Calcium Affects Phosphoinositide Turnover in Human Erythrocytes

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Abstract. Changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]$) were observed to affect $^{32}P_i$ incorporation into polyphosphoinositides (PPI) and phosphatidic acid (PA) of human erythrocytes. A decrease of extracellular $[Ca^{2+}]$ from 1.5 mmol/l to 0.04 μmol/l increased the specific radioactivity (S.A.) of phosphatidylinositol 4,5-bisphosphate to 182 % and that of phosphatidylinositol 4-phosphate to 120 % of controls. Simultaneously S.A. and concentration of PA decreased. Further decrease of the extracellular $[Ca^{2+}]$ from 0.04 μmol/l to lower values as well as depletion of intracellular Ca^{2+} using ionophore A 23187 in Ca^{2+}- free medium did not accelerate the PPI turnover rates any more. None of the above changes in extracellular $[Ca^{2+}]$ had any effect on the phosphorylation pattern of erythrocyte membrane proteins. Isolated erythrocyte membranes were incubated in the presence of [γ-^{32}P]ATP in media with various $[Ca^{2+}]$. The decrease of $[Ca^{2+}]$ from 0.04 μmol/l (physiological concentration inside the cell) to lower values did not influence the turnover of PPI and PA monoester phosphates. Only after $[Ca^{2+}]$ was increased to 1—5 μmol/l an increase of PPI and PA turnover was observed. Our data suggest that the changes in extracellular $[Ca^{2+}]$ affect the metabolism of PPI and PA (despite the intracellular location of the latter) and may thus influence the properties of red cell plasma membrane.

Key words: Calcium — Phosphoinositides — Human erythrocytes — Calcium ionophore

Introduction

The high turnover rates of PPI monoester phosphates observed in most mammalian tissues have been associated with the inositol lipid signalling system

Abbreviations: PPI, polyphosphoinositides; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PA, phosphatidic acid; $[Ca^{2+}]$, concentration of Ca^{2+}; S.A., specific radioactivity

coupled with intracellular calcium mobilization (Berridge 1986). The physiological function of mammalian erythrocytes is not connected with expressive receptor activation and until now, no physiological stimulus inducing phosphoinositide splitting was found. However, the turnover of monoester phosphate groups of phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidic acid (PA) in human erythrocytes is also very high (Allan 1982) and attempts were made to disclose the physiological function for this process (Maretzki et al. 1983; Ferrel and Huestis 1984). In contrast to the active metabolism of monoester phosphates the PPI specific phosphodiesterase does not show significant activity in the range of physiological calcium concentrations (Allan et al. 1976; Downes and Michell 1981). We studied the properties of the PPI metabolism in human erythrocytes and found that the turnover of monoester phosphates is a sensitive indicator of extracellular [Ca^{2+}].

Materials and Methods

[γ-^{32}P] ATP was prepared according to Walseth and Johnson (1979). Human erythrocytes from healthy adult male volunteers were used immediately after collection, washed, and membranes prepared according to Dodge et al. (1963).

Incubation of intact erythrocytes. Final concentrations in the incubation mixture were (in mmol/l): Hepes 40, adenosine 1, inosine 0.5, glucose 8, Na_2HPO_4 0.2, KCl 31.2, K_2EDTA 10, Mg^{2+} 0.15, Ca^{2+} as specified, and NaCl to isotonicity. The samples were well stirred in a water bath at 37 °C for the times indicated. [^{32}P]-orthophosphate (3.7 MBq) was added to 2 ml of incubation suspension (erythrocytes at 50 % hematocrit). At the end of incubation 5 vols. of ice cold NaCl-Hepes buffer were added, erythrocytes were quickly pelletted and washed in ice cold NaCl-Hepes buffer.

