Ca^{2+} stimulates polyphosphoinositide degradation vary widely. This is attributable to differences among the erythrocyte species used and/or to differences in the membrane preparations and labelling procedures applied.

We embarked on the study of these complex phenomena by using iso-osmotic saponin membranes prepared as described by Klinger *et al.* (1980). The peculiarities of these membranes are as follows. (i) They do not reseal during incubation at 37 °C in the presence of bivalent cations; (ii) the membrane-bound Ca^{2+} -

sensitive phosphodiesterase is almost completely absent after saponin treatment; (iii) the activities of membrane-bound PtdIns4P kinase and PtdIns(4,5) P_2 phosphomonoesterase, two of several tissue cytosolic enzymes (Roach & Palmer 1981; van Rooijen *et al.*, 1985), are negligibly small; (iv) the enzyme-catalysed cycle of phosphorylation and dephosphorylation of PtdIns is in the same range as that observed in intact erythrocytes (Müller *et al.*, 1986). This membrane preparation appeared convenient for investigating the effects of free Ca^{2+} and free Mg^{2+} on the concentration of PtdIns4P, as well as on the turnover of its phosphomonoester group. A better understanding of the mechanisms which control the level of PtdIns4P seems to be important, since this factor is thought to be involved in erythrocyte shape changes (Quist & Reece, 1980; Quist & Barker, 1983) and in the regulation of Ca^{2+} -transport ATPase (Buckley & Hawthorne, 1972).

MATERIALS AND METHODS

Materials

Carrier-free neutral [^{32}P] P_i solution [10 mCi (370 MBq)/ml] and neutral [γ - ^{32}P]ATP solution [2 mCi (74 MBq)/ml] were obtained from Zentralinstitut für Kernforschung (Rossendorf/Dresden, German Democratic Republic), ATP and the bivalent-cation ionophore A23187 from Boehringer (Mannheim, Germany), trypsin from Spofa (Prague, Czechoslovakia), trypsin inhibitor from Reanal (Budapest, Hungary), and silica-gel HR 'reinst' and saponin (white, pure) from Merck (Darmstadt, Germany). Calmodulin was prepared from ram testes as described by Wenz *et al.* (1987). Other chemicals were of the highest purity available from commercial sources. Organic solvents were glass-distilled before use.

Abbreviations used: PtdIns, phosphatidylinositol; 4P, 4-phosphate; (4,5) P_2 , 4,5-bisphosphate.

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Heparinized blood was withdrawn from healthy donors with informed consent and centrifuged for 10 min at 4 °C and at 1400 *g*. Plasma and buffy coat were removed and the packed erythrocytes washed three times with cold wash solution [50 mM-Tris/maleate/100 mM-NaCl/5 mM-KCl/1 mM- Na_2HPO_4 /5.5 mM-glucose, pH 7.4 (adjusted at 37 °C)]. The erythrocytes were used immediately after washing for experiments with intact cells.

Membrane isolation

Leucocytes of freshly drawn heparinized blood samples were removed by passage through a cotton column as described by Busch & Pelz (1966). The erythrocytes were then washed three times with cold wash solution as described above. Membranes were isolated after haemolysis of erythrocytes with saponin by the method of Klinger *et al.* (1980), modified as follows. The pelleted erythrocytes were lysed for 20 min at 4 °C with stirring in 9 vol. of haemolysing buffer containing 50 mM-Tris/HCl, 100 mM-KCl, 0.5 mM-EDTA and 0.3 mg of saponin/ml, the pH being adjusted at room temperature to 7.2. Cell debris was pelleted by 5 min centrifugation at 1400 *g* and at 4 °C. The membranes were sedimented from the supernatant by 10 min centrifugation at 5600 *g* and at 0 °C and then washed three or four times with ice-cold saponin-free haemolysing buffer. An additional wash of the membranes was performed in the cold with membrane incubation solution containing 50 mM-Tris/HCl, 100 mM-KCl, 1 mM-EGTA and 2 mM-ATP, with CaCl_2 and MgCl_2 as indicated in the Figures and Tables, the pH being adjusted at 37 °C to 7.2. The membranes were used immediately after isolation.

These membranes were almost completely devoid of Ca^{2+} -dependent phosphodiesterase when this enzyme's activity was determined by the method of Downes & Michell (1981). For the experiment depicted in Fig. 1, 10 ml of washed and pelleted erythrocytes were labelled with [^{32}P]P_i as described by Müller *et al.* (1986). Cells from one half of the incubation mixture were lysed in saponin-containing haemolysing buffer as described above, and cells from the other half in hypo-osmotic haemolysing buffer as described by Müller *et al.* (1986). Washed and pelleted membranes from both preparations were then incubated in parallel, with and without Ca^{2+} , as described in the legend to Fig. 1.

The activities of PtdIns4P kinase and PtdIns(4,5) P_2 phosphomonoesterase were also very low in these saponin membranes. Thus the turnover of the phosphomonoester groups of PtdIns(4,5) P_2 was very small in these membranes (see also Müller *et al.*, 1986), and the pool size of PtdIns(4,5) P_2 was very stable under all conditions employed.

