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Heat-induced alterations in monkey erythrocyte membrane phospholipid organization and skeletal protein structure and interactions *

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Rhesus monkey erythrocytes were subjected to heating at 50 °C for 5–15 min, and the heat-induced effects on the membrane structure were ascertained by analysing the membrane phospholipid organization and membrane skeleton dynamics and interactions in the heated cells. Membrane skeleton dynamics and interactions were determined by measuring the Tris-induced dissociation of the Triton-insoluble membrane skeletons (Triton shells), the spectrin-actin extractability at low ionic strength, spectrin self-association and spectrin binding to normal monkey erythrocyte membrane inside-out vesicles (IOVs). The Tris-induced Triton shell dissociation and spectrin-actin extractability were markedly decreased by the erythrocyte heating. Also, the binding of the heated erythrocyte membrane spectrin-actin with the IOVs was much smaller than that observed with the normal erythrocyte spectrin-actin. Further, the spectrin structure was extensively modified in the heated cells, as compared to the normal erythrocytes. Transbilayer phospholipid organization was ascertained by employing bee venom and pancreatic phospholipases A_2, fluorescamine, and Merocyanine 540 as the external membrane probes. The amounts of aminophospholipids hydrolysed by phospholipases A_2 or labeled by fluorescamine in intact erythrocytes considerably increased after subjecting them to heating at 50 °C for 15 min. Also, the fluorescent dye Merocyanine 540 readily stained the 15-min-heated cells but not the fresh erythrocytes. Unlike these findings, the extent of aminophospholipid hydrolysis in 5-min-heated cells by phospholipases A_2 depended on the incubation time. While no change in the membrane phospholipid organization could be detected in 10 min, prolonged incubations led to the increased aminophospholipid hydrolysis. Similarly, fluorescamine failed to detect any change in the transbilayer phospholipid distribution soon after the 5 min heating, but it labeled greater amounts of aminophospholipids in the 5-min-heated cells, as compared to normal cells, after incubating them for 4 h at 37 °C. These results have been discussed to analyse the role of membrane skeleton in maintaining the erythrocyte membrane phospholipid asymmetry. It has been concluded that both the ATP-dependent aminophospholipid pump and membrane bilayer–skeleton interactions are required to maintain the transbilayer phospholipid asymmetry in native erythrocyte membrane.

Introduction

Mature mammalian erythrocytes contain cytoskeleton (membrane skeleton) which is totally associated with the inner surface of the overlying membrane bilayer. It is composed of three major (spectrin, actin and polypeptide 4.1) and several minor proteins which upon specific associations form a reticulate type of structure [1]. This structure is attached to the overlying membrane bilayer primarily through the protein–protein interactions [1], and controls the membrane structure and function [2].

Erythrocyte membrane phospholipids are asymmetrically distributed in two surfaces of the membrane bilayer. While the choline-containing phospholipids (PC

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Abbreviations: RBC, red blood cells; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; ATP, adenosine 5′-triphosphate; GSH, glutathione; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraaceticacid; SDS, sodium dodecyl sulfate; Mc 540, Merocyanine 540; IOVs, inside-out vesicles; TLC, thin-layer chromatography.

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and SM) are located mainly in the outer monolayer, the aminophospholipids (PE and PS) are present almost exclusively in the inner monolayer [3]. This asymmetric phospholipid distribution in the erythrocytes is believed to be maintained by the membrane bilayer-skeleton associations [4]. Alternatively, it has been thought to originate from the ATP-dependent aminophospholipid pump that translocates PE and PS from the outer to the inner monolayer [5].

In order to further understand the role of membrane skeleton in maintaining the erythrocyte membrane phospholipid asymmetry, we have recently analysed the membrane phospholipid organization as well as the membrane skeleton structure and interactions in the human erythrocytes after subjecting them to heating at 50 ° C for 15 min [6]. Results of these studies revealed that inspite of the marked changes in the structure and interactions of the heated erythrocyte membrane skeleton, the membrane phospholipid asymmetry remained unaltered in these cells, questioning the exclusive role of membrane skeleton in genesis and maintenance of the phospholipid asymmetry. To further examine this problem, we now describe the results of our similar studies carried out on the rhesus monkey red cells.

Materials and Methods

Materials

Triton X-100, ATP, PMSF, pepstatin A, leupeptin, GSH, fluorescamine, and phospholipases A_2 from bee venom and hog pancreas were purchased from Sigma Chemical Company. Mc 540 was procured from Serva Feinbiochemica. Sodium [^{125}I]iodide was bought from Amersham. Precoated silica gel 60F-254 TLC plates (20 × 20 cm, 0.2 mm thickness) were obtained from E. Merck.

Erythrocytes

Blood was collected from normal rhesus monkeys in heparinized glass tubes. After removing plasma and buffy coat, the cells were filtered through a cellulose CF-11 column to completely remove the leucocyte contamination.

Heat treatment

Erythrocytes suspended (10% hematocrit) in 10 mM glycylglycine containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose and 1 mM adenosine (pH 7.4) were subjected to heating at 50 ° ± 0.1 ° C (or stated otherwise) under an atmosphere of humidified nitrogen. The cells were harvested, and washed at least four times to ensure complete removal of microvesicles, formed during heating. The absence of microvesicles was confirmed by both light and electron microscopy.

Erythrocyte ATP and GSH levels

ATP and GSH levels in cells were determined as described earlier [6].

Electron microscopy

Scanning electron microscopy on normal and heat treated erythrocytes was carried out on a Philips SEM 515 electron microscope at a magnification of 1550 × (30 ° tilt angle), essentially following the published procedure [7].

