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Abnormal transbilayer mobility of phosphatidylcholine in hereditary pyropoikilocytosis reflects the increased heat sensitivity of the membrane skeleton

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We determined whether the membrane defect in hereditary pyropoikilocytosis (HPP) is associated with thermally induced changes in the lipid bilayer, the stability of which was probed by the rate of translocation of phosphatidylcholine (PC) over the two leaflets. [^{14}C]PC was incorporated into the outer leaflet of the lipid bilayer of the intact erythrocytes using a PC-specific phospholipid exchange protein. The transbilayer equilibration of this PC was determined by measuring the time-dependent changes in its accessibility to exogenous phospholipase A_2. The rate of transbilayer equilibration of PC was increased in HPP cells at 37°C when compared to normal erythrocytes (rate constants, 0.07 ± 0.02 and 0.03 ± 0.01 h^{-1}, respectively). A further dramatic increase in PC transbilayer equilibration was noted in HPP cells incubated at 44°C (rate constant, 0.15 ± 0.02 h^{-1}). A similar marked acceleration in transbilayer movement of PC was also seen in normal erythrocytes when incubated at 46°C (rate constant, 0.13 ± 0.03 h^{-1}). Despite the enhanced transbilayer mobility of PC in HPP cells when compared to normal erythrocytes, no major alteration in the asymmetric distribution could be observed when probed with phospholipase A_2. Since changes in transbilayer mobility of PC and cell morphology occur in HPP cells at lower temperature than in normal red cells, it may be concluded that the enhanced thermal sensitivity of spectrin is the major factor responsible for these changes. Our results therefore support the view that the structural integrity of the skeletal network is essential for stabilization of the lipid bilayer of the red cell membrane.

Introduction

The red cell membrane is composed of a lipid bilayer and a meshwork of peripheral proteins located at the cytoplasmic interface. It is believed that this lattice of proteins is responsible for the shape and stability of the cell [1,2]. One of the major components of the membrane skeleton is spectrin, a long fibrous dimer, arranged in tetrameric form by head-to-head interactions. The te-

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; HPP, hereditary pyropoikilocytosis.

tramers are combined in a two-dimensional network, by interaction of the free ends with oligomers of actin and polypeptide 4.1 [3]. The membrane skeleton is linked to the membrane bilayer by lipid-protein as well as protein-protein interactions. The best-characterized linkage is that between spectrin-ankyrin and the bilayer spanning protein band 3 [3,4].

Phospholipids are localized in an asymmetric fashion in the two halves of the lipid bilayer of the red cell membrane. Choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin are predominantly found in the outer leaflet, whereas the amino-containing species, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are primary localized in the inner leaflet [5,6]. Evidence is accumulating that the skeleton interacts with the inner lipid leaflet of the membrane, thereby stabilizing this asymmetric distribution of phospholipids and perhaps limiting the translocation of phospholipids across the lipid bilayer [4,7–13].

The transbilayer movement of phospholipids within the human erythrocyte membrane is a slow process with half-time values for the flip-flop rate of 13–27 h [6,12–14]. A causal relationship between the structure of the membrane skeleton and stability of the lipid bilayer was demonstrated in normal red cells which had been treated with a sulfhydryl-oxidizing agent. This treatment resulted in crosslinking of membrane skeletal proteins, an accelerated transbilayer movement of phospholipids and an enhanced availability of PE and PS in the exterior leaflet to chemical and enzymatic probes [9,10,13,15]. It should be realized that this oxidant-induced modification of the membrane represents a harsh treatment to the cell, and it cannot be excluded that modifications other than those in the membrane skeleton were responsible for the observed alterations in the lipid bilayer.

Studies on red blood cells, characterized by abnormalities within the membrane skeleton, support the hypothesis that an intact membrane skeletal is required to stabilize the lipid bilayer. For instance, when reversibly sicklable cells are induced to sickle, increased amounts of the aminophospholipids become accessible for phospholipid localizing reagents [11,16], which is most likely the consequence of changes in the transversal dynamics rather than a static redistribution of these phospholipids across the bilayer [12]. The association between this phenomenon and the process of sickling is demonstrated by restoration of the original slow transbilayer movement of PC [12] following reoxygenation and return to the dscoid shape. Very recently, we have shown that this destabilization of the lipid bilayer in the deoxygenated reversibly sicklable cell is a consequence of uncoupling of the membrane skeleton from the bilayer, which uncoupling predominantly occurs in the long terminal spicules protruding from those cells [38].