Incubation of erythrocyte membranes. Erythrocyte membranes (10 mg of protein) were incubated at 37 °C in a final volume of 2 ml for 30 min. The incubation suspension contained (in mmol/l): Hepes 10, KH_2PO_4 0.2, K_2EDTA 10, Mg^{2+} 0.35, and Ca^{2+} as specified at pH = 7.4. The radionuclid represented 0.74 MBq of [γ-^{32}P]ATP with 0.21 mmol/l carrier ATP per sample. The incubation was stopped by the addition of acidified chloroform-methanol. The ionophore A 23187 (2 mmol/l solution in 2 % DMSO) was added to a final concentration of 10 μmol/l.

Phospholipids were extracted using acidified chloroform/methanol essentially as described by Strunecká and Folk (1988). The extracts were evaporated under vacuum at 30 °C and applied on silica gel precoated plates (Merck) impregnated with oxalate. The solvent system used was chloroform/methanol/acetone/acetic acid/water (40:15:13:13:7, v/v) as described by Jolles et al. (1979). The authenticity of PIP_2 was proved by cochromatography with commercial standards (Amersham, UK).

For SDS-PAGE the erythrocyte membranes were solubilized in SDS-dithiothreitol buffer and the proteins were separated on 5– 15 % gradient gels using the discontinuous buffer system of Laemmli (1970). The gels were dried and autoradiographed.

Table 1. PPI and PA concentrations in human erythrocyte membranes. Intact erythrocytes were incubated at 37°C for 5 hours in the presence of glucose and different concentrations of Ca^{2+} as described in Methods. The membranes were then isolated and the phospholipids extracted. Where indicated 10 mmol/l A 23187 was added 10 min before the end of the incubation. The values are means ± S.E.M., $n = 15$.

Phospholipid	Concentration (nmol phospholipid/ml packed erythrocytes)		
	1.5 mmol/l Ca^{2+}	0.04 μmol/l Ca^{2+}	1.5 mmol/l Ca^{2+} + A 23187
PIP_2	20.4 ± 5.6	20.9 ± 3.5	3.6 ± 0.2
PIP	32.5 ± 4.1	30.7 ± 2.4	23.0 ± 1.3
PA	61.3 ± 2.1	41.7 ± 3.2	109.1 ± 3.1

Phosphate was estimated by the modified method of Duck-Chong (1979). The composition of calcium buffers was calculated as described by Fabiato and Fabiato (1979) with modifications and using dissociation constants according to Fabiato and Fabiato (1979) and Sillen and Martell (1971). The results were compared by paired Student's *t*-test and by non-parametric Wilcoxon test.

Results and Discussion

According to our protocol, 20.4 ± 5.6 nmol PIP_2, 32.5 ± 4.1 nmol PIP, and 61.3 ± 2.1 nmol PA per ml packed erythrocytes were found. These values are based on extraction of membranes prepared from erythrocytes that were preincubated for 5 hours in glucose containing medium with 1.5 mmol/l Ca^{2+} (see Table 1). The estimates in the literature cover a broad range of values as the direct estimation is always influenced by the extraction method used. For example, the reported values for PIP_2 range between 6.4 (Allan and Michell 1978) and 58 (Christensen 1986) nmol phospholipid/ml packed cells.

$^{32}P_i$ incorporation into phospholipids of intact erythrocytes

The effects of varying [Ca^{2+}] on PPI and PA turnover in intact human erythrocytes were studied. It is evident that $^{32}P_i$ incorporation into both PPI and PA depends on extracellular [Ca^{2+}] (Fig. 1). A comparison of incorporation rates shows enhanced turnover of PIP_2 and PIP and simultaneous decrease of both PA concentration and turnover in 0.04 μmol/l Ca^{2+} medium as compared to 1.5 mmol/l Ca^{2+} medium (see Fig. 1 and Table 1).

