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CALCIUM-INDUCED CHANGES IN POLYPHOSPHOINOSITIDES AND PHOSPHATIDATE IN NORMAL ERYTHROCYTES, SICKLE CELLS AND HEREDITARY PYROPOIKILOCYTES

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Summary

A study of the effects of low concentrations of calcium on the polyphosphoinositides and phosphatidate of normal human erythrocytes, in the presence of ionophore A23187, was carried out. The metabolism of these phospholipids in pathologic erythrocytes (sickle cells and pyropoikilocytes) that are leaky to calcium was also investigated. In erythrocyte polyphosphoinositides prelabelled with $^{32}P_i$, decreases of 40 and 70% in the radioactivity associated with triphosphoinositide were observed in media containing 10 and 100 μM $CaCl_2$, respectively, in the presence of the ionophore, as compared to controls. Diphosphoinositide was comparatively less sensitive to calcium-induced breakdown. Incorporation of $^{32}P_i$ into phosphatidate increased as the concentration of calcium in the medium increased. When isolated erythrocyte membranes pre-labelled with $^{32}P_i$ were exposed to calcium, a marked loss of radioactivity associated with polyphosphoinositides was observed in medium containing 100 μM $CaCl_2$. However, radioactivity associated with phosphatidate showed little change under the above conditions. The general pattern of incorporation of $^{32}P_i$ (2 : 1 : 1 into triphosphoinositide : diphosphoinositide : phosphatidate) into normal erythrocytes was altered in pathological erythrocytes (sickle cells and hereditary pyropoikilocytes), with a higher incorporation of $^{32}P_i$ into diphosphoinositide or phosphatidate or both. Re-incubation of $^{32}P_i$ pre-labelled erythrocytes with $1 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$ M carbamylcholine had no effect on the polyphosphoinositides or phosphatidate metabolism.

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Introduction

Inositol lipids constitute less than 5% of the total phospholipid content of human erythrocytes. Under energy-generating conditions, these lipids can be phosphorylated to diphosphoinositide and triphosphoinositide. The role of these polyphosphoinositides in erythrocyte metabolism has been of some interest in recent years [1—6].

In human erythrocytes, the phospholipids associated with phosphate turnover are primarily polyphosphoinositides and phosphatidate. Several reports presenting information on the levels of polyphosphoinositides in erythrocytes of different mammalian species have appeared, and attempts have been made to correlate the levels of intracellular calcium with those of the membrane polyphosphoinositides. Wieneke and Woodin [7] have compared the levels of diphosphoinositide and triphosphoinositide in leukocytes, macrophages and erythrocytes. Buckley and Hawthorne [3] and Harrison and Long [8] have attempted to correlate the intracellular levels of calcium with those of membrane polyphosphoinositides in erythrocytes. Allan et al. [9] have reported an increased synthesis of phosphatidate in human erythrocytes in the presence of calcium and the divalent ionophore A23187. Reduced synthesis of polyphosphoinositides in erythrocytes in the presence of calcium has been reported by Lang et al. [1]. Garrett and co-workers [2] have observed rapid dephosphorylation effects of calcium on the polyphosphoinositides of rabbit erythrocytes. Recently, Akhtar and Abdel-Latif [10] have observed a calcium ion requirement for the acetylcholine-stimulated breakdown of triphosphoinositide in rabbit iris smooth muscle mediated through muscarine receptors. These studies have indicated a definite relationship between cellular polyphosphoinositides and calcium. Muscarinic receptors have also been reported to be present in human erythrocytes [11].

In the present study we have investigated the metabolism of polyphosphoinositides and phosphatidate in human erythrocytes (1) in the presence of calcium and the ionophore A23187, (2) in pathologic erythrocytes (sickle cells and pyropoikilocyte cells) with increased permeability to calcium and (3) when stimulated by the muscarinic agonist, carbamylcholine. Exposure of human erythrocytes to low concentrations of calcium in the presence of ionophore caused simultaneous dephosphorylation of polyphosphoinositides and increased phosphatidate synthesis. The sensitivity of these lipids to calcium was also shown in erythrocytes with higher-than-normal intracellular calcium content (sickle cells) [12] and in those with increased permeability to calcium (hereditary pyropoikilocytes) [13]. Levels of polyphosphoinositides and phosphatidate were unaffected by treatment with carbamylcholine. Our results substantiate the sensitivity of polyphosphoinositides to cellular calcium movement in human erythrocytes.

