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Membrane phospholipid organization in calcium-loaded human erythrocytes *

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Intracellular Ca^{2+} levels in human erythrocytes were increased by incubating them with variable concentrations of Ca^{2+} in the presence of ionophore A23187. Experiments were done to confirm that the Ca^{2+} loading did induce changes in the cell shape and membrane protein composition. The effect of the increased cytoplasmic Ca^{2+} levels on the membrane phospholipid organization was analysed using bee venom and pancreatic phospholipases A_2, Merocyanine 540 and fluorescamine as the external membrane probes. About 20% phosphatidylethanolamine (PE) and 0% phosphatidylserine (PS) were hydrolysed by the phospholipases in intact control cells, whereas in identical conditions these enzymes readily degraded, 20–30% PE and 7–30% PS, in Ca^{2+}-loaded erythrocytes, depending on the cytoplasmic Ca^{2+} concentration. Also, Merocyanine 540 failed to stain the fresh or control erythrocytes, but it labeled the cells loaded with Ca^{2+}. Furthermore, fluorescamine labeled approx. 20% PE in fresh or control erythrocytes while in identical conditions, significantly higher amounts of PE were modified in intact Ca^{2+}-loaded cells. These results demonstrate that Ca^{2+} loading in human erythrocytes leads to loss of the transbilayer phospholipid asymmetry, and suggest that, together with spectrin, polypeptides 2.1 and 4.1 may also play an important role in maintaining the asymmetric distribution of various phospholipids across the erythrocyte membrane bilayer.

Introduction

Red cell membrane phospholipids are unequally distributed in the two leaflets of the membrane bilayer. Choline-containing phospholipids (sphingomyelin and phosphatidylcholine (PC)) are located mainly in the outer monolayer, whereas aminophospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS)) tend to localize almost exclusively in the inner monolayer [1]. This typical asymmetric arrangement of phospholipids within the membrane bilayer seems to be stabilized by the interactions between the inner layer phospholipids and cytoskeletal proteins, especially spectrin [2–11].

Several studies have shown that loading of the red cells with Ca^{2+}, using ionophore A23187, leads to marked structural changes in the cytoskeletal proteins [12–19]. These changes are induced mainly by the activation of some Ca^{2+}-regulated enzymes, including transglutaminase [12–14] and Ca^{2+}-dependent proteinases [15–18]. It has been reported that transglutaminase catalyses cross-lin-

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; GSH, glutathione; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TLC, thin-layer chromatography.

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king of spectrin with other membrane proteins [12–14], while Ca^{2+}-dependent proteinases cause selective proteolysis of spectrin and polypeptide 4.1 [18].

To investigate further the role of the spectrin-inner layer phospholipid interactions in stabilizing the asymmetric distribution of various phospholipids across the erythrocyte membrane bilayer, we have analysed the effect of increased intracellular Ca^{2+} levels on the transbilayer organization of membrane phospholipids in human erythrocytes. Loading of the cells with Ca^{2+} was carried out by their incubation with variable concentrations of Ca^{2+} (2 μM–1.5 mM) and ionophore A23187. The membrane phospholipid organization was analysed by means of enzymatic, amino group-labeling and fluorescent probes. Experiments were done to confirm that Ca^{2+} loading in red cells did induce changes in cell shape and membrane protein compositions. Results of these studies suggest that, together with spectrin, the proteins which anchor cytoskeleton to the membrane bilayer could also play an important role in stabilization of the red cells membrane phospholipid asymmetry.

Materials and Methods

Materials

Normal human blood samples were collected from National Pathology Blood Bank, Lucknow. Ionophore A23187, phospholipases A_2 from porcine pancreas and bee venom, lactoperoxidase, fluorescamine, *N*,*N*-dimethylated casein, cystamine, putrescine, glutathione (GSH), bovine serum albumin, Mg^{2+}-ATP and ATP estimation kit were purchased from Sigma Chemical Company. Merocyanine 540 was bought from Serva Feinbiochemica. [^{3}H]Putrescine and sodium [^{125}I]iodide (carrier-free) were obtained from Amersham, U.K. $^{45}CaCl_2$ was from Bhabha Atomic Research Centre, Trombay, India. Pre-coated silica gel 60F-254 thin-layer chromatography (TLC) plates were purchased from E. Merck. Radiolabeling of pancreatic phospholipase A_2 with ^{125}I was carried out essentially according to the known procedure [20]. Erythrocytes from fresh human blood were isolated by removing the plasma and buffy coat.

Ca^{2+} loading

Red cells were suspended to 50% hematocrit in buffer A (5 mM Tris/100 mM KCl/60 mM NaCl/10 mM glucose (pH 7.4)). A stock solution (5 mM) of A23187 in dimethylsulfoxide was kept frozen, and diluted with buffer A on the day of use. A solution (20 mM) of $CaCl_2$ was prepared in buffer A. The loading of Ca^{2+} in cells was carried out essentially according to the published method [21]. While Ca^{2+} concentration in the incubation mixture was varied from 2 μM to 1.5 mM, the ionophore concentration was fixed at 10 μM throughout this study. About 1–3% erythrocytes were lysed by incubating them for 3 h under these conditions. The control experiments were run after including EGTA (5 mM) in the incubation mixture. Ionophore and Ca^{2+} were removed by washing ($900 \times g$) the cells three times with cold buffer A containing bovine serum albumin (0.4%) and EDTA (1 mM). This was followed by 4–5 washings with phosphate-buffered saline to ensure complete removal of albumin and EDTA. The cells so isolated were virtually free from microvesicles and ghosts, as judged by both light and electron microscopy.