After 5 h of incubation in 1.5 mmol/l and 0.04 μmol/l Ca^{2+} medium the

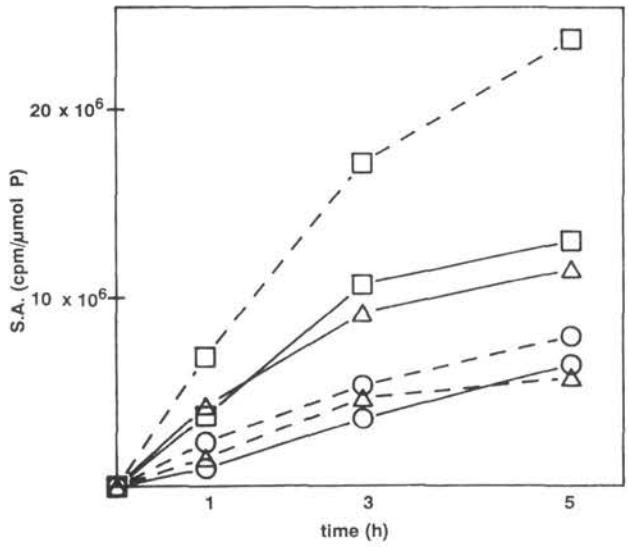

Fig. 1. The time course of $^{32}P_i$ incorporation into phospholipids of intact human erythrocytes incubated at different Ca^{2+} concentrations. The data presented are from a single representative experiment. The incubations were performed in 0.04 μmol/l Ca^{2+} (– – – –) or 1.5 mmol/l Ca^{2+} (————) medium. □ PIP_2, ○ PIP, △ PA.

PIP_2/PIP/PA concentration ratios were 1/1.6/3 and 1/1.5/2 respectively. Analogically, the respective PIP_2/PIP/PA ratios for S.A. were 1/0.5/0.9 in 1.5 mmol/l Ca^{2+} medium and 1/0.3/0.3 in 0.04 μmol/l Ca^{2+} medium. No difference was observed between 0.04 μmol/l Ca^{2+} medium and Ca^{2+} free medium. There were also no differences between Ca^{2+} free medium in combination with high concentration of A 23187 (10 μmol/l), where Ca^{2+}-depletion is expected to be more profound, and 0.04 μmol/l Ca^{2+} medium (Table 3, see also the results of Ponappa et al. (1980)). The incorporation pattern into phospholipids of intact erythrocytes incubated in 0.04 μmol/l Ca^{2+} medium observed in the present experiments is identical with that reported by Moore and Appel (1984) who used Ca^{2+} free medium.

The calcium channel blocker verapamil decreases, in 1.5 mmol/l Ca^{2+} medium, the S.A. of PA and increases the S.A. of both PIP_2 and PIP. The drug thus mimics the effect of low extracellular Ca^{2+} concentration (Fig. 2).

Changes in extracellular Ca^{2+} concentration have no effect on the phosphorylation pattern of erythrocyte membrane proteins as analyzed by SDS-PAGE and autoradiography (Fig. 3). However, protein phosphorylation in erythrocytes is sensitive to rises in intracellular [Ca^{2+}] (Fig. 3, line C) as well as