Storage of the membranes at 0 °C for several hours or at -70 °C for several days led to a more or less extended and irreversible decrease in the concentration of PtdIns4P, with a corresponding increase in PtdIns. This was probably due to proteolytic inactivation of PtdIns kinase, which seemed to be extremely sensitive to proteolysis. This supposition was derived from the following model experiment. Isolated membranes were treated for 10 min at 37 °C with an amount of trypsin just sufficient to transform the membrane-bound Ca^{2+} -transport ATPase into an activated and calmodulin-independent form exactly as described by Wetzker *et al.*

Fig. 1. Loss of Ca^{2+} -dependent phosphodiesterase activity in erythrocyte membranes after saponin treatment

Saponin-treated and hypo-osmotic ghosts were incubated simultaneously for 15 min at 37 °C (4 mg of ghost protein per incubation) in 2 ml of 50 mM-Tris/HCl, 50 mM-KCl, 50 μM -EGTA, pH adjusted at 37 °C to 7.2, with the addition of either 1 mM-EGTA (blanks) or 0.5 mM- CaCl_2 . The reactions were terminated by the addition of 0.1 vol. of 15% (w/v) trichloroacetic acid, the membranes pelleted by 5 min centrifugation at 10000 *g* and the supernatants submitted to anion-exchange chromatography as described by Downes & Michell (1981). The elution profiles of supernatants of the two incubation mixtures with the addition of 1 mM-EGTA were used as blanks in order to obtain the Ca^{2+} -induced release of inositol bisphosphate (0–10 ml elution volume) and inositol trisphosphate (20–34 ml elution volume). ●, Hypo-osmotic ghosts; ○, saponin-treated ghosts. This is one representative experiment out of three similar ones with three different samples of erythrocyte membranes.

(1982). After a subsequent 20 min incubation in the absence of trypsin, the PtdIns4P pool was decreased by more than 50% compared with a control treated in the same manner but without trypsin addition (results not shown).

Radiolabelling

Intact erythrocytes. Portions of washed and pelleted erythrocytes (2.2 ml; haematocrit about 90%) were preincubated for 15 min at 37 °C in the same volume of wash solution (see above) containing, in addition, compounds as indicated. The labelling was started by adding 0.2 ml of wash solution with the indicated concentrations of MgCl_2 and CaCl_2 and 0.1 mCi (3.7 MBq) of [^{32}P]P_i to the incubation mixture. After 15 min of incubation with shaking at 37 °C, 2 ml of the incubation mixture was pipetted into 15 ml of an ice-cold mixture of n-hexane/propan-2-ol/12 M-HCl (30:60:1,

by vol.) for termination of labelling and initiation of lipid extraction as described by Radin (1981), modified as described in detail below.

Isolated membranes. (a) Macro-assay. Portions of ghosts (about 10 mg of ghost protein) were preincubated for 20 min at 37 °C in 10 ml of membrane incubation solution (see above) containing, in addition, MgCl₂ and CaCl₂ as indicated. The labelling was started by the addition of 27–42 μ Ci (1–1.5 MBq) of [γ -³²P]ATP dissolved in 0.2 ml of membrane incubation solution. After 15 min of incubation with shaking, 2 ml of 6% (w/v) trichloroacetic acid was added to the incubation mixture to terminate labelling. After mixing, the membranes were pelleted by 3 min centrifugation at 14000 g, washed twice with about 16 ml of distilled water in order to remove trichloroacetic acid and then submitted to lipid extraction.

(b) Micro-assay. For the experiments depicted in Fig. 4 (below) we applied a miniaturized micro-assay for the determination of radiolabelling of polyphosphoinositides as follows. Portions of ghosts (about 0.2 mg of ghost protein) were preincubated in small stoppered plastic vessels for 20 min at 37 °C in 0.9 ml of membrane incubation solution (see above) with MgCl₂ and CaCl₂ as indicated. The labelling was started by the addition of 14 μ Ci (0.5 MBq) of [γ -³²P]ATP dissolved in 0.1 ml of incubation solution. After 15 min of incubation with shaking, the labelling was terminated by the addition of 0.3 ml of stop solution containing 100 mM-P_i, 4 mM-ATP and 10% (w/v) trichloroacetic acid. After mixing, the membranes were pelleted by 2 min centrifugation at 9400 g, washed twice with 1 ml of distilled water and then submitted to lipid extraction.

Lipid extraction

Intact erythrocytes. A mixture containing 2 ml of the incubation mixture and 15 ml of n-hexane/propan-2-ol/12 M-HCl (see under 'Radiolabelling' above) was stirred for 15 min under N₂ at room temperature. Then the finely dispersed protein precipitate was pelleted by 5 min centrifugation at 2500 g. The supernatant was removed and the extraction continued by stirring the sediment for 15 min with 10 ml of n-hexane/propan-2-ol/12 M-HCl (45:30:1, by vol.) under N₂ at room temperature. After pelleting the protein precipitate, the supernatants were combined and vigorously mixed with 15 ml of n-hexane and 8 ml of 2 M-KCl. Phase separation was performed by 10 min centrifugation at 2500 g. The upper n-hexane phase was washed twice with 20 ml of 0.1 M-HCl, neutralized with NH₃ vapour and evaporated to dryness in a stream of N₂ at 35 °C. The lipids were finally dissolved in 0.3 ml of benzene/ethanol (4:1, v/v) and stored in a small stoppered plastic vessel on solid CO₂. The contamination of this lipid extract with haem pigments was small and did not disturb the subsequent chromatographic lipid separation.