Kinetics and efficiency of Ca^{2+} loading

To determine the kinetics and efficiency of Ca^{2+} loading in red cells, the external Ca^{2+} was labeled with $^{45}Ca^{2+}$ by including traces (approx. $2 \cdot 10^6$ cpm) of $^{45}CaCl_2$ (spec. act., 67 mCi/g) in the incubation mixture. Cells were washed to remove the ionophore and excess radioactivity after completion of the incubation. The washed cells were lysed with water, and the lysates treated with cold 10% trichloroacetic acid. The precipitated material was removed by centrifugation. Measured aliquots of the supernate were counted for radioactivity.

Erythrocyte GSH and ATP levels

GSH levels in red cells were measured essentially according to the known procedure [22]. Red cell ATP levels were determined by an enzymatic test employing a Sigma Diagnostics Kit (procedure No. 366-UV), except that isolated red cells were used in the assay instead of whole blood.

Scanning electron microscopy

Electron microscopy was carried out in a Philips SEM 515 electron microscope, as described previously [23].

Erythrocyte transglutaminase activity

Transglutaminase activity was measured by using *N,N*-dimethylated casein and [^{3}H]putrescine, essentially according to the published procedure [24]. The effect of cystamine on the red cell transglutaminase activity was determined by including 10 mM cystamine in the incubation mixture.

Erythrocyte treatment with phospholipase A_2

A 0.25-ml aliquot of packed erythrocytes was suspended in 5.0 ml of 10 mM glycylglycine buffer containing 100 mM KCl/50 mM NaCl/0.25 mM $MgCl_2$/0.25 mM $CaCl_2$/44 mM sucrose (pH 7.4). Bee venom (10 IU) phospholipase A_2 was added, and the mixture incubated at 37°C. The enzyme reaction was stopped by washing the cells three times with saline containing 5 mM EDTA (pH 7.4). The extent of hemolysis was determined at the end of each incubation prior to EDTA wash by comparing the hemoglobin content in the supernate of each sample to that of a 100% hemolysed control.

Treatments with porcine pancreatic phospholipase A_2 (10 IU/0.25 ml packed cells) were carried out at 37°C in the above incubation medium, except that the concentration of $CaCl_2$ was 10 mM.

Unsealed erythrocyte ghosts were also treated with both the enzymes essentially under the above conditions.

Determination of phospholipid degradation

Lipids were extracted directly (without lysis) from the phospholipase-treated cells according to the method of Rose and Oklander [25]. Individual phospholipids were separated by two-dimensional TLC, as described earlier [26]. Spots for different phospholipids were identified after staining the plate with iodine vapor followed by ninhydrin spray. These were removed and eluted several times with a mixture of methanol/chloroform (1 : 1, v/v). Total phosphorus present in each spot was determined according to the method of Ames and Dubin [27]. The recoveries of various phospholipids from silica gel were over 95%.

Erythrocyte labeling with fluorescamine

Labeling of aminophospholipids with fluorescamine in intact red cells as well as in ghosts was carried out essentially according to the published procedure [28], except that Hepes was used instead of Tricine buffer. Lipids from cells were extracted [25], and separation between the labeled and unlabeled phospholipids affected by two-dimensional TLC [29].

Erythrocyte labeling with Merocyanine 540

Labeling of red cells with Merocyanine 540 was carried out as described earlier [30]. About 10^8 cells were suspended in 2 ml incubation medium (10 mM Tris/0.25 M sucrose/15 mM NaCl/5 mM KCl/3 mM $MgCl_2$ (pH 7.4)) containing 2.5% AB^+ human serum and Merocyanine 540 (20 μg), and incubated in the dark at 37°C for 10 min. The cells were rapidly washed three times with 10 mM Tris containing 140 mM NaCl/10 mM KCl/3 mM $MgCl_2$ (pH 7.4) to remove excess dye. Fluorescence microscopy was performed in a Polyvar Reichert Jung Microscope. Merocyanine 540-stained cells, after fluorescence micrography, were largely intact, as judged by phase-contrast light microscopy.

SDS/polyacrylamide gel electrophoresis

Membrane protein composition in erythrocyte was determined by SDS-polyacrylamide gel electrophoresis. The membranes were prepared according to the published procedure [31], except that phenylmethylsulfonyl fluoride (1 mM) was also included in the cell lysis medium. Electrophoresis was carried out on 5% polyacrylamide slab gels in the presence of SDS according to the method of Fairbanks et al. [32]. The gels were stained with Coomassie blue R250 and scanned on a Bio-Med soft laser TRLFF densitometer.