Erythrocyte membrane phospholipid hydrolysis by phospholipase A_2

Hydrolysis of membrane phospholipids in intact monkey erythrocytes and unsealed ghosts by bee venom and hog pancreatic phospholipases A_2 were carried out as described earlier [8]. The concentrations of bee venom and pancreatic phospholipases used in these experiments were 15 IU and 20 IU/0.25 ml packed cells, respectively. The phospholipid degradations were determined by our published method [9].

Erythrocyte labeling with fluorescamine

Aminophospholipid labeling in intact erythrocytes and ghosts with fluorescamine was carried out essentially according to Chandra et al. [8].

Erythrocyte labeling with Mc 540

Mc 540 labeling of erythrocytes was done following the published method [10]. The cells were suspended (about $5 \cdot 10^7$ cells/ml) in 10 mM Tris buffer containing 0.25 M sucrose, 15 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 3% pooled monkey serum. To it was added Mc 540 (40 μg/ml cell suspension) and the mixture incubated in dark for 10 min. The cells after washing quickly with 10 mM Tris containing 140 mM NaCl, 10 mM KCl and 3 mM $MgCl_2$ (pH 7.4) were visualised for fluorescence under Leitz Diavert Poleomak microscope.

Triton shells

Triton insoluble membrane skeletons (Triton shells) were prepared, and their Tris-induced dissociation measured, as described earlier [11]. The erythrocyte membranes were prepared by the published method [12].

Erythrocyte membrane vesicles preparation

IOVs from the erythrocyte membranes were prepared using the standard procedure [13]. Contamination of right-side-out vesicles and unsealed membranes was removed by Con A-Sepharose treatment. The purity of vesicles was judged by measuring acetylcholine esterase activity [14].

Spectrin-actin binding with IOVs

Spectrin-actin extracted [15] from the erythrocyte membranes at 4 ° C was radiolabeled with ^{125}I using the

known method [16]. The binding of the ^{125}I-labeled spectrin-actin with IOVs was carried out as described earlier [13].

Electrophoresis

Protein compositions in erythrocyte membranes, Triton shells and spectrin-actin extract were determined by SDS-polyacrylamide gel electrophoresis followed by densitometry of the Coomassie blue-stained gels. The electrophoreses were carried out essentially according to Hubbard and Lazarides [17] using 5% acrylamide as stacking and 10% acrylamide as running gels. The gels were stained with Coomassie brilliant blue R 250 and scanned on Shimadzu dual wavelength scanner CS-910 at 560 nm.

The spectrin self-association was analysed in the spectrin-actin 4° C extract by electrophoreses on agarose gels under non-denaturing conditions [18]. The protein compositions of the spectrin dimer and tetramer bands were determined by two-dimensional gel electrophoresis [19].

Spectrin tryptophan fluorescence measurements

Steady-state spectrin tryptophan fluorescence was measured in Tris-buffered saline (20 mM Tris containing 145 mM NaCl, pH 7.5) at 22–24° C on a Shimadzu RF-540 spectrofluorometer using excitation wavelength of 295 nm. The spectrin tryptophan fluorescence quenching measurements were made using iodide (KI) as the water soluble quencher. The concentration of spectrin used was $4.3 \cdot 10^{-8}$ M. The fluorescence data was analysed essentially as described earlier [6].

Results

Cell shape, and ATP and GSH levels

The cellular ATP (normal RBC: 0.92 ± 0.20; heated RBC: 0.75 ± 0.21 mmol/l packed cells) and GSH (normal RBC: 135 ± 2.3; heated RBC: 134 ± 5.0 mg/100 ml packed cells) levels were not appreciably affected by subjecting the monkey erythrocytes to heating at 50° C for 15 min. But this treatment led to the transformation of cell shape from discocytes to spherocytes and poikilocytes (Fig. 1).

Membrane phospholipid organization

Erythrocyte membrane phospholipid organization was analysed by using bee venom and pancreatic phospholipases A_2, fluorescamine and Mc 540 as the external membrane probes. Fig. 2 shows that accessibility of the various membrane glycerophospholipids to both bee venom and pancreatic phospholipases A_2 in intact monkey red cells was significantly increased by heating them at 50° C. Besides the increased PE hydrolysis, the procoagulant phospholipid PS was also degraded by the enzymes in 10–15 min heat-treated monkey erythrocytes. This was consistent with our finding that the amounts of PE labeled by fluorescamine in heated erythrocytes were greater than that observed in normal monkey cells (Fig. 3). These results indicate that membrane phospholipid organization in monkey red cells is altered by heating them at 50° C for 10–15 min. This was further confirmed by our finding that Mc 540 readily stained the heated monkey erythrocytes but not the normal cells (data not shown).

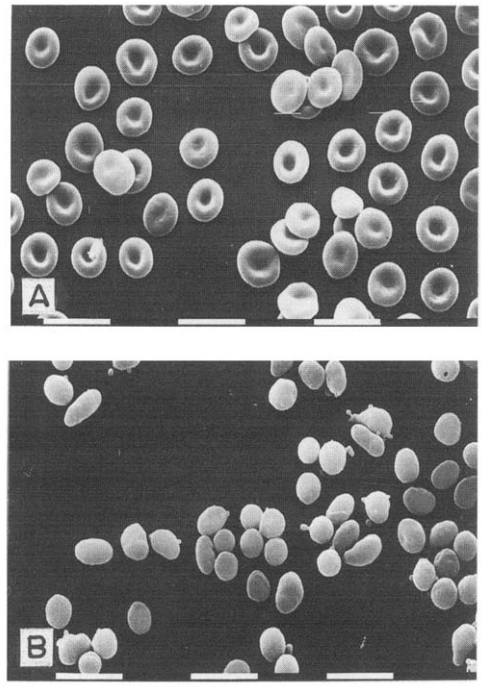

Fig. 1. Scanning electron micrographs of monkey erythrocytes. (A) Normal RBC; (B) RBC heated at 50° C for 15 min. Bar, 10 μm.