In three parallel experiments, we compared the pool sizes of different phospholipids and the specific radioactivities of polyphosphoinositides of this lipid extract with those found in extracts obtained by our standard procedure (Müller *et al.*, 1986). No significant differences could be detected in both lipid extracts. Thus the simple procedure of Radin (1981), modified as described above, can be recommended for lipid extraction of intact erythrocytes.

Isolated membranes. (i) Macro-assay. The lipid extraction was performed as described by Müller *et al.* (1986) with the following modification. The combined organic phases were washed once with about 1 volume of 0.1 M-HCl. This additional step removed impurities migrating between start and PtdIns(4,5)P₂ during the subsequent two-dimensional t.l.c. run.

(ii) Micro-assay. The pelleted membranes were vigorously mixed with 0.7 ml of chloroform/methanol/1 M-HCl (1.5:3:1, by vol.). After 5 min shaking at room temperature, phase separation was performed, after addition of 0.2 ml of chloroform, 0.2 ml of 2 M-KCl and vigorous mixing, by 2 min centrifugation at 9400 g. The lower organic phases were removed and the upper phase was washed once with 0.5 ml of chloroform. The combined organic phases were then washed once with 0.5 ml of 0.1 M-HCl and dried *in vacuo*. The lipids were finally dissolved in 50 μ l of benzene/ethanol (4:1, v/v) and stored in the same plastic vessel used for the whole-lipid-extraction procedure.

Lipid chromatography

Portions of the lipid extracts of intact erythrocytes and of isolated membranes submitted to the macro-assay (see above) were separated by two-dimensional t.l.c. as described by Müller *et al.* (1986). Portions of the lipid extracts of isolated membranes submitted to the micro-assay (about 0.2 μ mol of lipid phosphorus) were applied to silica-gel HR thin-layer plates (20 × 20 cm, 6 g of dry gel suspended in 20 ml of 0.07% potassium oxalate) previously heated at 110 °C for 60 min. The lipids were chromatographed in chloroform/methanol/acetic acid/water (40:7:22:5, by vol.). Then the major phospholipids [sphingomyelin (R_F 0.54), phosphatidylcholine (R_F 0.61), phosphatidylserine (R_F 0.67) and phosphatidylethanolamine (R_F 0.79)] were revealed by exposure to I₂ vapour. Strips of X-ray film (ORWO XR1, 16 h) were used for autoradiography in order to localize PtdIns4P (R_F 0.38) and PtdIns(4,5)P₂ (R_F 0.29).

Determination of pool sizes and of radiolabelling of inositides

The areas on the two-dimensional thin-layer chromatograms corresponding to the inositides as well as those corresponding to phosphatidylcholine and phosphatidylethanolamine were treated exactly as described by Müller *et al.* (1986) in order to obtain the pool sizes of inositides in nmol/ml of erythrocytes and the specific radioactivities in c.p.m./nmol of phosphorus in the indicated position, i.e. in the 4P of PtdIns4P and in the 4P plus 5P of PtdIns(4,5)P₂.

The areas on the one-dimensional thin-layer chromatograms (employed in the micro-assay) corresponding to PtdIns4P, PtdIns(4,5)P₂ and phosphatidylethanolamine were removed by scraping and submitted to wet ashing. The phosphorus contents of the phosphatidylethanolamine spots were then analysed by using the micro-method of Bartlett (1959). Corresponding blanks were run in parallel. The Bartlett (1959) procedure was also applied to the areas autoradiographically localized as PtdIns4P and PtdIns(4,5)P₂. Since their phosphorus contents were too low to be determined accurately, only the radioactivity was measured, by scintillation counting in a portion of the final solution in the P_i determination. Corresponding blanks were run in parallel. In order to compensate for different amounts of lipids applied on

the thin-layer plates, all measured radioactivities were related to an arbitrary unit of phosphatidylethanolamine.

Calculation of free Ca^{2+} and Mg^{2+} concentrations

The calculation of $[\text{Ca}^{2+}]_{\text{free}}$ and $[\text{Mg}^{2+}]_{\text{free}}$ from given total Ca^{2+} , Mg^{2+} , ATP and EGTA concentrations was carried out by the method of Perrin & Sayce (1967) by means of a Hewlett-Packard HP 20 desk calculator, with the association constants taken from Sillén & Martell (1964), Taqui Khan & Martell (1976) and Boyd *et al.* (1965). The reliability of the calculated free Ca^{2+} concentrations was controlled by measuring free Ca^{2+} in the absence of Mg^{2+} with a Ca^{2+} -sensitive electrode (F 2110 Ca selectrode; Radiometer, Copenhagen, Denmark) as described by Dinjus *et al.* (1984). Within a $\pm 25\%$ range there was a good agreement between calculated and measured values.