Results

Human erythrocytes were loaded with Ca^{2+} by incubating them with variable concentrations (2 μM–1.5 mM) of Ca^{2+}, in the presence of ionophore A23187 (10 μM), at 37°C under nitrogen atmosphere. Fig. 1 shows that the uptake of Ca^{2+} by the red cells increased with time upto 1 h, but after that it remained virtually constant, at least up to 3 h. The efficiency of Ca^{2+} loading in the cells was about 80–90% under our experimental conditions.

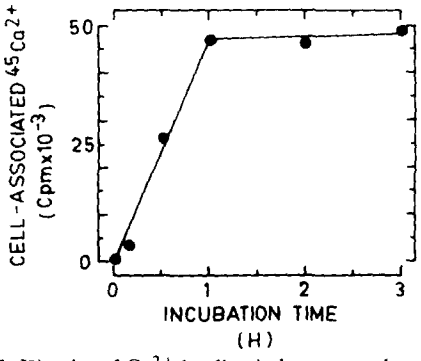

Fig. 1. Kinetics of Ca^{2+} loading in human erythrocytes.

The effects of Ca^{2+} loading on the erythrocyte ATP and GSH levels were measured by standard procedures. The ATP level in the cells decreased with an increase in the intracellular Ca^{2+} (Table I), while no effect was observed on the GSH levels (data not shown).

The effect of the cytoplasmic Ca^{2+} levels on the cell shape was ascertained by scanning electron microscopy. In agreement with the literature [33], the cells were transformed from discocytes to echinocytes and spheroechinocytes by loading them with Ca^{2+} (data not shown).

Effect of Ca^{2+} loading on the erythrocyte membrane protein composition

The effect of the increased cytoplasmic Ca^{2+} concentration on the erythrocyte membrane protein composition was determined by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows that an increase in the intracellular Ca^{2+} levels invariably resulted in the appearance of new membrane protein bands with a concomitant decrease in the

TABLE I

ATP LEVELS IN ERYTHROCYTES LOADED WITH Ca^{2+}

Loading of the cells with Ca^{2+} was carried out at 37 °C for 3 h. Values shown are means ± S.D. of 4–6 determinations.

Red cells	ATP levels (mmol/l packed cells)
Untreated fresh cells	1.14 ± 0.07
A23187-treated cells	1.02 ± 0.09
A23187 + 5 μM Ca^{2+}-treated cells	0.75 ± 0.23
A23187 + 1.5 mM Ca^{2+}-treated cells	0.19 ± 0.08

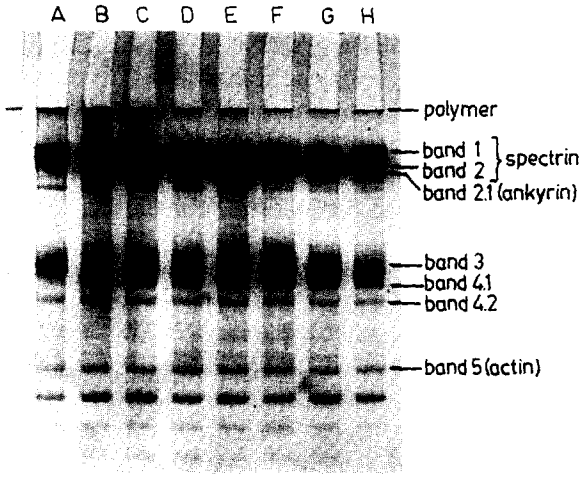

Fig. 2. Coomassie blue-stained SDS-polyacrylamide electrophoretograms of human erythrocyte membrane proteins. (A) Cells incubated with 1.5 mM Ca^{2+} plus ionophore, (B) cells incubated with 1.0 mM Ca^{2+} plus ionophore, (C) cells incubated with 0.5 mM Ca^{2+} plus ionophore, (D) cells incubated with 0.25 mM Ca^{2+} plus ionophore, (E) cells incubated with 0.1 mM Ca^{2+} plus ionophore, (F) cells incubated with 50 μM Ca^{2+} plus ionophore, (G) cells incubated with 25 μM Ca^{2+} plus ionophore, (H) cells incubated with ionophore only. The incubation was carried out for 3 h at 37 °C.

intensities of polypeptides 2.1 and 4.1, and to some extent spectrin and anion channel protein. A high-molecular-weight band, which failed to penetrate into the gels, appears to correspond to the transglutaminase-catalysed polymer formation, which is known to involve cross-linking of spectrin with other membrane proteins [12–14]. Formation of this polymer was apparent only when the red cells were loaded with over 0.25 mM Ca^{2+}. At lower Ca^{2+} concentrations, no high-molecular-weight band could be detected, even after overloading the gels. Also, polymer formation did not occur upon preincubating the cells with cystamine (10 mM) prior to their loading with higher Ca^{2+} concentrations (0.5 mM). These results were in accordance with the measurements of Ca^{2+}-dependent erythrocyte transglutaminase activity (data not shown). Very little activity could be detected at 25 μM Ca^{2+}, but it was significant at higher Ca^{2+} concentration (over 0.1 mM). However, the activity was completely blocked by including 10 mM cystamine in the incubation mixture.