Unlike the 10–15 min-heated cells, phospholipases A_2 failed to hydrolyse PS in the 5-min-heated erythrocytes in identical conditions (Fig. 2). Also, the amounts of PE degraded in these cells were similar to those observed in control cells (Fig. 2). However, these enzymes readily cleaved both PE and PS in intact 5-min-heated erythrocytes upon prolonged incubation (Fig. 4). In agreement with these findings, fluorescamine labeled higher amounts of PE (normal RBC: 21.5 ± 0.6%; 5-min-heated RBC: 19.5 ± 0.2%; 5-min-heated and then 4-h-incubated RBC: 31.7 ± 1.5%) in 5-min-heated 4-h-incubated cells as compared to normal or 5-min-heated erythrocytes.

Membrane protein composition

The membrane protein composition was not appreciably altered by heating the monkey red cells at $<$ 46° C (Fig. 5). But at 50° C several new protein bands could be seen in the membrane. Most of these bands corresponded to the proteins present in the monkey erythrocyte cytosol, and may thus be considered of the cytosolic origin. This was further supported by our

finding that intensity of these bands in the cytosol was reduced after heating the cells at 50 °C.

Heat-induced degradation, if any, of the major membrane proteins was assessed by analysing the protein compositions in normal and heat-treated monkey erythrocytes. As may be seen in Fig. 5, the relative intensities of the major erythrocyte proteins were not appreciably altered by subjecting the cells to the heat treatment. Also, the relative amounts of the major membrane proteins in heated erythrocyte ghosts were similar to those observed in the normal erythrocyte membrane. It may therefore be inferred that most of the new major protein bands seen in the heated erythrocyte membrane could result from the heat-induced associations of cytosolic proteins with the membrane. However, the possibility that some of the new minor protein bands could arise from some membrane protein degradation during membrane preparations can not be ruled out completely.

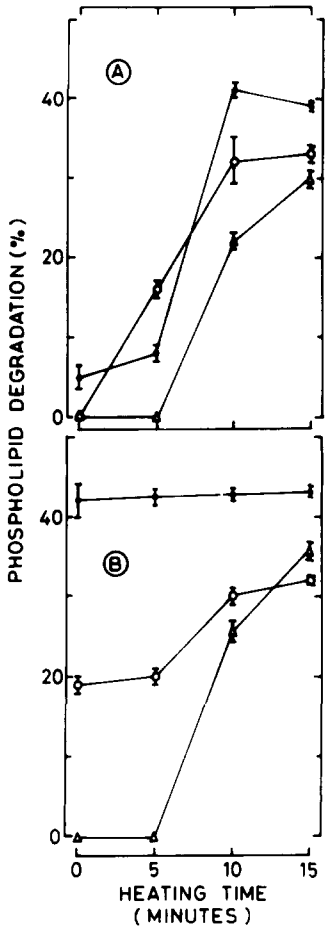

Fig. 2. Effect of preheating of monkey erythrocytes on their membrane phospholipid hydrolysis by phospholipase A_2. Panel A, hog pancreatic phospholipase A_2. Panel B, bee venom phospholipase A_2. Solid circles, PC; open circles, PE; open triangles, PS. The enzyme treatments were carried out for 10 min. Hemolysis was less than 1%. Values shown are means ± S.D. of 4–6 determinations. Under identical conditions, the amounts of various phospholipids hydrolysed by these enzymes in unsealed monkey erythrocyte ghosts were as follows: Bee venom: PC, 84.5 ± 0.4%; PE, 86.0 ± 0.3%; PS, 94.8 ± 0.6%. Pancreatic: PC, 94.6 ± 0.2%; PE, 94.5 ± 1.6%; PS, 94.3 ± 0.7%. The 10-min incubation time was selected as treatment of 15-min heated monkey RBC with 15 IU/0.25 ml cells of bee venom phospholipase A_2 for 1 h led to considerable hemolysis. However, less than 3% 15-min heated cells were lysed in 1 h when the enzyme quantity was reduced to 10 IU/0.25 ml cells. The amounts of phospholipids hydrolysed under these conditions were as follows: Normal RBC: PC, 45.5 ± 0.8%; PE, 21.3 ± 1.2%. 15-min-heated RBC: PC, 41.4 ± 4.6%; PE, 36.1 ± 0.6%; PS, 33.8 ± 2.5%. These amounts did not significantly increase by further increasing the incubation time to 2 h.

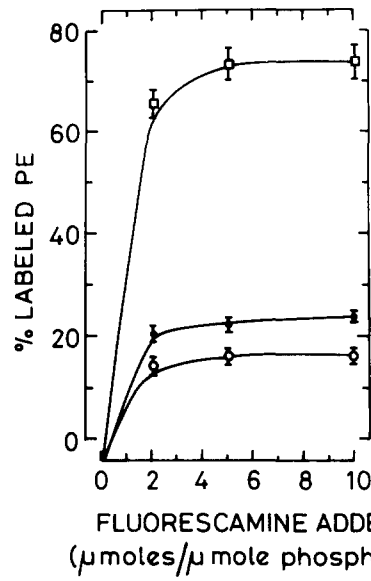

Fig. 3. Labeling of PE by fluorescamine in monkey erythrocytes. The labeling was carried out at 15–20 °C. Open circles, labeled PE in unheated normal RBC; closed circles, labeled PE in 15-min-heated RBC; open squares, labeled PE in unsealed erythrocyte ghosts. Values shown are means ± S.D. of 4–8 determinations. PS was also labeled in the heated cells but not in the normal erythrocytes. However, the amounts of the labeled PS could not be determined accurately, as the spot corresponding to this lipid did not completely separate from phosphatidylinositol on the TLC plates.