Hereditary pyropoikilocytosis is a congenital hemolytic anemia due to an abnormality in the membrane skeleton; the abnormality is characterized by enhanced thermal sensitivity of the cell, resulting in fragmentation and strangely shaped poikilocytes and microspherocytes [17–19]. Normal red cells undergo similar morphological changes when incubated at temperatures of 49°C or greater [20], whereas HPP cells fragment dramatically at 46°C. Chang et al. [21] showed by circular dichroism measurements that the thermal sensitivity in hereditary pyropoikilocytosis is due to an abnormality within spectrin. The spectrin dimers are altered in their ability to associate into tetramers, which could explain the in vivo instability and abnormal morphology [22]. Moreover, recent studies have shown that the spectrin content of HPP cells may be decreased [18,22,23]. In this paper we report the effect of thermal injury on transbilayer movement of PC in normal and HPP cells. Our results support the view that an intact membrane skeletal network is essential for optimal stabilization of the lipid bilayer.

Materials and Methods

Chemical material. Egg phosphatidylcholine (PC) was obtained from Sigma, and cholesterol was from Merck (Darmstadt, F.R.G.). Phosphatidic acid was prepared from egg PC by hydrolysis with phospholipase D. Egg [*methyl*-^{14}C]PC was synthesized according to Stoffel [24], using [^{14}C]methyliodide from Amersham International (Amersham, U.K.). Glycerol tri[^{3}H]oleate was also purchased from Amersham International. Fat-free bovine serum albumin and poly(ethylene glycol)

(Aquacide III) were both from Calbiochem-Behring, and BioSolv was obtained from Beckman Instruments. Bee venom phospholipase A_2 was purified according to Shipolini et al. [25].

Clinical material. Three adolescent non-black patients were available for study. The diagnosis of hereditary pyropoikilocytosis was based on the characteristic red cell morphology, increased osmotic fragility, fragmentation and poikilocytosis induced by incubation at 46°C. These patients, which are related via the asymptomatic fathers, who are brothers, had a history of hemolytic anemia since birth. All three patients were jaundiced immediately after birth. They required several blood transfusions, often following viral infections. The hemoglobin concentration ranged from 5.5 to 9.0 g/100 ml; hematocrit ranged from 19–26%; mean corpuscular volume ranged from 76–80 fl and reticulocyte count ranged from 13 to 15%. These patients (P.Z., W.Z. and E.Z.) underwent splenectomy at ages of 15, 5 and 3 years, respectively. They now enjoy excellent health and their hematologic parameters are as follows: hemoglobin concentration ranges from 12.0 to 12.2 g/100 ml; hematocrit ranges from 29 to 33%; mean corpuscular volume ranges from 64 to 75 fl, and reticulocyte count ranges from 3.3 to 5.6%. At the time of this investigation their ages were 19 (P.Z.), 17 (W.Z.) and 16 (E.Z.) years.

After informed consent was obtained, whole blood from hereditary pyropoikilocytosis and normal donors was collected into sterile tubes containing EDTA. The cells were pelleted by centrifugation for 5 min at $2500 \times g$ and the buffy coat was removed by aspiration. They were washed twice in an isotonic buffer containing 90 mM KCl, 45 mM NaCl, 44 mM sucrose, 10 mM glucose and 10 mM Tris-HCl (pH 7.4), referred to as buffer A.

PC-specific exchange protein. PC-specific exchange protein was purified from bovine liver according to Kamp and Wirtz [26], and prepared for use in our experiments as described before [12]. The final protein concentration was 150 μg/ml.

^{14}C-labeled PC donor vesicles. Unilamellar vesicles were prepared from equimolar amounts of cholesterol and phospholipids (97% egg PC and 3% egg phosphatidic acid). Trace amounts of egg [*methyl*-^{14}C]PC (91 μCi/mmol of vesicle PC) and glycerol tri[^{3}H]oleate (0.91 mCi/mmol vesicle PC) were added. The latter served as a nonexchangeable marker. The final vesicle suspension contained 6 μmol PC/ml. For full details, see Ref. 12.