RESULTS

Mg^{2+} dependence

The results depicted in Fig. 2 clearly agree with earlier reports (Peterson & Kirschner, 1970; Garrett *et al.*, 1976; Buckley & Hawthorne, 1972; Allan & Michell, 1978; Quist & Barker, 1983; Marche *et al.*, 1982) that the

Fig. 2. Dependence on Mg^{2+} of steady-state pool sizes of PtdIns and PtdIns4P and specific radioactivity of PtdIns4P in isolated erythrocyte membranes

Ghosts were incubated in an iso-osmotic medium at 37 °C and pH 7.2 in the absence ($< 10^{-8}$ M) of free Ca^{2+} and in the presence of 2 mM-ATP with various free Mg^{2+} concentrations. After 25 min preincubation with unlabelled ATP, sufficient to establish a new Mg^{2+} -dependent steady state of PtdIns and PtdIns4P pool sizes, the labelling was started by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and terminated after 15 min by the addition of trichloroacetic acid. All values shown are the percentages of the pool sizes and specific radioactivities determined in parallel at 0.5 mM free Mg^{2+} and are stated as means $\pm$ S.E.M. The data were derived from four experiments with four different samples of erythrocyte membranes. Absolute pool sizes (means $\pm$ S.E.M. for seven experiments) at 0.5 mM free Mg^{2+} were: PtdIns, 20 ± 1.6 ; and PtdIns4P, 12 ± 0.4 (both in nmol $\cdot$ ml of cells $^{-1}$). $\circ$, Pool size of PtdIns; $\triangle$, pool size of PtdIns4P; $\square$, specific radioactivity of the 4P of PtdIns4P.

membrane content of PtdIns4P rose with increasing Mg^{2+} , whereas that of PtdIns decreased correspondingly in our membrane preparation. In comparison with the physiological intracellular free Mg^{2+} concentration, which was found to be near 0.5 mM in human red cells (Till *et al.*, 1977; Achilles *et al.*, 1978), PtdIns4P had nearly doubled at 10 mM free Mg^{2+} at the cost of PtdIns. Only minor changes were found at a further increase in Mg^{2+} . The Mg^{2+} -induced changes were completed after about 15–20 min incubation by net synthesis of PtdIns4P. Afterwards a new steady state was established, with fairly constant inositide contents during the succeeding hour (results not shown).

The specific radioactivities of the phosphomonoester group of PtdIns4P were approximately identical at different Mg^{2+} concentrations when measured after completion of net synthesis in the presence of unlabelled ATP by a further 15 min incubation with labelled ATP (see Fig. 2). Taking into account the dependence of the turnover rate on pool size and specific radioactivity (see Müller *et al.*, 1986), the same specific radioactivity at a doubled pool size implies a doubled turnover rate. From this it follows that the steady-state turnover of the 4P of PtdIns4P increased with increasing Mg^{2+} proportionally to its increased pool size. This result simultaneously implies that Mg^{2+} activated the PtdIns kinase and the PtdIns4P phosphomonoesterase to about the same extent.

A rise in the PtdIns4P content was reported to activate Ca^{2+} -ATPase of hypo-osmotic pig erythrocyte membranes severalfold (Buckley & Hawthorne, 1972). In our iso-osmotic human erythrocyte membrane preparation, however, a Mg^{2+} -induced doubling of PtdIns4P content was without any effect on the activity of Ca^{2+} -ATPase (results not shown). The reasons for these differences remain to be elucidated.

Owing to the very low activities of PtdIns4P kinase and PtdIns(4,5) P_2 phosphomonoesterase in our membrane preparation, the pool size of PtdIns(4,5) P_2 remained unaltered by increasing Mg^{2+} concentrations. The very slow radiolabelling of its phosphomonoester group was likewise not changed by increasing Mg^{2+} (results not shown).

Ca^{2+} dependence

Fig. 3 shows that the concentration of PtdIns4P decreased and that of PtdIns increased correspondingly at increasing free Ca^{2+} . Simultaneously, specific radioactivities of 4P of PtdIns4P were decreasing at increasing free Ca^{2+} . In the presence of 0.5 mM free Mg^{2+} , half-maximum inhibition of labelling was observed at about 10 μM free Ca^{2+} and an almost complete inhibition at 50 μM . These results suggested that the decrease in PtdIns4P concentration and labelling was due primarily to a Ca^{2+} inhibition of PtdIns kinase activity, without any significant effect of Ca^{2+} on PtdIns4P phosphomonoesterase. This conclusion was further supported by the 'delabelling' experiment shown in Table 1. A 20 min incubation of prelabelled ghosts with unlabelled ATP and with Mg^{2+} in the absence of Ca^{2+} decreased the specific radioactivity of PtdIns4P by about 40%, whereas no decrease at all was observed in the presence of 0.1 mM free Ca^{2+} . This result suggested a complete inhibition of PtdIns kinase by the Ca^{2+} concentration employed.