Membrane skeleton dissociation / association

Heat-induced changes in erythrocyte membrane skeleton structure, dynamics, and interactions with membrane bilayer determined by measuring the Tris-induced dissociation of the Triton-insoluble membrane skeletons, spectrin-actin extractability, and spectrin-actin binding with normal erythrocyte membrane IOVs. Fig. 6 shows that the Tris-induced dissociation of membrane skeleton was considerably reduced by subjecting the cells to the heat treatment; only about 20% of the total heated erythrocyte membrane skeletal proteins were dissociated by 1 M Tris as compared to over 80% dissociation observed with the normal erythrocyte membrane skeletons under identical conditions. Also, the Tris concentration required for the half-maximal dissociation was increased from about 0.44 M to 0.65 M after the heat treatment. Besides the Tris-induced dissociation, we also analysed the protein compositions in

the Triton shells before and after the Tris treatment. As shown in Fig. 7 most of the new protein bands associated with the heated erythrocyte membrane were found to localize in the Triton-insoluble membrane skeletons, and were not completely dissociated from the skeleton by the Tris treatment (data not shown). However, the quantities of the band 3 protein present in the heated erythrocyte Triton shells are similar to those observed with the normal erythrocytes (Fig. 7). These findings indicate that the heat treatment does not probably affect the skeleton–bilayer interactions, but it seems to increase the protein-protein associations within the skeleton.

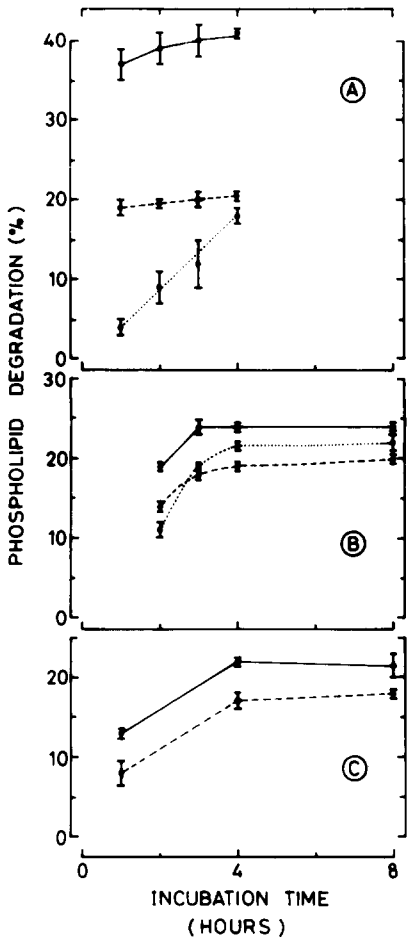

Fig. 4. Phospholipid hydrolysis in heated erythrocytes by phospholipase A_2. Panel A, hydrolysis of 5-min-heated monkey RBC by bee venom phospholipase A_2. Panel B, hydrolysis of 5-min-heated monkey RBC by pancreatic phospholipase A_2. Panel C, hydrolysis of 15-min-heated human RBC by pancreatic phospholipase A_2. Solid line, PC; dashed line, PE; dotted line, PS. Incubations with the enzymes were carried out as given in Materials and Methods. Normal monkey erythrocytes were also subjected to enzyme hydrolysis under identical conditions. No PS in these cells was hydrolysed at least upto 8 h. Also, the amounts of PC and PE hydrolysed by bee venom phospholipase A_2 in 1 h did not significantly increase by further increasing the incubation time upto 4 h. Hemolysis during the enzyme treatments was never more than 5%. Values shown are means of six determinations ± S.D.

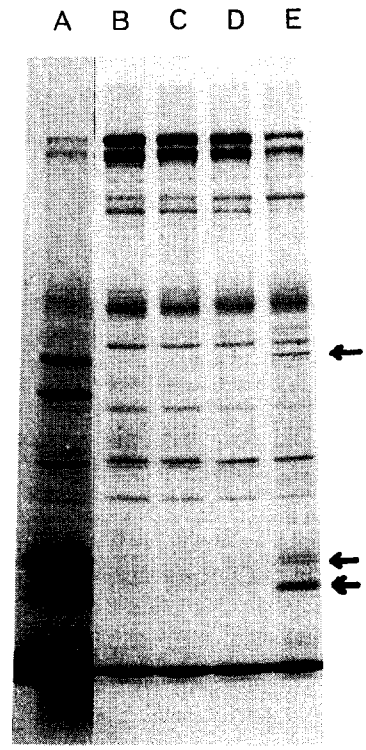

Fig. 5. Protein composition in monkey erythrocytes. A, normal intact RBC; B, normal RBC membrane; C–E, membranes derived from RBC after heating at 46 °C (C), 48 °C (D) and 50 °C (E). Heating was carried out for 15 min. The proteins were quantitated by scanning the gels as given in Materials and Methods. The amounts of protein that could not enter into the gels were negligible in case of both normal and heated erythrocyte ghosts. The weaker intensity of spectrin bands seen in lane E is not due to irreversible protein aggregation or degradation but should arise due to the presence of some cytosolic proteins (indicated by arrows) in the heated erythrocyte membrane, as equal amounts of protein were applied to lanes B–E.

To further confirm that the membrane skeleton structure and dynamics are altered in the heat-treated erythrocytes, we measured spectrin-actin extractability after treating the membranes with low ionic strength buffer. Fig. 8 shows that the extractability decreased by increasing the cell heating temperature; the amounts of extracted proteins from the membranes of 50 °C heated erythrocytes were about one-third of that extracted from the membranes of 37 °C heated cells. Analysis of the protein compositions in the extracts (Fig. 9) revealed that some of the heated erythrocyte membrane-associated cytosolic proteins were extracted with spectrin-actin at low ionic strength.