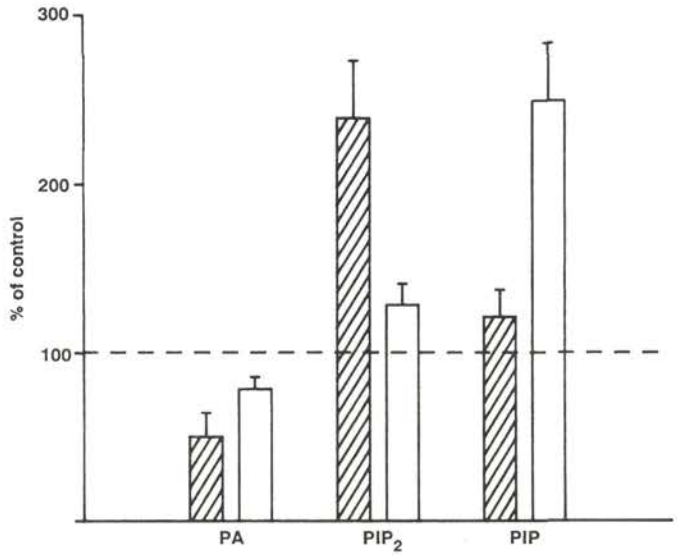

Fig. 2. Comparison of changes of $^{32}P_i$ incorporation into phospholipids of intact human erythrocytes induced by verapamil with the effect of decreased extracellular Ca^{2+} concentration. The S.A. of phospholipids from erythrocytes incubated for 2 h in 1.5 mmol/l Ca^{2+} medium were taken for 100 % (broken line). Bars represent percent changes of S.A. of phospholipids (± S.E.M.) as compared to controls. Open bars, erythrocytes incubated in 1.5 mmol/l Ca^{2+} medium with 10 μmol/l verapamil; hatched bars, erythrocytes incubated in 0.04 μmol/l Ca^{2+} medium. Data from 3 separate experiments.

to changes in S.A. of ATP γ-phosphate. The results from Fig. 3 thus suport the conclusion that nor the changes in intracellular [Ca^{2+}] nor those in S.A. of ATP could explain the observed effects on PPI and PA metabolism.

In 1.5 mmol/l Ca^{2+} medium the calcium pump-leak turnover rate and intracellular [Ca^{2+}] should be in the physiological range (Allan and Michell 1978; Lew et al. 1982; Schatzman 1973). The decrease of [Ca^{2+}] in extracellular medium from 1.5 mmol/l to 0.04 μmol/l reduces the Ca^{2+} concentration gradient across the plasma membrane nearly 40,000 times, as in intact erythrocytes cytosolic [Ca^{2+}] is in the range of 0.04—0.01 μmol/l (Murphy et al. 1986; Lew et al. 1982). This change is expected to influence Ca^{2+} influx and to eventually result in a new steady state, with the intracellular [Ca^{2+}] decreasing to a level at which the Ca^{2+}-ATPase catalyzed Ca^{2+} efflux is equal to total Ca^{2+} influx. As was shown by Lew et al. (1982) the Ca^{2+}-ATPase is almost inactive at intracellular [Ca^{2+}] below 5 nmol/l. Hence, the decrease in intracellular [Ca^{2+}] is likely in the order of 10 or less.

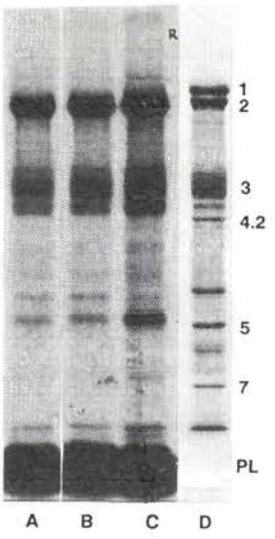

Fig. 3. The effect of increased intracellular Ca^{2+} concentration on the phosphorylation pattern of erythrocyte membrane proteins. Intact human erythrocytes were incubated for 3 h in the presence of $^{32}P_i$ and different Ca^{2+} concentrations, the membranes were isolated and subjected to SDS-PAGE followed by autoradiography. Where indicated, 10 μmol l A 23187 was added 10 min before the end of the incubation. The amount of protein applied was the same in all lanes. (*A*) 0.04 μmol l Ca^{2+}, (*B*) 1.5 mmol l Ca^{2+}, (*C*) 1.5 mmol l Ca^{2+} + A 23187, (*D*) pattern of Coomassie Blue stained proteins.

Table 2. Incorporation of ^{32}P into phosphoinositides and PA of human erythrocyte ghosts at various Ca^{2+} concentrations. Isolated erythrocyte membranes were incubated for 0.5 hours in the presence of [γ-^{32}P]ATP in Ca^{2+} buffered media as described in Methods. The data represent means ± S. E. M. of 9 determinations from 3 independent experiments. The numbers in brackets are relative deviations from values measured in 0.04 μmol l Ca^{2+} medium. Asterisk indicates statistically significant differences ($P < 0.01$).