Introduction of ^{14}C-labeled PC into the outer monolayer of intact erythrocytes. For introduction of ^{14}C-labeled PC into the outer membrane layer, a 33% suspension of washed erythrocytes was incubated for 1 h at 37°C in the presence of the egg [^{14}C]PC/cholesterol vesicles (137 nmol of PC/100 μl of packed cells) and the PC-specific exchange protein (1 nmol/100 μl of packed cells). The exchange reaction was stopped by centrifugation (5 min, $2500 \times g$), followed by two additional washes of the cells with buffer A to remove residual exchange protein and lipid vesicles. After resuspending the cells in buffer A (33% suspension), the samples were saturated with nitrogen to avoid oxidative stress during the subsequent incubations at various temperatures.

Equilibration of ^{14}C-labeled PC across the lipid bilayer at various temperatures and treatment of the intact cells with phospholipase A_2. The above suspensions of HPP and normal red cells, both labeled with [^{14}C]PC, were incubated at temperatures ranging from 37 to 49°C. Samples (930 μl) were removed at timed intervals and incubated at 37°C for 5 min to equilibrate them at this temperature. An equal volume of buffer B (90 mM KCl, 45 mM NaCl, 20 mM $CaCl_2$, 0.50 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4)) containing 80 I.U./ml bee venom phospholipase A_2 was added. Selective hydrolysis of the PC in the outer leaflet of the membrane lipid bilayer was achieved during the incubation of this suspension for 1 h at 37°C. The cells were subsequently collected by centrifugation, and the action of the phospholipase was terminated by resuspending the cells in 0.5 ml 100 mM EDTA in buffer B. The cells were lyzed by adding a cold, CO_2-saturated 10 mM EDTA solution. After 1 h at 4°C, the ghost membranes were spun down at $4500 \times g$ for 30 min and stored at −20°C until extraction and analysis of their lipids.

Lipid analysis. Lipids were extracted by the method of Rose and Oklander [27] and separated by two-dimensional thin-layer chromatography according to Broekhuyse [28]. Phosphate content of the different phospholipid spots, visualized by iodine staining, was determined by the method of Rouser et al. [29]. Cholesterol recovered from the

thin-layer plates was measured according to Zlatkis et al. [30].

Radioactivity in PC and lyso-PC was determined from corresponding spots after a one-dimensional separation performed in chloroform/methanol/25% ammonia/water (90 : 54 : 5.5 : 5.5, v/v). Spots were scraped from the plates and transferred into counting vials. The scintillation medium was toluene containing 2,5-diphenyloxazole (5 g/l), methyl-1,4-bis[2-(5-phenyloxazolyl)]benzene (0.25 g/l) and BioSolv (2%, v/v). Radioactivity was measured by the ^{14}C single-channel-ratio method or the $^{3}H/^{14}C$ dual-label method, using a Packard liquid scintillation spectrometer.

Determination of the PC flip-flop rate. During the incubation of the erythrocytes with the [^{14}C]PC-containing vesicles in the presence of the exchange protein, the radioactive PC is exclusively introduced into the outer monolayer of the membrane. The transbilayer movement of PC will lead to a redistribution of the labeled PC between both halves of the bilayer. Hence, a fall in specific radioactivity of the PC in the outer monolayer, determined as lyso-PC after nonlytic treatment of the cells with phospholipase A_2, will be observed upon subsequent incubation of the labeled cells in the absence of vesicles and exchange protein.

The half-time ($t_{1/2}$) value for equilibration of the labeled PC over both halves of the bilayer can easily be determined from a semi-logarithmic plot of the relative specific radioactivity of the (lyso-)PC in the outer monolayer versus the time of incubation. Full details about this procedure and its theoretical background have been published elsewhere [12,14].

Prior to their use in these calculations, the analytical data were corrected for contamination of the erythrocytes with the [^{14}C]PC/cholesterol vesicles, making use of the nonexchangeable marker glycerol tri[^{3}H]oleate they contained. Contamination never exceeded 2% of the total erythrocyte phospholipid.

Results

Morphology of heated HPP and normal cells

Fig. 1 shows the morphology of HPP and normal erythrocytes incubated for 30 min at different temperatures. HPP cells had abnormal morphology at 37°C when compared to normal discoid cells. The thermal instability of HPP cells was clearly demonstrated when cells were incubated at 46°C. At this temperature discoid cells became poikilocytes and simultaneously underwent vesiculation. Under similar conditions normal cells retained their discoid shape. However, at 49°C normal cells underwent similar morphological changes.

Composition and phospholipase A_2-induced hydrolysis of phospholipids in intact cells

The phospholipid composition of HPP cells was not significantly different from that found in normal red cells (Table I). Furthermore, no change in cholesterol/phospholipid ratio could be detected (0.81 ± 0.04 and 0.82 ± 0.05 for normal and HPP cells, respectively).