To ascertain the effect of erythrocyte heating on the membrane bilayer-skeleton associations, we measured the binding of ^{125}I-labeled spectrin-actin, extracted from both normal and heated cells, with normal erythrocyte membrane IOVs. Fig. 10 shows that association of the heated erythrocyte spectrin-actin with the IOVs was significantly reduced (K_a: 0.280) as compared to that

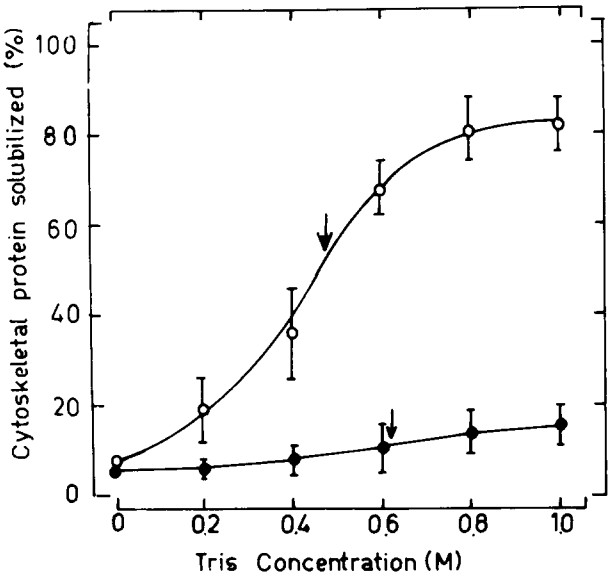

Fig. 6. Tris-induced dissociation of Triton-insoluble membrane skeletons (Triton shells). Open circles, normal monkey RBC; closed circles, monkey RBC heated at 50 °C for 15 min. Values are means ± S.D. of three determinations. Arrows indicate Tris concentrations required to affect the half-maximal dissociation.

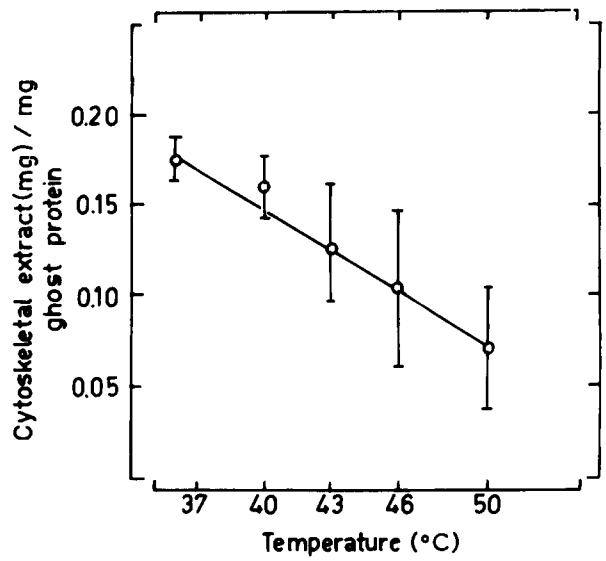

Fig. 8. Effect of monkey erythrocyte heating (15 min) on the membrane skeleton extractability at low ionic strength. Values shown are means ± S.D. of three determinations.

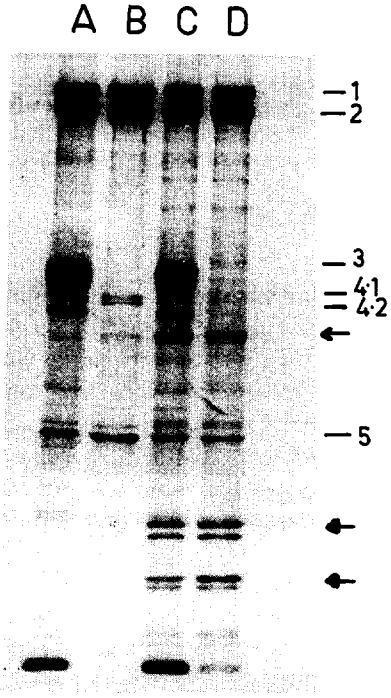

Fig. 7. Protein composition in Triton shells. A, normal monkey RBC membrane; B, Triton shells prepared from the normal RBC membrane; C, membranes derived from monkey RBC after heating at 50 °C for 15 min; D, Triton shells prepared from the heated RBC membrane. Major additional protein bands seen in the heated RBC Triton shells, as compared to normal RBC Triton shells, represent cytosolic proteins which are absorbed to the membrane during heating.

observed with the normal erythrocyte spectrin-actin (K_a: 0.610).

Spectrin structure

Heat-induced changes in the spectrin structure were determined by analysing the spectrin self-association in the crude 4 °C spectrin-actin extracts by native gel electrophoresis. As shown in Fig. 11, spectrin in normal monkey erythrocytes existed mainly as tetramers and oligomers and to a very little extent as dimers, which was similar to that observed earlier with human erythrocytes [6]. However, unlike the human erythrocytes [6], at least two bands in the spectrin dimer region could be seen after subjecting the monkey red cells to heating at 50 °C for 15 min (Fig. 11). These bands besides containing spectrin dimers, perhaps also contained spectrin monomers (α and β subunits) along with some cytosolic protein(s), as revealed by their electrophoretic analysis in the second dimension (Fig. 12b). These results indicate that spectrin in monkey erythrocytes has been extensively modified by the heat treatment.

To further investigate this problem, we attempted to purify spectrin [6] from the spectrin-actin extracts, obtained by treating the membranes of heated monkey erythrocytes with low ionic strength buffer. Inspite of our repeated efforts, we failed to isolate this protein in pure form; most of it eluted from the Sepharose 4B column as undissociated spectrin-actin complex or was contaminated with cytosolic proteins. We therefore could not further analyse the spectrin structure in heated monkey erythrocytes, as we have done earlier in case of the heated human cells [6].