The disappearance of band 2.1 seems to correlate with the appearance of a new polypeptide of molecular weight approx. 180000. Earlier studies have suggested that this polypeptide is formed by proteolysis of band 2.1 [12], but a polypeptide of similar molecular weight is known to result also from a limited degradation of spectrin by Ca^{2+}-dependent proteinases [18]. As we observed no loss of spectrin at relatively lower Ca^{2+} concentrations (up to 50 μM), formation of the new polypeptide from spectrin appears less likely, at least at these concentrations.

Another membrane protein which appears to be sensitive to the Ca^{2+}-induced changes is polypeptide 4.1. Its intensity was not affected up to 100 μM external Ca^{2+} concentration, but above this concentration the intensity of the polypeptide decreased with an increase in the external level of Ca^{2+}. Our data, however, does not allow us to conclude whether the observed loss of polypeptide 4.1 is due to its irreversible cross-linking with spectrin [16] or its degradation by Ca^{2+}-dependent proteinases [18].

Effect of Ca^{2+} loading on the erythrocyte membrane phospholipid organization

Membrane phospholipid organization in the Ca^{2+}-loaded erythrocytes was analysed using bee venom and pancreatic phospholipases A_2, Merocyanine 540, and fluorescamine as the external membrane probes. Fig. 3 shows that pancreatic phospholipase A_2 hydrolysed PE and PS in the Ca^{2+}-loaded erythrocytes but not in the control cells. The amount of degraded PS increased with the intracellular Ca^{2+}. About 25% PE and 28% PS were cleaved by this enzyme in the red cells that were preincubated with 1.5 mM Ca^{2+} in the presence of ionophore A23187. These results were consistent with our observation that bee venom phospholipase A_2 failed to attack PS in the intact control erythrocytes, but it readily hydrolysed this aminophospholipid in the Ca^{2+}-loaded cells (Fig. 3). However, both the enzymes were found to degrade almost completely (85–100%) the various glycerophospholipids in the unsealed erythrocyte ghosts, under identical conditions.

To examine whether the 30 min enzyme treatment, used in the above studies, is optimal for effecting the maximum phospholipid hydrolysis,

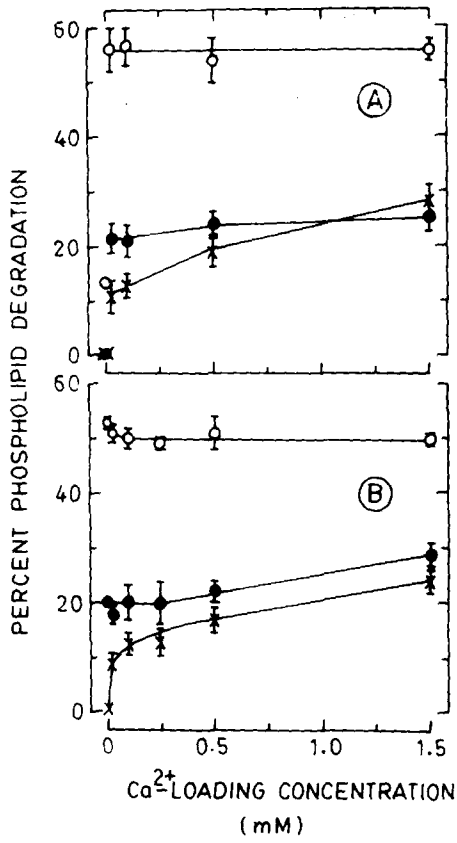

Fig. 3. Membrane phospholipid hydrolysis by pancreatic (A) and bee venom (B) phospholipases A_2 in human erythrocytes preincubated with variable concentrations of Ca^{2+} in the presence of ionophore A23187. Preincubations with Ca^{2+} and ionophore were carried out for 3 h at 37 °C. The phospholipase treatment was done for 30 min. ○, PC; ●, PE; ×, PS. Values shown are mean ± S.D. of 4–6 determinations. Hemolysis during enzyme reactions was less than 3%. Unsealed ghosts prepared from normal human erythrocytes were also treated with both bee venom and pancreatic phospholipases A_2 in identical conditions. Bee venom phospholipase A_2 hydrolysed almost completely (98–100%) all the glycerophospholipids (PC, PE and PS), whereas pancreatic phospholipase A_2 degraded 89.0 ± 2.1% PC, 83.5 ± 1.9% PE and 100% PS under these conditions.

we determined the extent of phospholipid degradation after treating the red cells with both pancreatic and bee venom phospholipases A_2 for varying periods of time. Fig. 4 shows that the extent of phospholipid hydrolysis increased with the incubation time up to 30 min, but after this period it remained virtually constant, at least upto 60 min. This indicates that 30 min enzyme treat-