The hydrolysis pattern of glycerophospholipids following incubation of intact red cells with phospholipase A_2 at 37°C for 1 h is shown in Table I. The portion of PE which was available in intact HPP cells for hydrolysis by bee venom phospholipase A_2 was identical to that in control cells. The phospholipase A_2-induced hydrolysis of PC was 63 and 69% in HPP and normal red cells, respectively. No detectable amounts of PS were degraded under these conditions.

It should be realized that a complete degradation of the glycerophospholipids situated in the outer monolayer can only be achieved when sphingomyelin, present in that layer, is also degraded by sphingomyelinase C [5]. However, more than 10% lysis of HPP cells was noted when they were subjected to the combined action of phospholipase A_2 and sphingomyelinase C. Therefore, a complete picture of the glycerophospholipids situated in the outer monolayer cannot be obtained by using this combination of enzymes.

Preincubation of HPP cells and normal erythrocytes for 30 min at 46 and 49°C, respectively, did not result in discernable differences in the availability of any of the glycerophospholipids to phospholipase A_2 hydrolysis, and minimal hemolysis was noted.

Transbilayer mobility of phosphatidylcholine in HPP and normal erythrocytes

To study this process, we followed the redistri-

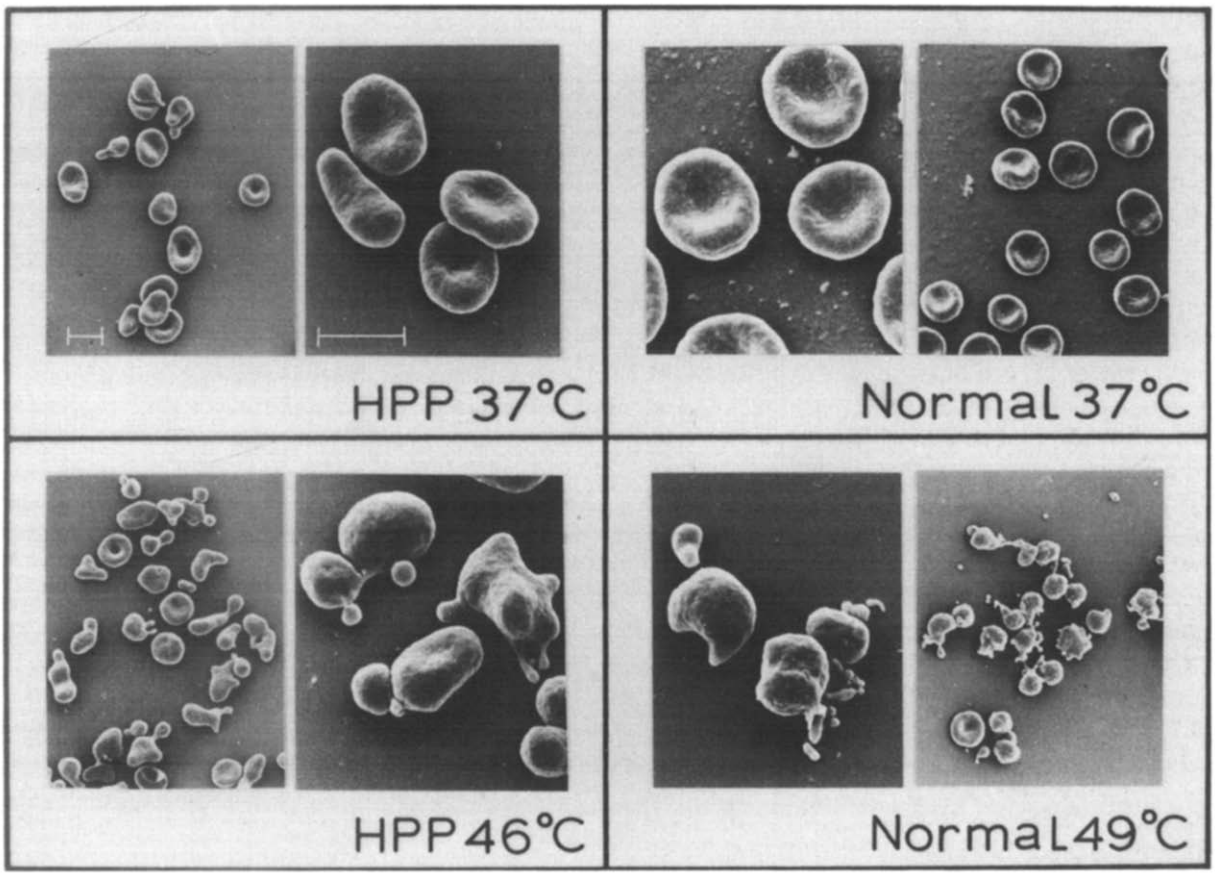

Fig. 1. Scanning electron micrographs of HPP and normal red cells, incubated at different temperatures for 30 min. Erythrocytes, incubated in buffer A, were sampled and fixed in 10 vol. of 0.4% formaldehyde in isotonic buffer A for 30 min at room temperature. The fixed cells were subsequently incubated in 1% osmium tetraoxide dissolved in buffer A for 30 min, dehydrated with graded ethanol and finally suspended in isopropanol. Erythrocytes were air-dried on glass coverslips and covered with a thin layer of coal and gold. Bars represent a length of 5 μm.

TABLE I

COMPOSITION AND PHOSPHOLIPASE A_2-INDUCED DEGRADATION OF THE MAJOR PHOSPHOLIPID CLASSES IN INTACT NORMAL AND HPP ERYTHROCYTES

150 μl packed red cells in 1.0 ml buffer B were incubated with or without bee venom phospholipase A_2, as described in Materials and Methods. Sph, sphingomyelin; n.d., not detectable.