Alternatively, we purified spectrin from normal monkey erythrocytes and then subjected the pure pro-

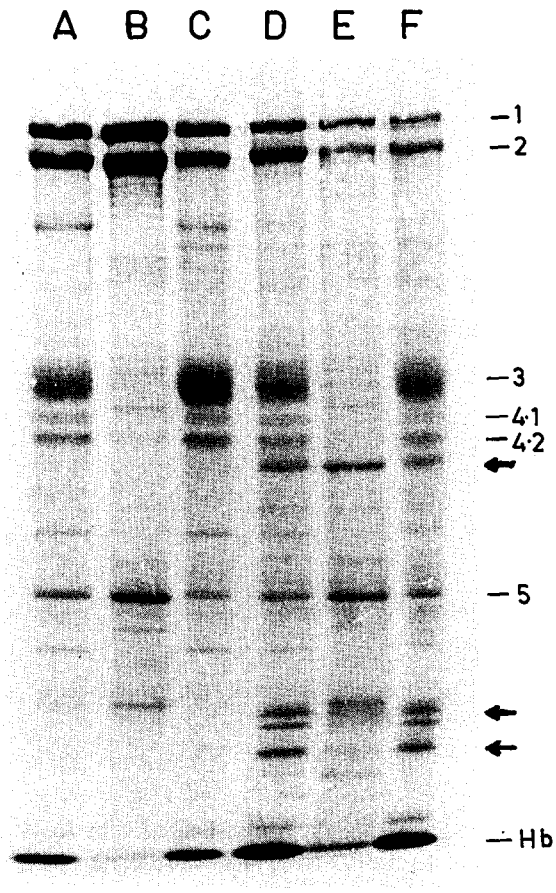

Fig. 9. Protein composition of membrane skeleton extracts obtained from the monkey erythrocytes membrane at low ionic strength. A, normal RBC membrane; B, skeletal extract from normal RBC membrane; C, residual normal RBC membrane pellet; D, membranes derived from RBC after heating at 50 °C for 15 min; E, skeletal extract from heated RBC membrane; F, residual heated RBC membrane pellet. The arrows indicate the cytosolic proteins that get adsorbed to the membranes during heating, and are extracted with spectrin-actin by treating the heated RBC membrane with low ionic strength buffer.

tein to heating at 50 °C for 15 min. Under these conditions, most of the protein (about 85%) formed aggregates which could not be solubilized for native gel electrophoresis.

It would seem that under identical conditions, spectrin in monkey erythrocytes experiences greater heat effects than in human cells. To find out whether this difference is related to a difference, if any, in the gross structural features of these two spectrins, we analysed structures of normal human and rhesus spectrins by measuring the spectrin tryptophan fluorescence in the presence as well as in absence of a water-soluble quencher. The rhesus spectrin, like the human spectrin [6], exhibited an emission maxima at 342 nm, but the fluorescence quantum yield in case of monkey spectrin was higher (rhesus: 0.107; human: 0.086) than that observed for human spectrin. Besides this, there was a marked difference between the accessibilities of the tryptophan residues to the quencher in these two protein samples; only about 70% of the total fluorescence of human spectrin tryptophan residues was accessible to the quencher [6], as apposed to 100% in rhesus spectrin (Fig. 13).

Discussion

This study shows that spectrin in monkey erythrocytes is structurally modified by subjecting the cells to the heat treatment. Also, it demonstrates that membrane skeleton dynamics and bilayer interactions are altered in the heated cells. These results are consistent with the earlier study that has been carried out on the heated human erythrocytes [6].

Unlike the human erythrocytes [6], significantly greater amounts of aminophospholipids became accessible to both bee venom and pancreatic phospholipases A_2 in intact monkey red cells after subjecting them to heating at 50 °C for 10–15 min. This increased accessibility of these phospholipids to the enzymes could be interpreted [20] to suggest that the transbilayer phospholipid distribution is altered in the heated monkey erythrocytes. But it is difficult at the moment to ascertain whether this change represents a change only in the transbilayer phospholipid mobility [21] or also in the transbilayer phospholipid distributions. To further analyse this problem, we studied the erythrocyte membrane phospholipid organization employing Mc 540 and fluorescamine as the external membrane probes.

Mc 540 is a fluorescent dye which has earlier [22–25] been used to analyse the transbilayer phospholipid distribution in modified erythrocytes. This dye in the presence of serum does not stain the normal erythrocytes, but in identical conditions, it brightly stains the cells that have an altered transbilayer phospholipid distribution [22]. As Mc 540 readily labeled the monkey erythrocytes after heating them at 50 °C for 15 min, it may be envisaged that the equilibrium transbilayer phospholipid distribution is perhaps disturbed by the heat treatment. That this could indeed be the case, is further evidenced by our observation that fluorescamine [26] labeled greater amounts of PE in heated erythrocytes as compared to the normal cells.

It is interesting that soon after heating the monkey cells at 50 °C for 5 min, we could not detect any change in the membrane phospholipid organization by exposing these cells to phospholipases A_2 for only 10 min. However, upon prolonged incubation, these enzymes degraded not only the greater amounts of PE but also PS in the same cells, suggesting that the membrane phospholipid organization is altered during the phospholipase A_2 treatment. To analyse whether this change is caused by phospholipase A_2 [21] or by just incubation, we carried out fluorescamine labeling in the monkey erythrocytes soon after the 5 min heating as