Materials and Methods

$H_3{}^{32}PO_4$ was purchased from ICN Radiochemicals. Triphosphoinositide and diphosphoinositide (as sodium salts), phosphatidate and other lipid standards were purchased from Sigma (St. Louis, MO). Silica gel H was purchased from

Merck, and the divalent cation ionophore (A23187) was a gift from Eli Lilly Co. (Indianapolis, IN). All other chemicals used were of reagent grade.

Preparation of erythrocytes. Heparinized human blood was centrifuged to remove plasma and buffy coat, washed once with phosphate-buffered (25 mM) saline (0.9%), and passed through a cotton column to remove platelets and leukocytes [14]. Erythrocytes were washed twice with modified Krebs-Ringer phosphate buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \cdot 7\,H_2O$, 1 mM glucose and 5 mM Na_2HPO_4 plus NaH_2PO_4, pH 7.45) and finally suspended in the same buffer (hereafter referred to as solution K) and stored for 1 day at 0—4°C. Immediately before use, the cells were diluted with solution K, washed once, and resuspended at 30% hematocrit.

Labelling of erythrocyte phospholipids. Erythrocytes were incubated at 30% hematocrit for 3 h at 37°C with 25—50 μCi $^{32}P_i$/ml suspension. At the end of incubation, cells were centrifuged (1500 × *g*, 5 min), washed twice more with solution K, and reconstituted to 30% hematocrit.

Re-incubation. For experiments involving intact erythrocytes and the ionophore, 1 ml of the above suspension was mixed with 1 ml of medium containing calcium or EGTA in the presence of ionophore A23187, and re-incubated as indicated in the footnotes to Tables I—IV. The ionophore was removed from erythrocytes prior to hemolysis by washing once with 0.5% albumin in 0.16 M KCl (pH 7.4). Subsequently, cells were washed twice with solution M (145 mM NaCl; 5 mM KCl; 20 mM Tris or imidazole, pH 7.4).

Preparation of erythrocyte membranes. Packed erythrocytes were hemolysed in 30 vols. of a solution containing 20 mM Tris or imidazole and 0.5 mM EDTA (pH 7.6), and centrifuged at 20 000 × *g* for 20 min in a Sorvall RC-2B centrifuge. The membranes were collected and washed twice with the same buffer without EDTA. The resultant erythrocyte membranes were pale-pink or creamy-white in color. Samples were used for lipid, protein and hemoglobin determinations.

Lipid extraction and thin layer chromatography. Erythrocyte membrane lipids were extracted according to the procedure of Buckley and Hawthorne [3] and chromatographed on TLC as detailed by Gonzales-Sastre and Folch-Pi [15] with slight modifications. The membranes were extracted with 15 vols. of chloroform/methanol/conc. HCl (100 : 100 : 0.6, v/v/v), and kept overnight in the cold. Extra $CHCl_3$ was added to produce a final ratio of chloroform : methanol of 2 : 1 (v/v) and washed with 1/5 vol. of 1 M HCl. The lower phase was washed twice with theoretical upper phase according to the procedure of Folch et al. [16], substituting 0.1 M HCl for the aqueous component. The resultant lower phase was neutralized with dilute ammonium hydroxide and rendered homogeneous by adding methanol. Concentrated lipid extracts were spotted on TLC coated with silica gel H (500 μm thick) containing 0.4% potassium oxalate, and lipids were separated using $CHCl_3/CH_3OH$/4 N NH_4OH (9 : 7 : 2, v/v/v) as the solvent system. Reference standards were used to identify triphosphoinositide, diphosphoinositide and phosphatidate after exposure to iodine vapor. Autoradiography (not shown) demonstrated only three spots corresponding to triphosphoinositide, diphosphoinositide and phosphatidate that were distinctly separated (R_F values 0.22, 0.4 and 0.6, respectively). Since Redman [4], who used similar conditions for the extraction of these lipids, found after suitable checks on purity that $^{32}P_i$ was incorpo-

rated only into diphosphoinositide, triphosphoinositide and phosphatidate, we performed no further check on purity on these three lipid classes that separated distinctly on TLC in the solvent system used. The spots corresponding to the lipids were scraped, transferred to vials and counted for Cerenkov radiation in 10 ml $CHCl_3$ in a Packard scintillation spectrometer.

Protein was estimated according to the procedure of Lowry et al. [17] using bovine serum albumin as standard. Hemoglobin was estimated by the pyridine hemochromogen method [18].