Phospholipid class	Phospholipid composition (% of total ± S.D.)		Hydrolysis of each phospholipid class (% ± S.D.)	
	Normal ($n = 6$)	HPP ($n = 3$)	Normal ($n = 3$)	HPP ($n = 3$)
Sph	26.0 ± 0.4	26.8 ± 0.1	– [a]	– [a]
PC	28.8 ± 0.5	29.2 ± 0.2	68.9 ± 1.1	63.4 ± 1.9
PE	29.0 ± 0.4	28.6 ± 0.4	5.8 ± 0.8	6.0 ± 1.1
PS + PI	16.1 ± 0.3	15.4 ± 0.4	n.d.	n.d.

[a] Sphingomyelin is not a substrate for phospholipase A_2.

bution of [^{14}C]PC, previously introduced into the outer half of the bilayer, as a function of time. This redistribution of radioactive PC was determined by measuring the relative specific radioactivity of this phospholipid in the outer layer, as determined after its modification into lyso-PC by phospholipase A_2 treatment. Fig. 2 represents the results obtained from three independent experiments performed with blood samples from one patient. From the slope of the lines depicted in this figure, the half-time value for the transbilayer movement of PC can be calculated to be 24 and 10 h for normal and HPP cells, respectively. This indicates that the rate of PC flip-flop in HPP cells is 2.4-times faster than that in normal erythrocytes. Similarly enhanced transbilayer movements of PC were observed in the cells obtained from the two other patients (results not shown).

Influence of heat on transbilayer movement of PC

Both HPP and normal cells, their native PC replaced in part by egg [^{14}C]PC, were exposed to temperatures varying from 37 to 46°C. During an 8-h incubation, samples were taken and equilibrated at 37°C for 5 min, after which phospholipase A_2 treatment was started. Unfortunately, incubations for several hours at higher temperatures (up to 49°C) could not be applied in these studies, because not only HPP cells but even normal erythrocytes underwent considerable hemolysis upon subsequent treatment with phospholipase A_2.

Fig. 3 shows the temperature dependency of the rate constants of PC transbilayer equilibration in both normal and HPP cells. At 37°C, normal erythrocytes have a slow rate of PC equilibration (constant, 0.03 ± 0.01 h^{-1}). Incubating these cells at 42 or 44°C slightly enhanced the equilibration rate, corresponding constants being 0.04 ± 0.02