The pool size of PtdIns4P decreased during 20 min

Fig. 3. Dependence on Ca^{2+} of steady-state pool sizes of PtdIns and PtdIns4P and of specific radioactivity of PtdIns4P in isolated erythrocyte membranes

Ghosts were incubated in an iso-osmotic medium at 37 °C and pH 7.2 in the presence of 2 mM-ATP and 0.5 mM free Mg^{2+} with various free Ca^{2+} concentrations. After 20 min preincubation with unlabelled ATP, the labelling was started by the addition of [$\gamma\text{-}^{32}\text{P}$]ATP and terminated after 15 min by trichloroacetic acid. All values shown are the percentages of the pool sizes and specific radioactivities determined in parallel in the absence of free Ca^{2+} . For absolute pool sizes of PtdIns and PtdIns4P in the absence of Ca^{2+} , see the legend to Fig. 1. Each point is the mean for duplicate t.l.c. runs of the same lipid extract. The data were derived from three experiments with three different samples of erythrocyte membranes. ○, Pool size of PtdIns; △, pool size of PtdIns4P; □, specific radioactivity of the 4P of PtdIns4P.

incubation in the absence and in the presence of Ca^{2+} by 27 and 64% respectively when compared with unincubated control membranes. The small decrease of the membrane content of PtdIns4P in the absence of Ca^{2+} , observed in all experiments with membranes isolated

Table 1. Effect of Ca^{2+} on the loss of label of PtdIns4P in membranes isolated from erythrocytes prelabelled with [^{32}P]P_i

Erythrocyte membranes prelabelled with ^{32}P were incubated for 20 min at 37 °C with 1.5 mM free Mg^{2+} , 2 mM-ATP, 1 mM-EGTA, 100 mM-KCl and 50 mM-Tris/HCl, pH 7.2, in the presence or absence of 0.1 mM-free Ca^{2+} . The values are expressed as percentages of pool size and specific radioactivity of PtdIns4P associated with unincubated control membranes. Results are representative of three similar experiments.

Incubation conditions	Pool size (% of control)	Specific radioactivity (% of control)
Control	100	100
With 1.5 mM- Mg^{2+}	73	58
With 1.5 mM- Mg^{2+} and 0.1 mM- Ca^{2+}	36	100

from erythrocytes after about 1 h incubation in the presence of [^{32}P]P_i, was probably the result of a partial proteolytic inactivation of PtdIns kinase. This did not obscure, however, the drastic decrease of the PtdIns4P pool as a result of a complete inhibition of PtdIns kinase by Ca^{2+} .

Taking into account the incubation time applied and a steady-state turnover rate of the 4P of PtdIns4P in the absence of Ca^{2+} (35 nmol·h⁻¹·ml of cells⁻¹; cf. Müller *et al.*, 1986), then the decrease of the pool size of PtdIns4P at complete inhibition of PtdIns kinase by Ca^{2+} (see Fig. 3 and Table 1) approximately corresponded to the activity of PtdIns4P phosphomonoesterase observed in the absence of Ca^{2+} under steady-state conditions. This, on the other hand, suggested that the phosphomonoesterase would not be influenced significantly by Ca^{2+} .

The pool size of PtdIns(4,5)P₂ also remained fairly constant in this series of experiments under all conditions applied, whereas its specific radioactivity mimicked that of PtdIns4P (see Fig. 4), indicating that

Fig. 4. Dependence on Ca^{2+} and Mg^{2+} of labelling of PtdIns4P and PtdIns(4,5)P₂ in isolated erythrocyte membranes

Ghosts were incubated in an iso-osmotic medium at 37 °C and pH 7.2 in the presence of 2 mM-ATP and various concentrations of free Ca^{2+} and Mg^{2+} . After 15–20 min preincubation with unlabelled ATP, the labelling was started by the addition of [$\gamma\text{-}^{32}\text{P}$]ATP and terminated after 15 min by trichloroacetic acid. All values shown are the percentages of radiolabelling of PtdIns4P and PtdIns(4,5)P₂ determined in parallel at the corresponding free Mg^{2+} concentration in the absence of free Ca^{2+} . The data at 0.5 mM-free Mg^{2+} were derived from six experiments with six different samples of erythrocyte membranes and stated as means ± S.E.M. The other data points are means of duplicate determinations with one or two samples of erythrocyte membranes. △, ± S.E.M., radiolabelling of PtdIns4P at 0.5 mM free Mg^{2+} ; □, radiolabelling of PtdIns(4,5)P₂ at 0.5 mM free Mg^{2+} without S.E.M., which was in the same range as that shown for PtdIns4P; △—△, radiolabelling of PtdIns4P at 10 mM free Mg^{2+} ; △---△, radiolabelling of PtdIns4P at 28 mM free Mg^{2+} .

the very small activity of PtdIns4P kinase present in our membrane preparation responded to Ca^{2+} in a similar manner to PtdIns kinase.