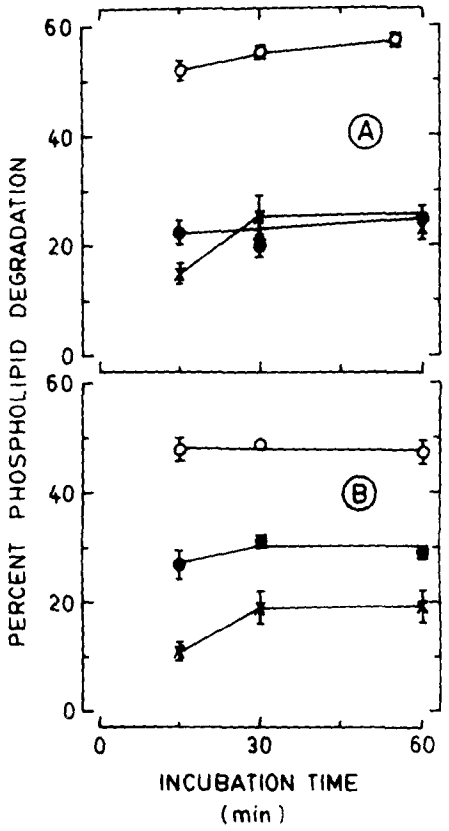

Fig. 4. Time-dependent hydrolysis of membrane phospholipids by pancreatic (A) and bee venom (B) phospholipases A_2 in human erythrocytes preincubated with 1.5 mM Ca^{2+} plus ionophore A23187 for 3 h. ○, PC; ●, PE; ×, PS. Values shown are mean ± S.D. of 3–5 determinations.

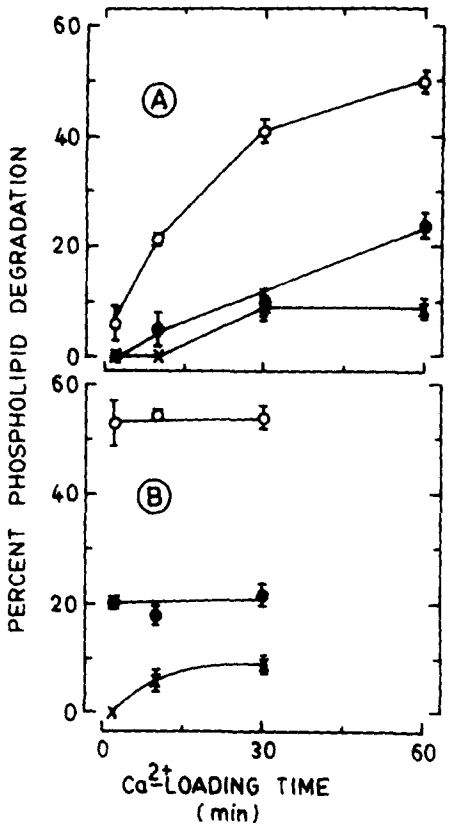

Fig. 5. Membrane phospholipid hydrolysis by pancreatic (A) and bee venom (B) phospholipases A_2 in human erythrocytes preincubated with 25 μM Ca^{2+} plus ionophore A23187 for variable periods of time. The hydrolysis was carried out for 30 min at 37°C. ○, PC; ●, PE; ×, PS. Values shown are mean ± S.D. of 3–6 determinations.

ment is sufficient to effect the maximum phospholipid hydrolysis under our experimental conditions.

These results demonstrate that accessibility of the membrane glycerophospholipids to both bee venom and pancreatic phospholipases A_2 is markedly enhanced upon loading the erythrocytes with Ca^{2+}. About 20% PE and 0% PS were cleaved by the phospholipases in the intact control cells, whereas in identical conditions these enzymes could readily degrade 20–30% PE and 7–30% PS in the Ca^{2+}-loaded erythrocytes, depending on the cytoplasmic Ca^{2+} concentration. To establish further that the extent of the membrane aminophospholipid hydrolysis in erythrocytes depends on the intracellular Ca^{2+} levels, we examined the accessibility of the membrane glycerophospholipids to both bee venom and pancreatic phospholipases A_2 in human red cells preincubated with 25 μM Ca^{2+}, in the presence of ionophore A23187, for varying periods of time (up to 60 min). Fig. 5 shows that the erythrocyte membrane phospholipid hydrolysis increased with the preincubation time. Since we have shown that 60 min incubation time is required for effecting the maximum Ca^{2+} loading (Fig. 1), it may be inferred that the above observed time-dependent increase in the phospholipid hydrolysis is due to increase in the cytoplasmic Ca^{2+} levels.

The observed enhanced hydrolysis of membrane aminophospholipids in Ca^{2+}-loaded erythrocytes is not due to penetration of the phospholipases into these cells, since these enzymes in identical conditions could degrade significantly

higher amounts of the various glycerophospholipids in the unsealed erythrocyte ghosts (Fig. 3). This was further confirmed by determining the residual amounts of cell-associated pancreatic phospholipase A_2 after the enzyme treatments. Erythrocytes were loaded with 1.5 mM Ca^{2+}, and both the loaded and control cells incubated with ^{125}I-labeled pancreatic phospholipase A_2 essentially under the conditions as given in Materials and Methods. The enzyme-treated washed cells were counted for radioactivity and then lysed with distilled water. The lysates were centrifuged at $100\,000 \times g$ (60 min), and the radioactivity determined in both the pellet and supernate. About 0.4 and 0.25% of the total ^{125}I were found to be associated with the control and Ca^{2+}-loaded cells, respectively. Of these amounts, approx. 70% ^{125}I was present in the pellet and approx. 30% in the supernate of both the types of cells. From these findings, we infer that the phospholipases did not penetrate into the Ca^{2+}-loaded erythrocytes, and that only the external glycerophospholipids were hydrolysed in both the control and loaded cells, under our experimental conditions.