PL	Activity (dpm)			S. A. (dpm μmol P)		
	without Ca^{2+}	0.04 μmol l Ca^{2+} Control	5 μmol l Ca^{2+}	without Ca^{2+}	0.04 μmol l Ca^{2+} Control	5 μmol l Ca^{2+}
PIP_2	11360 ± 350 (97.3%)	11670 ± 1170	17745 ± 945 (152.1%)*	131080 ± 3165 (94.5%)	138680 ± 13753	209600 ± 13470 (151.5%)*
PIP	1800 ± 880 (83.7%)	2150 ± 160	2885 ± 390 (134.2%)*	28425 ± 1106 (115.6%)	24593 ± 3279	30028 ± 4148 (122.1%)
PA	2165 ± 90 (98.2%)	2205 ± 35	6725 ± 295 (305.0%)*	42786 ± 1738 (107.4%)	39850 ± 1336	91921 ± 7948 (230.7%)*

$^{32}P_i$ incorporation into PPI and PA of erythrocyte membranes

Isolated membranes were incubated in the presence of [γ-^{32}P] ATP in media with various [Ca^{2+}]. The contents, activities, and specific activities of PPI and PA in Ca^{2+} free medium, in 0.04 μmol/l Ca^{2+} medium (which approaches the putative cytosolic [Ca^{2+}]) and in 5 μmol/l Ca^{2+} medium (representing the increased cytosolic [Ca^{2+}]) were compared (Table 2).

The increase of [Ca^{2+}] from 0.04 μmol/l to 5 μmol/l results in increased turnover of both PPIs (S.A. of PIP_2 increased to 155% of control). Furthermore, PA turnover and content increased (S.A. increased to 231%, the concentration to 132% of control). The same results were obtained using Ca^{2+}-free instead of 0.04 μmol/l Ca^{2+} medium.

The PIP_2/PIP/PA concentration ratios were 1/1.3/2 for the 0.04 μmol/l Ca^{2+} medium and 1/1.7/2.6 for the 5 μmol/l Ca^{2+} medium. The respective values for S.A. were 1/0.2/0.3 in 0.04 μmol/l Ca^{2+} medium and 1/0.1/0.4 in 5 μmol/l Ca^{2+} medium. In comparison with PIP_2, the rise in [Ca^{2+}] led to relative increases in PA and PIP contents and to a relative increase in PA turnover.

The concentration ratios as well as S.A. ratios for PIP_2, PIP, and PA from ghosts incubated in 0.04 μmol/l Ca^{2+} medium were similar to those obtained for intact erythrocytes after 5 h incubation at the same Ca^{2+} concentration. Replacing 0.04 μmol/l Ca^{2+} medium by Ca^{2+} free medium caused no significant change in erythrocyte membrane phospholipids (Table 2). This supports the conclusion that the effect of decreased extracellular [Ca^{2+}] on PPI metabolism in intact erythrocytes is not simply a consequence of decreased intracellular [Ca^{2+}].

The effect of calcium ionophore A 23187

A 23187 was used to induce Ca^{2+} overload and PPI splitting in human erythrocytes.

Isolated human erythrocytes were incubated in media with 10 mmol/l EDTA (to buffer divalent cations), 0.15 mmol/l Mg^{2+}, 75 mmol/l K^+, and various Ca^{2+} concentrations. The composition of the media was chosen according to Lew and Garcia-Sancho (1985) to prevent undesirable side effects of the ion movements. The concentrations of Mg^{2+} and K^+ were set to levels at which no net changes in the intracellular content occur.