In order to define more precisely the effect of Ca^{2+} on radiolabelling of polyphosphoinositides in the presence of physiological free Mg^{2+} , we applied a simplified assay (see the Materials and methods section) that greatly speeded up the analyses. In addition, we applied this method to the examination of the Ca^{2+} - Mg^{2+} antagonism. The results are shown in Fig. 4. At 0.3 and 1.0 μM free Ca^{2+} there was no noticeable inhibition of radiolabelling of PtdIns4P and PtdIns(4,5) P_2 . Significant inhibitions by about 30 and 50% were observed at 3.0 and 10 μM free Ca^{2+} respectively, in accordance with the results of Fig. 3. Increasing free Mg^{2+} from 0.5 to 10 and 28 mM (broken lines in Fig. 4) greatly diminished the inhibitory effects of Ca^{2+} on the radiolabelling of PtdIns4P [and PtdIns(4,5) P_2 ; result not shown], thus confirming and extending the literature data on Ca^{2+} - Mg^{2+} antagonism (Buckley & Hawthorne, 1972; Quist, 1982; Ferrell & Huestis, 1984; Quist, 1985).

Dependence on Ca^{2+} fluxes across the membrane

Although polyphosphoinositides do not appear to play an active part in erythrocyte Ca^{2+} fluxes across the membrane (Lang *et al.*, 1977), recent results have not completely discounted this possibility. Strunecká & Folk (1984) reported on increased turnover rates of polyphosphoinositides in human erythrocytes, either in the presence of the Ca^{2+} channel blocker verapamil or at very low Ca^{2+} concentrations in the external medium. With regard to the Ca^{2+} -transport ATPases, results of Varsanyi *et al.* (1983) indicated a formation of PtdIns4P associated with the sarcoplasmic-reticular Ca^{2+} -ATPase that correlated with an activation of the enzyme. The results of Buckley & Hawthorne (1972) indicated an activation of the Ca^{2+} -ATPase of erythrocyte membranes when the content of PtdIns4P was increased.

These data, together with our results on Ca^{2+} inhibition of PtdIns kinase, prompted us to re-examine the influence of Ca^{2+} fluxes across the membrane on the steady-state turnover of polyphosphoinositides.

Changes of Ca^{2+} fluxes across the membrane were induced in the following way. Decreasing the Ca^{2+} concentration in the external medium by EGTA to less than 10^{-8} M was thought to decrease the influx of Ca^{2+} through the membrane of intact erythrocytes. Adding

EGTA plus the bivalent-cation ionophore A23187 to the external medium was thought to decrease, in addition, the intracellular Ca^{2+} concentration as well as the efflux of Ca^{2+} through the membrane. Changes of the intracellular Mg^{2+} concentration were avoided in this series of experiments by the presence of 0.17 mM- Mg^{2+} in the external medium, which is in equilibrium with the intracellular free Mg^{2+} concentration (Till *et al.*, 1977). Neither reduced Ca^{2+} influxes nor reduced Ca^{2+} effluxes exerted significant effects on the concentration and labelling of polyphosphoinositides (results not shown). The intracellular free Ca^{2+} concentration is apparently far too low in normal erythrocytes to induce inhibitory effects on both inositide kinases, as was demonstrated in the foregoing section at Ca^{2+} concentrations in the micromolar range for PtdIns kinase in the isolated erythrocyte membranes.

The effect of an about 5-fold activation of Ca^{2+} -transport ATPase by calmodulin (see Klinger *et al.*, 1980) on concentration and labelling of polyphosphoinositides in isolated membranes is shown in Table 2. Calmodulin did not alter pool sizes and radiolabelling significantly when compared with the effects induced by Ca^{2+} alone as a consequence of an inhibition of PtdIns kinase and PtdIns4P kinase (see also Figs. 3 and 4). This indicated that the turnover of polyphosphoinositides does not seem to be linked with the activity of Ca^{2+} -transport ATPase or the enzyme-catalysed Ca^{2+} movement through the membrane.

DISCUSSION

The results of this investigation have revealed a number of characteristic effects of Ca^{2+} and Mg^{2+} on membrane-bound PtdIns kinase and PtdIns4P phosphomonoesterase of human erythrocyte membranes which have not previously been described. In addition, they have confirmed and extended the literature data on the involvement of Ca^{2+} and Mg^{2+} in the regulation of the inositide metabolism.

Erythrocyte membranes contained an Mg^{2+} -dependent PtdIns kinase which, at physiological free Mg^{2+} concentration, is half-maximally inhibited by 10 μM -free Ca^{2+} (Figs. 3 and 4). A Ca^{2+} -activated PtdIns4P phosphomonoesterase, as is thought to occur in rabbit erythrocyte membranes and to be involved in the Ca^{2+} -dependent breakdown of polyphosphoinositides

Table 2. Effect of calmodulin on pool sizes and specific radioactivities of inositides in isolated erythrocyte membranes

Erythrocyte membranes were preincubated for 20 min at 37 °C with 1.5 mM free Mg^{2+} , 2 mM-ATP, 1 mM-EGTA, 100 mM-KCl and 50 mM-Tris/HCl, pH 7.2, in the presence or absence of 20 μM -free Ca^{2+} and 1 μM -calmodulin. The labelling was started by the addition of 0.15 MBq of [γ - ^{32}P]ATP/ml of incubation mixture and terminated after 10 min by the addition of trichloroacetic acid. The data are the means for duplicate t.l.c. runs of the lipid extracts from one sample of erythrocyte membranes.