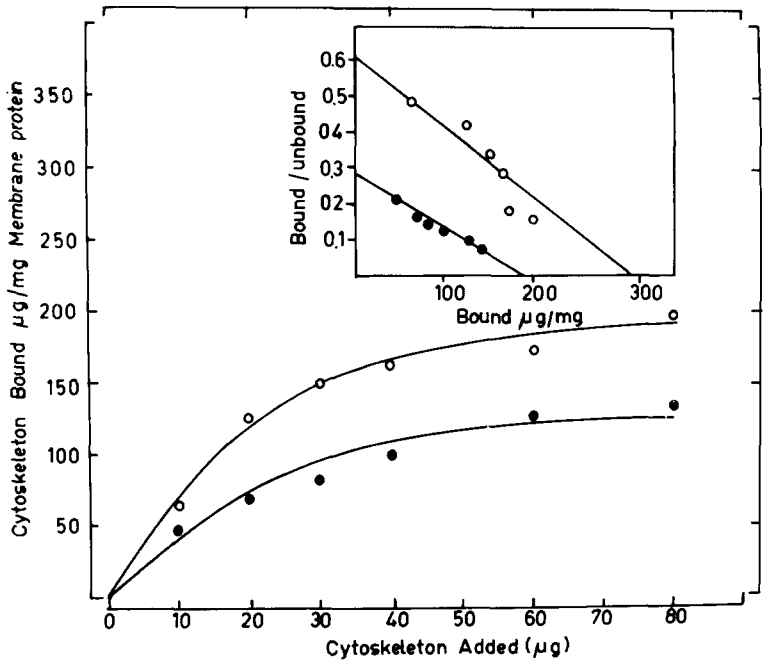

Fig. 10. Spectrin-actin binding with normal monkey erythrocyte membrane inside-out vesicles. Open circles, normal RBC spectrin-actin; closed circles, heated RBC spectrin-actin. Inset: Scatchard analysis of the binding data.

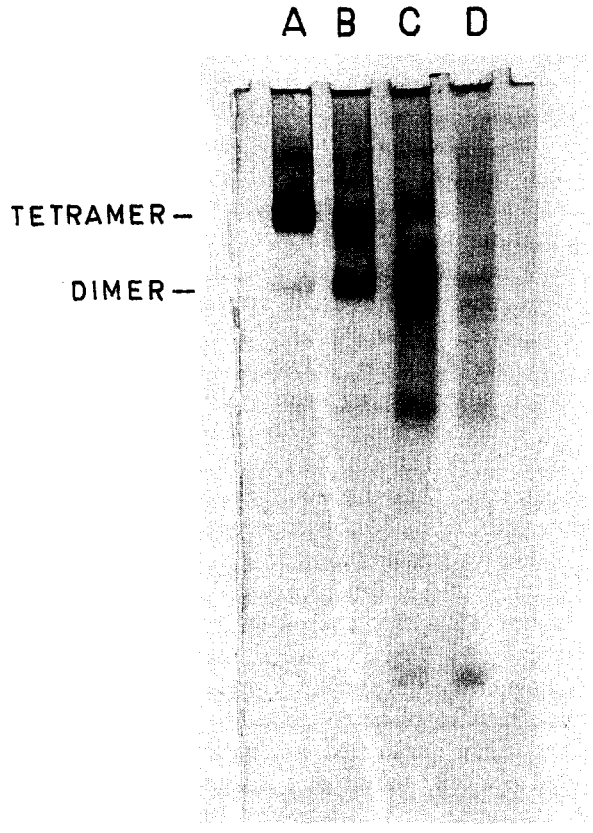

Fig. 11. Analysis of the spectrin self-association in membrane skeleton extracts obtained under low ionic conditions. A, normal monkey RBC membrane 4°C extract; B, normal RBC membrane 4°C extract incubated at 37°C (10 min); C, heated monkey RBC membrane 4°C extract; D, heated RBC membrane 4°C extract incubated at 37°C.

well as after incubating the 5 min-heated cells for 4 h at 37°C. As the amounts of PE labeled in 5 min heated 4 h incubated cells were significantly greater than that observed in the normal or 5-min-heated cells, it may be inferred that the membrane phospholipid organization is altered by incubating the 5-min-heated cells at 37°C.

It would seem that heating of the monkey erythrocytes at 50°C for 15 min leads to marked changes in both the membrane phospholipid organization and the membrane skeleton. This is quite different than that observed in the heated human red cells [6]. In the human cells, the membrane skeleton structure and interactions were altered after heating at 50°C for 15 min, but the transbilayer phospholipid distributions remained virtually unchanged. Considering these two results together, it becomes quite clear that the observed loss of transbilayer phospholipid asymmetry in the heated monkey erythrocytes is not entirely due to the heat-induced alterations in the structure and interactions of the membrane skeleton, and that besides the membrane skeleton, there must be another factor that plays a major role in maintaining the erythrocyte membrane phospholipid asymmetry.

Several studies have shown that the erythrocyte membrane contains an ATP-dependent aminophospholipid pump that translocates PE and PS from the outer to the inner monolayer [5,27–29]. It has been suggested that this phospholipid pump, rather than the membrane bilayer-skeleton association is the major factor that determines the asymmetric transbilayer phospholipid distribution in the native erythrocyte membrane [30]. As