The A 23187 treatment induced significant changes of phospholipid metabolism in the 1.5 mmol/l Ca^{2+} medium only (Table 3): a decrease in PIP_2 and PIP concentrations (17.6 and 70.9% of controls, respectively). With $^{32}P_i$ as precursor, the S.A. of PIP_2 remained unchanged, whereas that of PIP decreased to 30.3% of control. Thus the breakdown of PIP must be accompanied by the

Table 3. Effect of A 23187 on phosphoinositides and PA turnover in intact human erythrocytes at various Ca^{2+} concentrations. Intact erythrocytes were incubated for 4 hours in the presence of $^{32}P_i$ in Ca^{2+} buffered media as described in Methods. Ten μmol/l A 23187 was added 10 min before the end of the incubation. The results are expressed as relative values as compared to control incubations ± S. E. M. The data are from 4 separate experiments, $n = 12$. Significance levels: *($P < 0.01$), +($P < 0.001$).

PL	Activity (% of controls)			S. A. (% of controls)		
	without Ca^{2+}	0.04 μmol/l Ca^{2+}	1.5 mmol/l Ca^{2+}	without Ca^{2+}	0.04 μmol/l Ca^{2+}	1.5 mmol/l Ca^{2+}
PIP_2	94.6 ± 7.1	99.2 ± 3.6	17.8 ± 3.7*	108.2 ± 9.6	96.7 ± 11.2	100.9 ± 4.1
PIP	104.3 ± 10.0	94.5 ± 10.1	21.5 ± 10.7*	100.1 ± 6.4	90.9 ± 8.1	30.3 ± 12.4+
PA	121.9 ± 23.9	105.3 ± 9.1	425.6 ± 23.5+	94.5 ± 6.1	89.7 ± 11.1	238.5 ± 35.0+

replacement of PIP with a lower specific activity, probably originating from monoesteric breakdown of PIP_2. Alternatively, when two metabolic pools of this phospholipid are postulated, the breakdown of the pool with high S.A. would explain the decrease in total S.A. (for related results see Muller et al. (1986)). A simultaneous activation of PA synthesis was found, resulting in a 178 % increase in the concentration of this phospholipid.

The treatment with A 23187 in Ca^{2+}-free medium induced no changes in PPI and PA metabolism as compared to control without the ionophore (Table 3). It is likely, however, that the intracellular [Ca^{2+}] obtained in Ca^{2+}-free medium after ionophore permeabilization of the membrane is well below that in the intact cell.

The phosphorylation pattern of erythrocyte membrane proteins was not influenced by the addition of A 23187 to Ca^{2+}-free medium (not shown), but in 1.5 mmol/l Ca^{2+} medium a marked increase in labelling of individual bands was seen (Fig. 3). It agrees with the general assumption that elevation of [Ca^{2+}] stimulates protein phosphorylation in erythrocytes (Enyedi et al. 1982).

From our results we conclude that decrease of extracellular [Ca^{2+}] modulates the PPI turnover. The enzymes of PPI and PA metabolism might be directly or indirectly exposed to extracellular space, serving as receptors for extracellular [Ca^{2+}]. The enzymes may be influenced by changes of membrane surface charge caused by dissociation of Ca^{2+} from the Ca^{2+}-binding sites on the membrane. Furthermore, changes in PPI metabolism were shown to modulate Ca^{2+}-ATPase activity (Sarkadi et al. 1982) and direct feedback regulation could exist in the opposite direction as well.

Decrease in extracellular [Ca^{2+}] activates the turnover of PPI to a level,

which is not reached under physiological plasma [Ca^{2+}]. Together with the changes in the PA metabolism this can influence the properties of red cell plasma membrane. Depletion of the PPI pool is one of the first degradation-associated changes during Ca^{2+} overload and cell lysis in human erythrocytes (Allan 1982; Ponnappa et al. 1980). Thus the high turnover of monoester phosphates of PPI together with low levels of PA may prove beneficial for long-term preservation of red blood cells.

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