Incubation conditions	Pool size (nmol/ml of cells)			Specific radioactivity (c.p.m./nmol of labelled phosphorus)	
	PtdIns	PtdIns4P	PtdIns(4,5) P_2	PtdIns4P	PtdIns(4,5) P_2
Without Ca^{2+}	9	18	45	532	16
With Ca^{2+}	19	14	50	135	13
With Ca^{2+} plus calmodulin	20	14	52	134	12

(Quist, 1985), was not detectable in our membrane preparation. There was no significant effect of Ca^{2+} on PtdIns4P phosphomonoesterase activity, which was activated only by Mg^{2+} and/or increasing PtdIns4P concentration in the membrane (Fig. 2). A Ca^{2+} -induced decrease of PtdIns4P concentration and labelling was thus due primarily to inhibition of PtdIns kinase. The apparent Ca^{2+} -affinity of this enzyme was reduced by increasing Mg^{2+} concentrations (Fig. 4).

The inhibition of PtdIns kinase by Ca^{2+} may not be a factor at the very low Ca^{2+} levels often reported for normal erythrocytes, but probably contributes to the deleterious effects of a Ca^{2+} load in erythrocytes.

The data of Table 2 lead to the conclusion that the turnover of polyphosphoinositides does not seem to be linked with the activity of Ca^{2+} -transport ATPase.

A comparable inhibition of PtdIns kinase by micromolar Ca^{2+} concentrations has been reported to occur in insulinoma-cell membranes and secretory granules (Tooke *et al.*, 1984). Although possibly premature at this stage, one could speculate that the inhibition of the PtdIns kinase of erythrocytes by low Ca^{2+} concentrations could be important in the preservation of inositides from the breakdown of polyphosphoinositides by Ca^{2+} -dependent and polyphosphoinositide-specific phosphodiesterase during a Ca^{2+} transient.

The apparent Ca^{2+} -independence of PtdIns4P phosphomonoesterase found in our membrane preparation agrees with the results of Allan & Michell (1978) and Mack & Palmer (1984) obtained with hypo-osmotic erythrocyte membranes. The latter authors also found the enzyme to be Mg^{2+} -independent, which we could not confirm, since we observed an increased steady-state turnover of PtdIns4P with increasing Mg^{2+} concentration (Fig. 2).

With regard to the effects of Mg^{2+} in the absence of Ca^{2+} , the findings presented confirm and extend results of earlier studies (Buckley & Hawthorne, 1972; Marche *et al.*, 1982) on rat and pig erythrocyte membranes, namely that the concentration of Mg^{2+} influences the proportions of inositides found in isolated erythrocyte membranes (Fig. 2). The PtdIns content was shown to decrease in an amount equivalent to the quantity of PtdIns4P synthesized when the free Mg^{2+} concentration rose. After 20 min a new steady state was reached during which the turnover of the phosphomonoester group was increased.

It is easy to double the content of PtdIns4P at the cost of PtdIns in unsealed ghosts by increasing the Mg^{2+} concentration in the external medium. An increase of PtdIns4P is apparently correlated with the discoid morphology of erythrocytes and ghosts (Shukla *et al.*, 1978; Quist & Reece, 1980; Quist & Barker, 1983). Thus an increase in erythrocyte PtdIns4P *in vivo* might be achieved in cases of decreased PtdIns4P levels by an increase in Mg^{2+} in blood plasma. This, however, does not seem to be possible, because intact erythrocytes have a very limited permeability for Mg^{2+} , and a drastic rise of Mg^{2+} in blood plasma is lethal.

The very low labelling of PtdIns(4,5) P_2 in our membrane preparation in comparison with that in intact erythrocytes appears to be due to low activities of (a) PtdIns(4,5) P_2 phosphomonoesterase, which undoubtedly is a cytosolic enzyme in erythrocytes (Koutouzov & Marche, 1982) and (b) PtdIns4P kinase, which is almost certainly cytosolic in brain, but of unclear subcellular

distribution in erythrocytes (see Irvine, 1982). Another possibility could be that PtdIns4P kinase, although present in the membranes, is inactive because of product inhibition consequent on a lack of PtdIns(4,5) P_2 phosphomonoesterase. The latter, however, seems to be less probable, because the radiolabelling of PtdIns(4,5) P_2 mimicked exactly, under all conditions applied, that of PtdIns4P (shown in Fig. 4), although at a very low level (see Table 2). This finding could imply an identical Ca^{2+} - and Mg^{2+} -dependency of the enzyme pairs involved in labelling of PtdIns4P and PtdIns(4,5) P_2 which, however, appears to be less probable. Another, and more probable, possibility could be that the enzyme pair responsible for the labelling of PtdIns4P is also involved in the labelling of PtdIns(4,5) P_2 , although unspecifically and with low activity. This important question remains to be addressed by further experimentation.