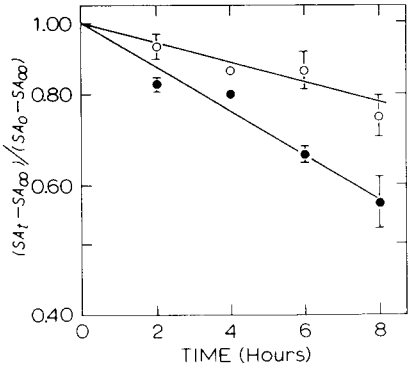

Fig. 2. Transbilayer movement of PC in normal and HPP red cells. Transbilayer movement of PC was determined by following the transbilayer equilibration of [^{14}C]PC, previously introduced into the outer monolayer. The redistribution of this radioactively labeled PC was followed by determining the relative specific radioactivity of PC in the outer layer, modified to lyso-PC by phospholipase A_2, which was calculated as $(SA_t - SA_\infty)/(SA_0 - SA_\infty)$. This relative specific radioactivity of lyso-PC is plotted semilogarithmically for normal (○) and HPP (●) red cells. Data represent the means (±S.D.) of three independent experiments performed with blood samples from three healthy individuals and three blood samples from one patient (P.Z.). Each experiment with HPP cells was run simultaneously with an experiment with normal erythrocytes. SA_0, SA_t and SA_∞, specific radioactivity of the lyso-PC at zero time, time t and infinite time, respectively. For more details of the method and the calculations involved, see Ref. 14.

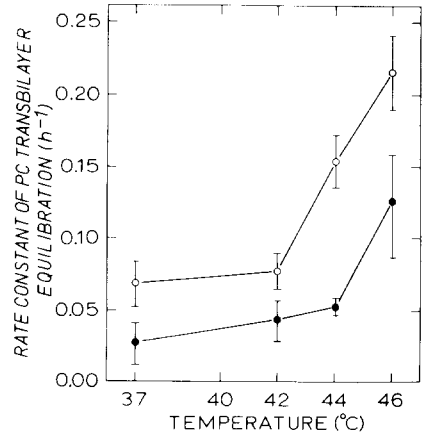

Fig. 3. Rate constants for PC transbilayer equilibration in normal and HPP red cells as a function of temperature. After introduction of [^{14}C]PC into the outer monolayer, intact cells were incubated for up to 8 h at the temperatures indicated. Two independent sets of experiments were performed with blood samples from two healthy individuals and two blood samples from one patient (P.Z.). Each set of experiments with normal (●) cells was run simultaneously with a set of experiments with HPP (○) cells. Samples were taken at zero time and after 2, 4, 6 and 8 h, except for the incubations at 46°C from which samples were taken at zero time, 1.5, 3.0 and 4.5 h. In each sample, specific radioactivity of the PC in the outer monolayer was determined, as indicated in the legend to Fig. 2. From these data, the rate constants of PC transbilayer equilibration were calculated as $\ln[(SA_t - SA_\infty)/(SA_0 - SA_t)]$ divided by the time of incubation (see Ref. 14). Data represent the means ±S.D. For both normal and HPP cells, $n = 8$, 9, 6 and 7, at 37, 42, 44 and 46°C, respectively.

and 0.05 ± 0.01 h^{-1}, respectively. When normal cells were exposed to 46°C for up to 4 h, the rate constant of PC equilibration was 0.13 ± 0.03 h^{-1} which means a 4.3-fold acceleration when compared to the rate at 37°C. During incubations longer than 4 to 5 h at 46°C, an additional acceleration in PC flip-flop was observed (results not shown). However, the time-scale at which the latter process took place is too short for accurate measurements of flip-flop rates. Furthermore, normal cells start to lyze when incubated at 46°C for more than 9 h.

The PC flip-flop rate in HPP cells increased only slowly when the temperature was raised from 37 to 42°C. However, further increase in temperature by only 2 degrees (up to 44°C) caused the equilibration rate in HPP cells to double, corresponding constants being 0.08 ± 0.01 and 0.15 ± 0.02 h^{-1}, respectively. This is in marked contrast to the small increase in the rate of this process in normal cells at these temperatures. At 46°C, the PC translocation rate constant in HPP cells reached a value of 0.22 ± 0.03 h^{-1} during the first 4 h of incubation. As in normal cells, though less drastic, prolonged incubation of the HPP cells at 46°C seemed to result in further enhancement of the PC flip-flop rate. However, prolonged incubation at this temperature was associated with hemolysis which complicated interpretation of these findings.

Discussion

Hereditary pyropoikilocytes are characterized by drastic morphological changes when exposed to 46°C (Fig. 1), a temperature at which normal erythrocytes keep their discoid shape. The transformation of HPP cells into poikilocytes and their vesiculation at this temperature, has been ascribed to an enhanced thermal sensitivity of spectrin in these cells [18,21,23]. Normal erythrocytes undergo similar changes, but only when incubated at a temperature of 49°C or higher, and such changes are also believed to be due to a transition of spectrin [21,31–34].