Results

Human erythrocytes incubated with $^{32}P_i$ for 3 h showed incorporation of the label into three lipid components of the membrane, triphosphoinositide, diphosphoinositide and phosphatidate. In normal erythrocytes, the general pattern of incorporation was 2 : 1 : 1 (see footnote to Table I) into triphoinositide: diphosphoinositide : phosphatidate when the incorporated radioactivity was expressed as cpm/mg Hb-free membrane protein. Erythrocyte polyphosphoinositides and phosphatidate pre-labelled with $^{32}P_i$ showed marked changes when intact cells were incubated with calcium and ionophore A23187 (Table I). Decreases of 40 and 70% in the radioactivity associated with triphosphoinositide were observed when the media contained 10 and 100 μM $CaCl_2$, respectively, in comparison to calcium-free controls. A slight increase in the radioactivity associated with diphosphoinositide was observed at 10 μM calcium, but at 100 μM calcium radioactivity had decreased substantially. Incorporation of the label into phosphatidic acid, however, consistently increased with increasing calcium concentration. With EGTA in the medium no appreciable difference from the control values was seen.

When erythrocyte membrane lipids pre-labelled with $^{32}P_i$ were incubated in the absence of either calcium or magnesium, a uniform loss of approx. 30% of the label was seen. However, in the presence of 100 μM calcium there was a further breakdown of triphosphoinositide and diphosphoinositide (Table II). Phosphatidic acid was less sensitive to calcium under these conditions. The presence or absence of magnesium had very little effect on the calcium-depen-

TABLE I

CALCIUM INDUCED CHANGES IN POLYPHOSPHOINOSITIDES AND PHOSPHATIDATE IN HUMAN ERYTHROCYTES

Erythrocytes pre-labelled with $^{32}P_i$ were incubated for 30 min in the presence of the ionophore A23187 (4 μM) and calcium or EGTA at 37°C. Values indicated are expressed as percent of control membranes in calcium-free medium and are the averages of three determinations ±S.D. The radioactivity associated with controls were 6200, 2800 and 3100 cpm/mg non-Hb protein in triphosphoinositide, diphosphoinositide and phosphatidate, respectively.

Phosphatide	Ca^{2+}		EGTA (2 mM)
	10 μM	100 μM	
Triphosphoinositide	60 ± 2	27 ± 2	91 ± 8
Disphosphoinositide	128 ± 28	55 ± 13	111 ± 7
Phosphatidate	134 ± 11	209 ± 14	71 ± 7

TABLE II

EFFECT OF DIVALENT METAL IONS ON $^{32}P_i$-PHOSPHOLIPID BREAKDOWN IN ISOLATED ERYTHROCYTE MEMBRANE

Erythrocyte membranes pre-labelled with $^{32}P_i$ were re-incubated in a final volume of 0.25 ml buffer containing 20 mM Tris-HCl (pH 7.45) for 30 min at 37°C, with $CaCl_2$ in the presence or absence of 1 mM $MgCl_2$. The values are expressed as percent of radioactivity (cpm) associated with control membranes (unincubated membranes without any additions) and are the means of four determinations, ±S.D. Radioactivity associated with controls, in cpm/mg non-Hb protein, was 6800, 3600 and 3100 for triphosphoinositide, diphosphoinositide and phosphatidate, respectively. One set of pre-labelled membranes was boiled at 100°C for 2 min and cooled before re-incubation.

Phosphatide	Ca^{2+} (10 μM)		Ca^{2+} (100 μM)			EGTA (2 mM)
	Mg^{2+} absent	Mg^{2+} present	Mg^{2+} absent	Mg^{2+} present	Mg^{2+} present 100°C, 2 min	
Triphosphoinositide	54 ± 3	62 ± 5	31 ± 2	29 ± 2	68 ± 6	74 ± 6
Diphosphoinositide	53 ± 3	57 ± 3	26 ± 2	31 ± 2	57 ± 5	57 ± 5
Phosphatidate	70 ± 8	70 ± 6	59 ± 6	62 ± 7	69 ± 8	69 ± 8

dent breakdown of these lipids. When the pre-labelled membranes were heated at 100°C for 2 min before the addition of calcium and magnesium and subsequently re-incubated, the breakdown of both triphosphoinositide and diphosphoinositide was greatly reduced (Table II).