Phospholipases have been extensively used to ascertain the membrane phospholipid organization in human erythrocytes (reviewed in Ref. 34). Although the degree to which these enzymes hydrolyse the various glycerophospholipids in intact cells has usually been correlated with the extent of their localization in the outer monolayer [1,34], it has recently been suggested that the phospholipase treatment of the modified erythrocytes may induce reorganization of the membrane phospholipids [28,35]. It is therefore difficult to ascertain whether the Ca^{2+} loading in the erythrocytes leads to partial migration of PE and PS to the external monolayer or simply enhances the flip-flop rates of the various phospholipids, without altering the transbilayer lipid distribution. To distinguish between these two possibilities, we analysed the erythrocyte membrane phospholipid organization by means of Merocyanine 540 [9] and fluorescamine [28] as the external membrane probes.

Merocyanine 540 staining has been claimed to be a very reliable method for assaying the transbilayer phospholipid asymmetry in mammalian erythrocytes and their derivatives [9,36–38]. It has been suggested [9] that this dye in the presence of serum (or plasma) stains only those red cells which have an abnormal transbilayer phospholipid distribution, Therefore, we examined the Merocyanine 540 labeling of both the control and Ca^{2+}-loaded erythrocytes. Fig. 6 shows that Merocyanine 540 failed to stain the control erythrocytes, but it readily labeled the Ca^{2+}-loaded cells. The intensity of this staining appeared to increase with the intracellular Ca^{2+} concentration. These findings are consistent with our observation that phospholipase A_2-catalysed membrane glycerophospholipid hydrolysis is increased with the cytoplasmic Ca^{2+} levels. Therefore, it would appear that Ca^{2+} loading in human erythrocytes probably induces an alteration in the transbilayer organization of the membrane phospholipids.

To confirm further the above suggestion, we

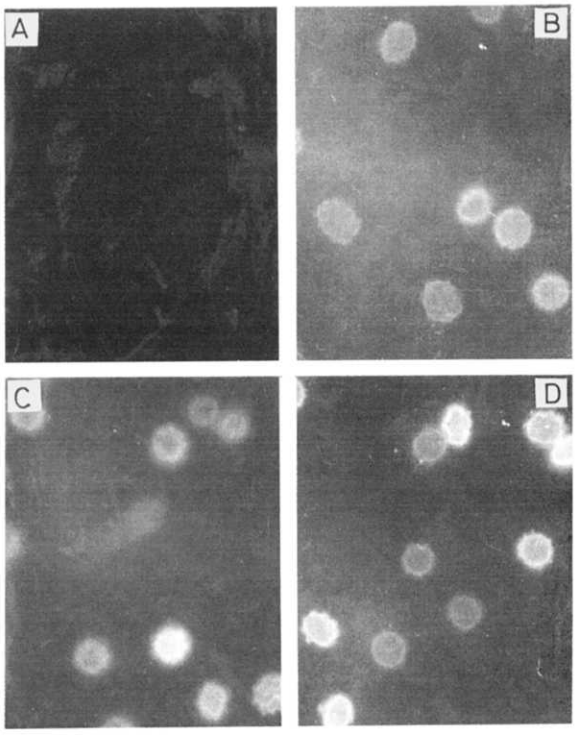

Fig. 6. Merocyanine 540 staining of human erythrocytes preincubated with variable concentrations of Ca^{2+} in the presence of ionophore A23187. Preincubation was done for 3 h at 37 °C. (A) Cells preincubated with ionophore only, (B) cells preincubated with 25 μM Ca^{2+} plus ionophore, (C) cells preincubated with 0.5 mM Ca^{2+} plus ionophore, (D) cells preincubated with 1.5 mM Ca^{2+} plus ionophore.

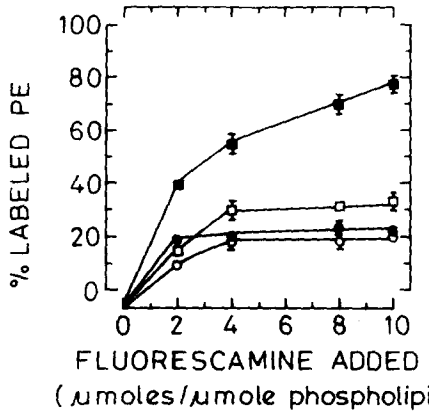

Fig. 7. Labeling of PE by fluorescamine in human erythrocytes. The PE labeling was carried out at 15–20 ° C. ○, Labeled PE in fresh cells; ●, labeled PE in cells preincubated with ionophore A23187; □, labeled PE in cells preincubated with 0.5 mM Ca^{2+} and ionophore for 3 h at 37 ° C; ■, labeled PE in unsealed erythrocyte ghosts. Values shown are mean ± S.D. of 3–5 determinations. The amounts of labeled PS in Ca^{2+}-loaded erythrocytes and unsealed ghosts could not be determined accurately, as the spot corresponding to this lipid did not completely separate from the phosphatidylinositol on the TLC plates.