The composition of the red cell phospholipids (Table I), as well as the cholesterol/phospholipid ratio, in our patients with hereditary pyropoikilocytosis are in agreement with the observation of Zarkowsky et al. [19] and similar to that seen in normal red cells and cells from splenectomized patients [35]. Nevertheless, membrane material, including lipids, may have been lost by vesiculation of the HPP cells in vivo and resulted in the small mean corpuscular volumes observed. A 1-h treatment of intact HPP cells with phospholipase A_2 revealed a degradation of the glycerophospholipids in those cells which was not significantly different from that in normal erythrocytes (Table I). These results were obtained with both HPP and normal erythrocytes at 37°C, as well as after preincubation at 46 and 49°C, respectively. Although the above observations suggest that no major changes occur in the asymmetric distribution of the glycerophospholipids in these cases, it should be recalled that complete information on this distribution can only be achieved when sphingomyelin in the outer monolayer is also degraded by using sphingomyelinase C [5,6]. The combined treatment with phospholipase A_2 and sphingomyelinase C, however, cannot be applied to HPP cells, due to the considerable hemolysis which follows this treatment even at 37°C. Since normal red cells tolerate this combined phospholipase treatment, hemolysis in HPP cells reflects a weakened membrane structure.

A significant temperature-induced alteration in the stability of the lipid bilayer was, however, found in HPP cells, which is particularly expressed by an enhanced mobility of PC between the two leaflets (Figs. 2 and 3).

In normal red cells, the flip-flop of PC is a slow process with a half-time value of 24 h. The results depicted in Fig. 3 demonstrate that the translocation of PC over the bilayer in normal cells was increased as a function of temperature from 37–46°C. Mohandas et al. [13] have similarly demonstrated a temperature dependency for the rate of transbilayer movement of lyso-PC in normal erythrocytes. Although the flip-flop rate of PC increased slowly when temperature was raised from 37 to 44°C, a considerable acceleration was observed when the temperature was further increased. Incubation at 46°C resulted in enhanced flip-flop of PC, with a constant of transbilayer equilibration of 0.13 ± 0.02 h^{-1}, which is 4.3-times faster than at 37°C.

In contrast, the transbilayer mobility of PC was

accelerated in HPP cells. Even at 37°C, the rate for this process was 2,3-times faster than that in normal cells (Figs. 2 and 3). As in normal cells, increasing temperature from 37 up to 42°C hardly induced an increase in the PC translocation rate. Again in contrast to normal erythrocytes, a marked acceleration in the PC flip-flop was observed at 44°C (Fig. 3). Although this temperature is two degrees below the transition temperature of spectrin in these cells [21], it is speculated that this marked acceleration in PC flip-flop reflects early major changes in the structure of this skeletal protein.

However, incubation of normal red cells at 46°C for periods appreciably longer than 4 h, also resulted in a dramatic increase in the transbilayer mobility of PC molecules. Such prolonged incubations were similarly accompanied by changes in morphology (results not shown), and these changes were almost immediately observed at 49°C (cf. Fig. 1). The transition of spectrin obtained from normal red cells has its optimum at 49°C, but this process begins at lower temperature [21,31–33]. Like in HPP cells, denaturation of spectrin might explain the dramatic changes in morphology and considerable acceleration of PC flip-flop (results not shown) observed in normal erythrocytes when incubated at 46°C for periods longer than 4 h.

In addition to this temperature-dependent denaturation of spectrin, the spectrin in HPP cells is likely to have an additional defect, which is responsible for the marked abnormalities in cell morphology and PC flip-flop rate noted at 37°C. Decreased mechanical stability is found in both resealed ghosts [36] and 'Triton shells' derived from HPP cells [22], and in the latter study this instability was associated with abnormal spectrin dimer self-association. A possibly decreased spectrin content which has been reported to exist in HPP erythrocytes [18,22,23] may also contribute to the enhanced transbilayer movement of PC we observed in those cells. Considerably enhanced transbilayer movements of lyso-PC have been reported to occur in two types of mouse red cells that were markedly deficient in spectrin [37]. Elucidation of the composition of the (skeletal) membrane proteins of the HPP cells used in our studies, however, has to await further investigations. Moreover, it cannot be precluded with certainty whether heat induced alterations in other membrane proteins may contribute to the effects described above [33,34].