REFERENCES

- Achilles, W., Klinger, R., Scheidt, B. & Frunder, H. (1978) *Acta Biol. Med. Germ.* **37**, 1161–1166
- Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Boyd, S., Bryson, A., Nancollas, G. H. & Torrance, K. (1965) *J. Chem. Soc.* 7353–7358
- Buckley, J. T. & Hawthorne, J. N. (1972) *J. Biol. Chem.* **247**, 7218–7223
- Busch, D. & Pelz, K. (1966) *Klin. Wochenschr.* **44**, 983–984
- Dinjus, U., Klinger, R. & Wetzker, R. (1984) *Biomed. Biochim. Acta* **43**, 1067–1072
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Downes, C. P. & Michell, R. H. (1982) *Biochem. J.* **202**, 53–68
- Ferrell, J. E., Jr. & Huestis, W. H. (1984) *J. Cell. Biol.* **98**, 1992–1998
- Garett, N. E., Garrett, R. J. B., Talwalkar, R. T. & Lester, R. L. (1976) *J. Cell. Physiol.* **87**, 63–69
- Giraud, F., M'Zali, H., Chaillet, B. & Mazet, F. (1984) *Biochim. Biophys. Acta* **778**, 191–200
- Irvine, R. F. (1982) *Cell Calcium* **3**, 295–309
- Klinger, R., Wetzker, R., Fleischer, I. & Frunder, H. (1980) *Cell Calcium* **1**, 229–240
- Koutouzov, S. & Marche, P. (1982) *FEBS Lett.* **144**, 16–20
- Lang, V., Pryhitka, G. & Buckley, J. T. (1977) *Can. J. Biochem.* **55**, 1007–1012
- Mack, S. E. & Palmer, F. B. St. C. (1984) *J. Lipid Res.* **25**, 75–85
- Marche, P., Koutouzov, S. & Meyer, P. (1982) *Biochim. Biophys. Acta* **710**, 332–340
- Moore, R. B. & Appel, S. H. (1984) *Can. J. Biochem.* **62**, 363–368
- Müller, E., Hegewald, H., Jaroszewicz, K., Cumme, G. A., Hoppe, H. & Frunder, H. (1986) *Biochem. J.* **235**, 775–783
- Perrin, D. D. & Sayce, J. G. (1967) *Talanta* **14**, 833–842
- Peterson, S. C. & Kirschner, L. B. (1970) *Biochim. Biophys. Acta* **202**, 295–304
- Ponnappa, B. C., Greenquist, A. C. & Shohet, S. B. (1980) *Biochim. Biophys. Acta* **598**, 494–501
- Quist, E. E. (1980) *Biochem. Biophys. Res. Commun.* **92**, 631–637
- Quist, E. E. (1982) *Arch. Biochem. Biophys.* **219**, 58–64
- Quist, E. E. (1985) *Arch. Biochem. Biophys.* **236**, 140–149
- Quist, E. E. & Barker, R. C. (1983) *Arch. Biochem. Biophys.* **222**, 170–178
- Quist, E. E. & Reece, K. L. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1023–1030
- Radin, N. S. (1981) *Methods Enzymol.* **72**, 5–7
- Roach, P. D. & Palmer, F. B. St. C. (1981) *Biochim. Biophys. Acta* **661**, 323–333

- Shukla, S. D., Billah, M. M., Coleman, R., Finean, J. B. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **509**, 48–57
- Sillén, L. G. & Martell, A. E. (1964) *Stability Constants of Metal-Ion Complexes* (Chem. Soc. Spec. Publ. 17), The Chemical Society, London
- Strunecká, A. & Folk, P. (1984) FEBS Meet. 16th, Moscow (Abstr.) 355
- Taqi Khan, M. M. & Martell, A. E. (1976) *J. Am. Chem. Soc.* **88**, 668–671
- Till, U., Petermann, H., Wenz, I. & Frunder, H. (1977) *Acta Biol. Med. Germ.* **36**, 597–610
- Tooke, N. E., Hales, C. N. & Hutton, J. C. (1984) *Biochem. J.* **219**, 471–480
- van Rooijen, L. A. A., Rossowska, M. & Bazun, N. G. (1985) *Biochem. Biophys. Res. Commun.* **126**, 150–155
- Varsanyi, M., Tölle, H.-G., Heilmeyer, L. M. G., Jr., Dawson, R. M. C. & Irvine, R. F. (1983) *EMBO J.* **2**, 1543–1548
- Wenz, J., Reißmann, R., Börnig, H., Hoppe, H. & Cumme, G. (1987) *Biomed. Biophys. Acta* **46**, 125–133
- Wetzker, R., Klinger, R., Cumme, G., Hoppe, H. & Frunder, H. (1982) *Biochem. Int.* **4**, 385–390

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