Two types of pathologic erythrocyte, one with a higher-than-normal cellular calcium content (sickle cells) [12], the other with increased permeability to calcium (pyropoikilocytes) [13], were tested for the pattern of incorporation of $^{32}P_i$ into their membrane polyphosphoinositides and phosphatidic acid components. Table III shows that incorporation of the label into all the lipid components of pyropoikilocytic red cell membranes was greater in comparison to normal erythrocyte membranes. With sickle cells, incorporation of the label into both the diphosphoinositide and phosphatidate was greater than that into the control erythrocytes. On the other hand, incorporation into the triphosphoinositide was slightly less. In general, the normal $^{32}P_i$ incorporation pattern

TABLE III

DISTRIBUTION OF $^{32}P_i$ INCORPORATION INTO THE PHOSPHOLIPIDS OF NORMAL AND PATHOLOGIC ERYTHROCYTES

Incorporation of $^{32}P_i$ into normal and pathologic erythrocytes was carried out in solution K (pH 7.45) at 30% hematocrit for 3 h at 37°C. Reticulocyte-free cells labelled with $^{32}P_i$ were washed and erythrocyte membranes prepared. Radioactivity associated with each of the lipid fractions was determined, The mean values (±S.D.) of four determinations are indicated in the table and are expressed as percent of radioactivity (cpm/mg non-Hb membrane protein) in the corresponding lipids from normal erythrocyte membranes. Radioactivity associated with normal membrane triphosphoinosotide, diphosphoinositide and phosphatidate was 7400, 2900 and 2100 cpm/mg non-Hb protein, respectively. These values are the means of three determinations.

Phosphatide	Normal	Hereditary pyropoikilocytes	Sickle cells
Triphosphoinositide	100	123 ± 10	73 ± 10
Diphosphoinositide	100	225 ± 32	254 ± 14
Phosphatidate	100	587 ± 31	196 ± 20

TABLE IV

EFFECT OF DEOXYGENATION ON THE POLYPHOSPHOINOSITIDES AND PHOSPHATIDATE OF NORMAL AND SICKLE ERYTHROCYTES EXPOSED TO CALCIUM

Normal erythrocytes and reticulocyte-free sickle cells were incubated with $^{32}P_i$ under identical conditions for 3 h at 37°C in a shaking water bath. The pre-labelled cells were reincubated at 15% hematocrit in the presence of Ca^{2+} or EGTA for 30 min under anaerobic conditions (100% N_2) at 37°C. Erythrocyte membranes were subsequently preapred and radioacitivty associated with each lipid component determined as previously described. Values indicated are the means of four determinations (±S.D.) expressed as percent of radioactivity in the corresponding lipids from normal erythrocytes treated as above in the presence of 1 mM EGTA. Radioactivity associated with normal erythrocyte membrane triphosphoinositide, diphosphoinositide and phosphatidate was 850, 250 and 260 cpm/mg non-Hb membrane protein, respectively.

Phosphatide	Normal Ca^{2+} (1.5 mM)	Sickle cells	
		EGTA (1 mM)	Ca^{2+} (1.5 mM)
Triphosphoinositide	92 ± 2	113 ± 9	99 ± 4
Diphosphoinositide	119 ± 6	508 ± 30	411 ± 9
Phosphatidate	129 ± 17	265 ± 11	334 ± 22

(2 : 1 : 1 into triphosphoinositide : diphosphoinositide : phosphatidate) was disturbed in the pathologic erythrocytes.

When normal erythrocytes and sickle cells pre-labelled with $^{32}P_i$ were incubated in the absence of oxygen (100% N_2), sickle cells, in medium containing EGTA, showed higher radioactivity associated with triphosphoinositide and diphosphoinositide during deoxygenation and re-incubation than did those in calcium-containing medium (Table IV). However, the $^{32}P_i$ label associated with phosphatidate was lower in the samples that contained EGTA in the medium. Only marginal changes were observed in the radioactivity associated with the lipids of normal erythrocytes exposed to calcium under the above conditions, in contrast to those in medium containing EGTA.

Reincubation of $^{32}P_i$ pre-labelled erythrocytes with carbamylcholine in the range $1 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$ M for 20 min at 37°C had little effect on the polyphosphoinositides or phosphatidate metabolism.