However, when our results are considered in the light of what is known about the heat sensitivity of spectrin in normal and HPP red cells, it seems reasonable to assume that the observed changes in both cell morphology and PC flip-flop rates find their primary basis in a thermal injury of the skeletal network. Therefore, the studies presented in this paper provide additional support for the view that an intact membrane skeleton is essential for stabilizing the lipid bilayer in the red cell membrane.

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References

1 Mohandas, N., Chasis, J.A. and Shohet, S.B. (1983) Semin. Hematol. 20, 225–242
2 Sheetz, M.P. (1983) Semin. Hematol. 20, 175–188
3 Cohen, C.M. (1983) Semin. Hematol. 20, 141–158
4 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
5 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
6 Op den Kamp, J.A.F (1979) Annu. Rev. Biochem. 48, 47–71
7 Mombers, C., Verkleij, A.J., De Gier, J. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 271–281
8 Sato, S.B. and Ohnishi, S.I. (1983) Eur. J. Biochem. 130, 19–25
9 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. biophys. Acta 509, 21–32

10 Franck, P.F.H., Roelofsen, B. and Op den Kamp, J.A.F. (1982) Biochim. Biophys. Acta 687, 105–108
11 Lubin, B., Chiu, D., Bastacky, J., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649
12 Franck, P.F.H., Chiu, D.T.Y., Op den Kamp, J.A.F., Lubin, B., Van Deenen, L.L.M. and Roelofsen, B. (1983) J. Biol. Chem. 258, 8435–8442
13 Mohandas, N., Wyatt, J., Mel, S.F., Rossi, E. and Shohet, S.B. (1982) J. Biol. Chem. 257, 6537–6543
14 Van Meer, G. and Op den Kamp, J.A.F. (1982) J. Cell Biochem. 19, 193–204
15 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.R. and Schlegel, R.A. (1982) Cell 30, 725–732
16 Chiu, D., Lubin, B. and Shohet, S.B. (1979) Br. J. Haematol. 41, 223–234
17 Lux, S.E. (1979) Semin. Hematol. 16, 21–51
18 Palek, J. and Lux, S.E. (1983) Semin. Hematol. 20, 189–224
19 Zarkowsky, H.S., Mohandas, N., Speaker, C.B. and Shohet, S.B. (1975) Br. J. Haematol. 29, 537–543
20 Coakley, W.T., Bater, A.J. and Deeley, J.O.T. (1978) Biochim. Biophys. Acta 512, 318–330
21 Chang, K., Williamson J.R. and Zarkowsky, H.S. (1979) J. Clin. Invest. 64, 326–328
22 Liu, S.C., Palek, J., Prchal, J. and Castleberry, R.P. (1981) J. Clin. Invest. 68, 597–605
23 Palek, J., Liu, S.C., Liu, P.Y., Prchal, J. and Castleberry, R.P. (1981) Blood 57, 130–139
24 Stoffel, W. (1975) Methods Enzymol. 35, 533–541
25 Shipolini, R.A., Callewaert, G.L., Cottrell, R.C., Doonan, S., Vernon, C.A. and Banks, B.E.C. (1971) Eur. J. Biochem. 20, 459–468
26 Kamp, H.H. and Wirtz, K.W.A. (1974) Methods Enzymol. 32, 140–146
27 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
28 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461
29 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
30 Zlatkis, A., Zak, B. and Boyle, A.F. (1953) J. Lab. Clin. Med. 41, 486–492
31 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 16, 3450–3454
32 Tomaselli, M.B., John, K.M. and Lux, S.E. (1981) Proc. Natl. Acad. Sci. USA 78, 1911–1915
33 Kodicek, M., Marvik, T., Brabec, V. and Vodrazka, Z. (1981) Acta Biol. Med. Germ. 40, 369–371
34 Ballas, S.K. and Miguel, O. (1981) Transfusion 21, 537–541
35 Cooper, R.A. and Jandle, J.H. (1969) J. Clin. Invest. 48, 736–744
36 Knowles, W.J., Morrow, J.S., Speicher, D.W., Zarkowsky, H.S., Mohandas, N., Mentzer, W.C., Shohet, S.B. and Marchesi, V.T. (1983) J. Clin. Invest. 71, 1867–1877
37 Rossi, M.E., Mohandas, N., Bernstein, S.E., Mentzer, W.C., Clark, M.R. and Shohet, S.B. (1982) Blood 60 (Suppl. 1) 26a (Abstr.)
38 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.-Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190