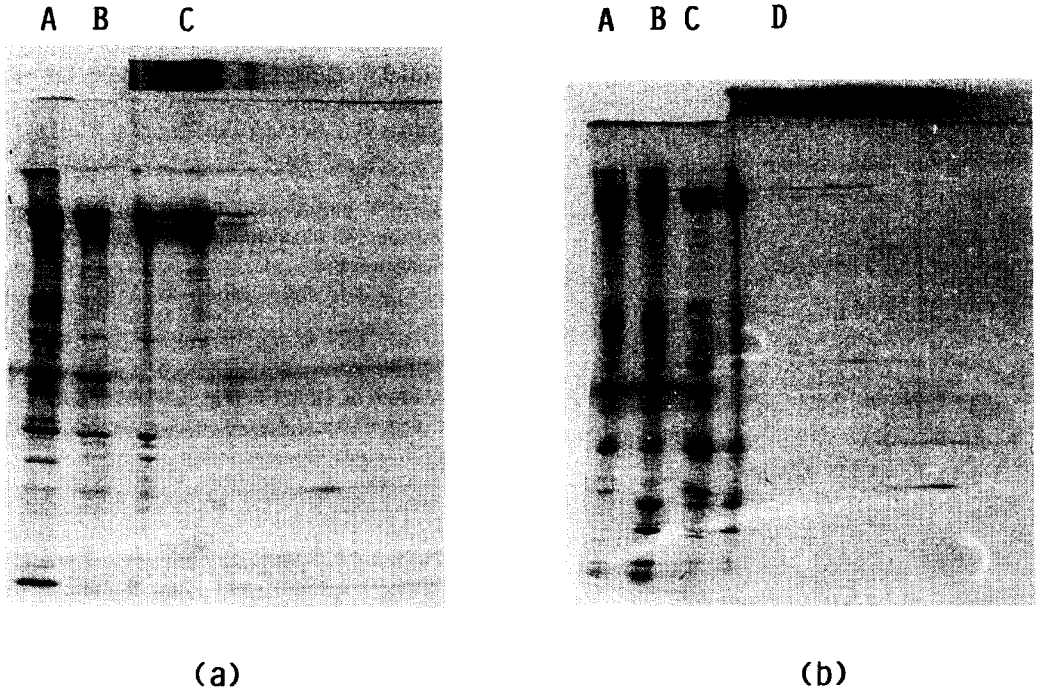

Fig. 12. Analysis of protein composition in spectrin dimer-tetramer bands by two-dimensional gel electrophoresis. The first dimension was run on agarose gels under nondenaturing conditions, whereas the second dimension was on polyacrylamide gels under denaturing conditions. (a) Normal monkey RBC membrane 4°C spectrin-actin extract: A, normal monkey RBC membrane; B, normal monkey RBC membrane spectrin-actin extract; C, strip from nondenaturing gel showing spectrin dimer and tetramer bands. (b) Heated monkey RBC membrane 4°C spectrin-actin extract: A, normal monkey RBC membrane; B, heated monkey RBC membrane; C, heated monkey RBC spectrin-actin extract; D, strip from nondenaturing gel showing spectrin dimer-tetramer pattern.

this pump has been shown to remain largely unaffected by heating the human erythrocytes at 50°C for 15 min [31], preservation of the membrane phospholipid asymmetry in these cells during the heat treatment [6] appeared quite logical. Keeping this in view, we speculate that besides the membrane skeleton, the aminophospholipid pump could have also been damaged by heating the monkey erythrocytes at 50°C. This would conveniently explain not only the observed loss of phospholipid asymmetry in the 15 min-heated monkey cells but also our data on 5 min-heated monkey erythrocytes wherein the transbilayer phospholipid distribution appeared to undergo changes gradually with time.

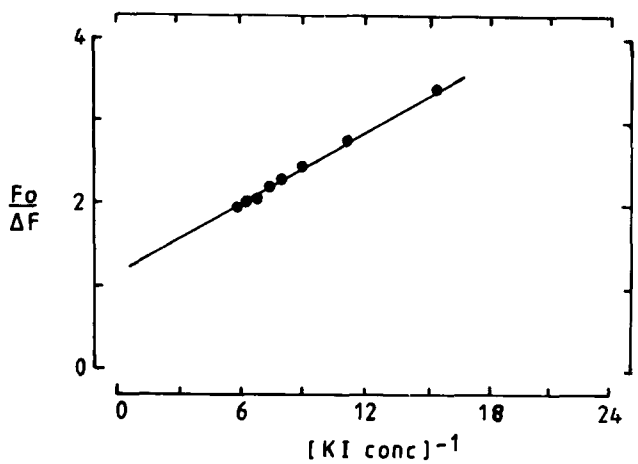

Fig. 13. Modified Stern-Volmer plot for iodide quenching of rhesus spectrin tryptophan fluorescence. F_0 and 'F' denote fluoresence intensities in absence and presence of quencher, respectively. ΔF represents $F_0 - F$. Percentage of total tryptophan fluorescence accessible to the quencher was obtained upon multiplying by hundred the inverse of the intercept of the modified Stern-Volmer plot on the Y-axis [6].

Alternatively, the altered membrane phospholipid organization in 15 min-heated rhesus erythrocytes may be related to the differences between the heat-induced structural changes in rhesus and human spectrins. Since rhesus spectrin experienced greater heat effects than the human spectrin in identical conditions, it may be inferred that the membrane skeleton in heated rhesus erythrocytes was more disorganized than in the heated human cells, which in turn might have resulted in generation of sites at which phospholipid flip to the outer leaflet could be faster than the ATP-dependent aminophospholipid translocation to the inner monolayer.

The ATP-dependent aminophospholipid pump would seem to be the major determinant of the transmem-

brane phospholipid asymmetry in the native erythrocytes, but the membrane skeleton could play an important role in stabilizing the aminophospholipid distribution in the inner monolayer by virtue of its interactions with PS [32–38]. That this may indeed be the case, was further evident from the results of our fluorescamine labeling experiments carried out on the human red cells that were ATP depleted prior to their heating. The cells were depleted (over 90%) of ATP by starving them at 37 °C for 18 h [39]. Half of these cells were subjected to heating at 45 °C for 15 min. These cells along with the ATP depleted (without heating) and fresh erythrocytes were labeled with fluorescamine as given in Materials and Methods. The amounts of labeled PE (Fresh RBC: 21.1 ± 0.8%; ATP depleted RBC: 20.4 ± 0.3%; Heated RBC: 20.7 ± 0.2%; ATP depleted and then heated RBC: 33.3 ± 0.4%) in ATP depleted and then heated erythrocytes were significantly greater than that observed in the simply ATP depleted or fresh cells, suggesting that both ATP-dependent aminophospholipid pump and membrane bilayer-skeleton associations are required for maintaining the phospholipid asymmetry in native erythrocytes. This is quite consistent with the earlier studies [40,41].

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