Discussion

Our results indicating that polyphosphoinositides and phosphatidate are influenced by intracellular calcium corroborate the results of others. Allan et al. [9] have shown that incorporation of $^{32}P_i$ into human erythrocyte phosphatidate was increased in the presence of calcium and ionophore A23187 as a result of increased 1,2-diacylglycerol formation. They also reported their observation of marginal decreases in polyphosphoinositide levels. Recently, Lang et al. [1] have shown a notable breakdown of polyphosphoinositides when human erythrocytes were incubated with calcium and the ionophore. They observed a trend towards a greater breakdown of triphosphoinositide and a lesser breakdown of diphosphoinositide. Our results using $^{32}P_i$ pre-labelled erythrocytes showed similar effects, particularly on triphosphoinositide (Table I). However, the slight increase in the level of diphosphoinositide observed at 10 μM Ca^{2+} with the decrease at higher calcium concentration is in

agreement with the results of Lang et al. [1] who suggested that breakdown of triphosphoinositide might lead to accumulation of diphosphoinositide. In isolated erythrocyte membranes pre-labelled with $^{32}P_i$ and exposed to similar amounts of calcium (Table II), the breakdown of triphosphoinositide was not associated with an increase in the label associated with diphosphoinositide. The fact that heating the pre-labelled erythrocyte membranes to 100°C for 2 min before re-incubation prevented the calcium-dependent breakdown of polyphosphoinositides indicates that the dephosphorylation system was heat-labile and suggests that the process is enzymatic and membrane-bound. The label associated with phosphatidate remained practically unaffected in the presence of either calcium or EGTA; these results are in agreement with those of Allan et al. [9]. Magnesium had little effect on the calcium-dependent dephosphorylation of polyphosphoinositides in the isolated membrane system.

Alterations in the metabolism of pathologic erythrocyte phospholipids have been reported [19,20]. Jacob and Karnovsky [19] observed increased $^{32}P_i$ labelling of phosphatidylserine in erythrocyte membranes derived from patients with hereditary spherocytosis, whereas Reed [20] observed labelling of phosphatidate only. A greater turnover of $^{32}P_i$ in the lipids of erythrocytes from patients with this hematologic disorder was attributed to the somewhat lower age of the cells. In our experiments with normal human erythrocytes we have consistently observed only three radioactive spots on TLC corresponding to triphosphoinositide, diphosphoinositide and phosphatidate, with the highest incorporation of $^{32}P_i$ into the triphosphoinositide. Interesting results were obtained with sickle cells which were separated from reticulocytes (Table III). The incorporation of label into the triphosphoinositide was slightly less than that into the normal erythrocytes with a marked increase into the diphosphoinositide and phosphatidate. This finding may have been obscured in previous studies where the complicating effects of the active reticulocyte fraction were present. These results are also similar to the observations obtained when normal erythrocytes were exposed to low concentrations of calcium in the presence of ionophore (Table I). Since sickle cells have been reported to contain higher levels of calcium than normal erythrocytes [12], the sensitivity of polyphosphoinositides and phosphatidate to calcium in sickle cells is consistent with a role for calcium in mediating polyphosphoinositides turnover in vivo. However, the possibility that agents other than calcium might affect the turnover of these lipids in pathologic erythrocytes cannot be ruled out.

It is known that sickle cells take up calcium during deoxygenation [12]. In an attempt to study the possible role of polyphosphoinositides and phosphatidate, normal erythrocytes and sickle cells pre-labelled with $^{32}P_i$ were deoxygenated in the presence and absence of calcium (1.5 mM). Although no drastic change in the turnover of these lipids was observed, the results (Table IV) were somewhat analogous to those shown in Table I where erythrocytes were exposed to low amounts (10 μM) of calcium in the presence of ionophore. In normal erythrocytes maximal incorporation of $^{32}P_i$ was associated with triphosphoinositide, and this lipid species was more susceptible to calcium-induced dephosphorylation than diphosphoinositide. In contrast, in sickle cells, the greatest incorporation of $^{32}P_i$ was associated with diphosphoinositide and was more susceptible to calcium-induced dephosphorylation. In hereditary

pyropoikilocytosis, a disease in which erythrocytes have increased permeability to calcium, relatively higher incorporation of $^{32}P_i$ into the diphosphoinositide with highest incorporation into the phosphatidate was observed. Thus, the normal incorporation pattern of $^{32}P_i$ into lipids associated with phosphatidate turnover in erythrocyte membranes is disturbed in the pathologic erythrocytes.

These results confirm and extend previous observations of the effects of calcium in red cells in vitro and add a new dimension to the possible role of polyphosphoinositides and phosphatidate during the trans-membrane movement of calcium ions in vivo.

The lack of effect of carbamylcholine on erythrocytes in the metabolism of polyphosphoinositides and phosphatidate suggests that these lipids are not involved in cellular reactions to muscarinic agents.

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