finally employed fluorescamine as the amino group-specific chemical probe for analysing the PE distribution in both the control and Ca^{2+}-loaded erythrocytes, since this reagent has recently been claimed to be the probe of choice to study the membrane phospholipid asymmetry [28]. Our results are shown in Fig. 7. The amounts of PE labeled in the control cells were similar to those observed in the fresh erythrocytes, but these amounts were appreciably greater in the Ca^{2+}-loaded cells. This increase in the PE labeling was not due to penetration of the probe into the Ca^{2+}-loaded erythrocytes, as in identical conditions we observed enormously higher labeling of this aminophospholipid in the unsealed erythrocyte ghosts. Similarly, the greater PE labeling in the control erythrocytes, as compared to the fresh cells, at 2 μmol fluorescamine/μmol phospholipid concentration was not due to permeation of the reagent across the membrane, but could be attributed to the ionophore-induced changes in the lipid-protein interactions within the membrane bilayer [39].

The above results clearly show that loading of the human red cells with Ca^{2+} leads to alterations in transbilayer distribution of the membrane glycerophospholipids. This is quite in accordance with the earlier studies which showed that Ca^{2+}-loaded red cells can elicit an increased rate of clotting when added as a reagent in the Russel's Viper Venom clotting time assay, and bind more acidic phospholipid-containing liposomes [40]. Also, it corroborates the previous conclusions which were drawn by using bee venom phospholipase A_2 [40] or Merocyanine 540 [36] as the external membrane probe. Our results on Merocyanine 540 labeling, however, differ from those of Williamson et al. [36], in that these authors did not observe fluorescence up to 3 mM external Ca^{2+} concentration. This may primarily be attributed to the differences in the experimental conditions, especially the Ca^{2+} loading time, used in the two studies. A difference in Ca^{2+} loading time would influence the ATP levels as well as the extent of membrane protein degradation [14], which in turn may affect the Merocyanine 540 staining efficiency. Also, the staining efficiency may have been influenced by the differences in the conditions used for Merocyanine 540 labeling.

Discussion

The present study demonstrates that an increase in the erythrocyte cytoplasmic Ca^{2+} level induces marked alterations in the transbilayer organization of the membrane phospholipids. This change was accompanied by changes in cell shape, membrane protein composition, intracellular ATP levels and transglutaminase activity. The degree to which these changes were induced in erythrocytes depended on the cytoplasmic Ca^{2+} concentration.

Alterations in the membrane phospholipid organization were apparent even upon loading the red cells with up to 25 μM Ca^{2+} (Fig. 5). However, at these Ca^{2+} concentrations spectrin did not appear to cross-link with other membrane proteins (Fig. 2), nor did we observe significant activation of the transglutaminase. Therefore, we suggest that changes in the phospholipid organization are induced, not by the transglutaminase activation, but by some other Ca^{2+}-dependent biochemical event(s). This finds further support from our observation that pancreatic phospholipase A_2 could readily degrade 59.8 ± 1.2% PC, 25.7 ± 1.5% PE and 11.5 ± 1.3% PS in the red cells that were

loaded with 25 μM Ca^{2+} in the presence of cystamine (10 mM), whereas the same enzyme in identical conditions failed to hydrolyse the erythrocytes preincubated with A23187 and cystamine in the absence of Ca^{2+}.

Another Ca^{2+}-induced biochemical event is known to be the activation of the red cell proteinases [15–18]. As compared to transglutaminase, these enzymes become active presumably at lower Ca^{2+} concentrations, and degrade several membrane proteins including spectrin, anion channel protein, ankyrin and polypeptide 4.1 [12,16,18]. Since ankyrin appears to be the most sensitive membrane protein to proteolytic cleavage [12,16,41], it may be inferred that this protein, together with spectrin, probably plays an important role in stabilization of the erythrocyte membrane phospholipid asymmetry. This is consistent with our finding that at 25 μM Ca^{2+} loading concentration the intensity of band 2.1 was slightly decreased, with a concomitant increase in intensity of the 180000 molecular weight band (Fig. 2).

Enhanced cytoplasmic Ca^{2+} levels besides activating the transglutaminase and proteinases, also activate the erythrocyte protein kinase C [42,43]. It has been shown that this kinase is responsible for Ca^{2+}-dependent phosphorylation of the polypeptide 4.1 [19]. Since phosphorylated 4.1 protein is known to have reduced binding with spectrin-actin [19,44], it is expected that Ca^{2+} loading in erythrocytes should result in a decreased binding of cytoskeleton to the membrane bilayer [45,46], which in turn may cause destabilization of the membrane phospholipid asymmetry [47].

In conclusion, this study suggests that ankyrin and 4.1 protein may play an important role in stabilization of the erythrocyte membrane phospholipid asymmetry. As these proteins are primarily responsible for attaching spectrin to the membrane bilayer [45,46,48], our suggestion is consistent with the existing belief that diffusion of PE and PS to the outer monolayer is restricted mainly by their interactions with cytoskeletal proteins, especially spectrin [2–11].

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References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
3 Mombers, C., Verkleij, A.J., De Gier, J. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 271–281
4 Sato, S.B. and Ohnishi, S.I. (1983) Eur. J. Biochem. 130, 19–25
5 Bonnet, D. and Begard, E. (1984) Biochem. Biophys. Res. Commun. 120, 344–350
6 Kumar, A. and Gupta, C.M. (1983) Nature 303, 632–633
7 Gupta, C.M., Alam, A., Mathur, P.N. and Dutta, G.P. (1982) Nature 299, 259–261
8 Kumar, A. and Gupta, C.M. (1984) Biochim. Biophys. Acta 769, 419–428
9 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.-R. and Schlegel, R.A. (1982) Cell 30, 725–733
10 Cohen, A.M., Liu, S.-C., Derick, L.H. and Palek, J. (1986) Blood 68, 920–926
11 Gupta, C.M., Kumar, A. and Joshi, P. (1985) Proc. Int. Symp. Biomolec. Struct. Interactions, Suppl., J. Biosci. 8, 355–362
12 Anderson, D.R., Davis, J.L. and Carraway, K.L. (1977) J. Biol. Chem. 252, 6617–6623
13 Palek, J., Liu, P.A. and Liu, S.C. (1978) Nature 274, 505–507
14 Lorand, L., Bjerrum, O.J., Hawkins, M., Lowe-Krentz, L. and Siefring, G.E., Jr. (1983) J. Biol. Chem. 258, 5300–5305
15 King, L.E., Jr. and Morrison, M. (1977) Biochim. Biophys. Acta 471, 162–168
16 Allen, D.W. and Cadman, S. (1979) Biochim. Biophys. Acta 551, 1–9
17 Siegel, D.L., Goodman, S.R. and Branton, D. (1980) Biochim. Biophys. Acta 598, 517–527
18 Croall, D.E., Morrow, J.S. and De Martino, G.N. (1986) Biochim. Biophys. Acta 882, 287–296
19 Cohen, C.M. and Foley, S.F. (1986) J. Biol. Chem. 261, 7701–7709
20 Litman, D., Hsu, C.J. and Marchesi, V.T. (1980) J. Cell Sci. 42, 1–22
21 Smith, B.D., La Celle, P.L., Siefring, G.E., Lowe-Krentz, L. and Lorand, L. (1981) J. Membrane Biol. 61, 75–80
22 Beutler, E., Duron, O. and Kelly, B.M. (1963) J. Lab. Clin. Med. 61, 882–888
23 Kaul, R., Bajpai, V.K., Shipstone, A.C., Kaul, H.K. and Kirshna Murti, C.R. (1981) Exp. Mol. Pathol. 34, 290–298
24 Lorand, L., Weissman, L.B., Epel, D.L. and Bruner-Lorand, J. (1976) Proc. Natl. Acad. Sci. USA 73, 4479–4481
25 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
26 Gupta, C.M. and Mishra, G.C. (1981) Science 212, 1047–1049

27 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775
28 Franck, P.F.H., Op den Kamp, J.A.F., Roelofsen, B. and Van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 857, 127–130
29 Pollet, S., Ermidou, S., LeSaux, F., Monge, M. and Baumann, B. (1978) J. Lipid Res. 19, 916–921
30 Schlegel, R.A., Phelps, B.M., Waggoner, A., Tereda, L. and Williamson, P. (1980) Cell 20, 321–328
31 Dodge, J.T., Mitchell, C., Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
32 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
33 White, J.G. (1974) Am. J. Pathol. 77, 507–518
34 Roelofsen, B. (1982) J. Toxicol. (Toxin. Rev.) 1, 87–197
35 Op den Kamp, J.A.F., Roelofsen, B. and Van Deenen, L.L.M. (1985) Trends Biochem. Sci. 10, 320–323
36 Williamson, P., Algarin, L., Bateman, J., Choe, H.-R. and Schlegel, R.A. (1985) J. Cell Physiol. 123, 209–214
37 Choe, H.-R., Schlegel, R.A., Rubin, E., Williamson, P. and Westerman, M.P. (1986) Br. J. Haemat, 63, 761–773
38 McEvoy, L., Williamson, P. and Schlegel, R.A. (1986) Proc. Natl. Acad. Sci. USA 83, 3311–3315
39 Klausner, R.D., Fishman, M.C. and Karnovsky, M.J. (1979) Nature 281, 82–83
40 Schwartz, R.S., Chiu, D.T.-Y. and Lubin, B. (1984) in Erythrocyte Membranes, Vol. 3: Recent Clinical and Experimental Advances, pp. 89–122, Alan R. Liss, New York
41 Land, R.D.A., Wickenden, C., Wynne, J. and Lucy, L.A. (1984) Biochem. J. 218, 295–305
42 Ling, E. and Sapirstein, V. (1984) Biochem. Biophys. Res. Commun. 120, 291–298
43 Horne, W.C., Leto, T.L. and Marchesi, V.T. (1985) J. Biol. Chem. 260, 9073–9076
44 Eder, P.S., Soong, C.J. and Tao, M. (1986) Biochemistry 25, 1764–1770
45 Anderson, R.A. and Lovrien, R.E. (1984) Nature 307, 655–658
46 Pasternack, G.R., Anderson, R.A., Leto, T.L. and Marchesi, V.T. (1985) J. Biol. Chem. 260, 3676–3683
47 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
48 Gratzer, W.B. (1981) Biochem. J. 198, 1–8