Biochimica et Biophysica Acta 905 (1987) 181–194
Elsevier

BBA 73782

Effects of intracellular Ca^{2+} and proteolytic digestion of the membrane skeleton on the mechanical properties of the red blood cell membrane

M. Shields *, P. La Celle **, R.E. Waugh **, M. Scholz, R. Peters and H. Passow

Max-Planck-Institut für Biophysik, Frankfurt am Main (F.R.G.)

(Received 15 May 1987)

Key words: Erythrocyte membrane; Membrane skeleton; Surface viscosity; Elasticity; Calcium ion, intracellular; Potassium channel; Lateral diffusion; (Human erythrocyte)

Intracellular Ca^{2+} at concentrations ranging from 0 to 10 μmol/l increases the shear modulus of surface elasticity (μ) and the surface viscosity (η) of human red blood cells by 20% and 70%, respectively. K^+ selective channels in the red cell membrane become activated by Ca^{2+}. The activation still occurs to the same extent when the membrane skeleton is degraded by incorporation of trypsin into resealed red cell ghosts, suggesting that the channel activation is not controlled by the proteins of the membrane skeleton and is independent of μ and η. Incorporation of trypsin at concentrations ranging from 0 to 100 ng/ml into red cell ghosts leads to a graded digestion of spectrin, a cleavage of the band 3 protein and a release of the binding proteins ankyrin and band 4.1. These alterations are accompanied by an increase of the lateral mobility of the band 3 protein which, at 40 ng/ml trypsin, reaches a plateau value where the rate of lateral diffusion is enhanced by about two orders of magnitude above the rate measured in controls without trypsin. Proteolytic digestion by 10–20 ng/ml trypsin leads to a degradation of more than 40% of the spectrin and increases the rate of lateral diffusion to about 20–70% of the value observed at the plateau. Nevertheless, μ and η remain virtually unaltered. However, the stability of the membrane is decreased to the point where a slight mechanical extension, or the shear produced by centrifugation results in disintegration and vesiculation, precluding measurements of η and μ in ghosts treated with higher concentrations of trypsin. These findings indicate that alterations of the structural integrity of the membrane skeleton exert drastically different effects on μ and η on the one hand and on the stability of the membrane on the other.

Introduction

Cell membranes exhibit both viscous and elastic properties. The latter are essential for cells which, like red cells, are subject to continuous deformations in the course of their journey through the circulation. It is thought that the proteinaceous meshwork underneath the lipid bilayer, the

* Permanent address: 1/15 George Street, Mortdale, New South Wales, Australia 2223.
** Permanent address: Department of Biophysics and Radiation Biology, School of Medicine and Dentistry, Rochester, NY, U.S.A.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecylsulfate; FITC, fluorescein isothiocyanate; DTAF, dichlorotriazinylaminofluorescein.

Correspondence: H. Passow, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, 6000 Frankfurt am Main 71, F.R.G. (or to the author most convenient).

so-called membrane skeleton, is essentially responsible for the elastic properties of the cell membrane. The composition of the membrane skeleton [1,2] and the disposition of its many constituents (for a recent review, see Ref. 3) have received much attention in the past. Nevertheless, little is known about the details of the correlation between the organization of the membrane skeleton and the mechanical properties of the red cell membrane.

The present paper provides some observations on the effects of experimental modification of the membrane skeleton in red cell ghosts on the viscoelastic properties of the red cell membrane. The membrane skeleton was modified in two different ways: (1) by the incorporation of known concentrations of Ca^{2+} and (2) by a graded tryptic digestion of the major membrane skeletal proteins. The corresponding changes of elasticity were derived from measurements of the deformation of red cell ghosts in a flow chamber as described by Hochmuth (1982) [4] and the viscosity was calculated from the rate at which a deformed red cell returned to the undeformed state under the influence of its own elasticity. The progress of tryptic degradation was followed (1) by SDS-polyacrylamide gel electrophoresis of the membrane skeletal proteins and (2) by employing fluorescence microphotolysis ('photobleaching') to measure the rate of lateral diffusion of the band 3 protein. The band 3 protein is almost immobile in the intact red cell [5] but becomes diffusable when the membrane skeleton is detached from the lipid bilayer [6]. The lateral mobility of band 3 represents, therefore, a convenient and sensitive measure of the modification of the skeletal meshwork.

The results show that considerable modifications of the red cell membrane skeleton produce surprisingly little changes of the viscoelastic properties of the red cell membrane. This finding is discussed in terms of 2 different hypotheses; (1) a small fraction of the membrane skeleton suffices to maintain the mechanical properties of the red cell membrane and (2) even after degradation of the protein 4.1 and actin and of much of the spectrin, the surviving fragments of the peptide chain of the latter convey to the lipid bilayer almost the same mechanical properties as the original, intact membrane skeleton.

The modified red cell membranes show a decreased stability as evidenced by a tendency to disintegrate into small vesicles under the influence of mechanical forces. Thus our observations suggest that stability and viscoelastic properties are independently controlled quantities. While our work was in its finishing stages, there appeared a paper by Chasis and Mohandas (1986) [6a] who reached the same conclusion on the basis of an entirely different experimental approach.

The lipid bilayer contains integral membrane proteins, some of which are involved in the mediation of transport processes. It is an open question, whether or not the activity of such transport proteins is controlled by association with the membrane skeleton. The present experiments shed some light on this question with respect to the Ca^{2+}-activated K^+ channels [7,8]. These channels are responsible for the so-called Gárdos effect, a selective increase of K^+ efflux as a consequence of an increase of intracellular Ca^{2+} [9] or Pb^{2+} [10]. We supplemented, therefore, the study of the mechanical properties of the red cell membrane by (1) looking for parallel effects of Ca^{2+} on the membrane skeleton and the activation of the K^+-selective channels and by (2) inquiring whether or not the tryptic digestion of the membrane skeleton would affect the susceptibility of the K^+-selective channels to activation by Ca^{2+}.

Some of the findings described in this paper have been presented at an International Conference on Environmental Toxicity in Rochester, NY in 1984 [11].

Materials and Methods

(a) Red cells and ghosts

Human blood was supplied by the Frankfurt Blood Transfusion Service and used within 5 days of withdrawal. Prior to use the cells were washed three times in isotonic NaCl. Resealed pink ghosts were prepared essentially according to Schwoch and Passow (1973) [12]. A 50% cell suspension in isotonic NaCl was hemolyzed in a medium containing (mM): $MgSO_4$ (4), KCl (2.5), acetic acid (0.7) (pH 3.9) at 0 °C. The hemolysis ratio was 1 ml red cell suspension in 20 ml hemolysis medium, to give a final dilution ratio cells: medium of

1 : 40. The hemolysates were left for 5 min and then isotonicity was restored by adding sufficient concentrated KCl and Tris to obtain final concentrations of 120 and 20 mM, respectively (pH 7.7). After another 10 min at 0 °C the ghosts were resealed by incubation at 37 °C for 60 min.

Trypsin was incorporated into the ghosts by adding it to the 50% cell suspension prior to hemolysis. This avoids prolonged exposure of the enzyme to the acidic hemolysis medium. The trypsin concentration indicated in the table and figure legends refers to the dilution in the final hemolysate. After resealing the ghosts were washed three times in a medium containing 120 mM NaCl and 20 mM Tris (pH 7.6).

White ghosts were prepared from washed human red cells by hemolysis of one volume of cells in 15 volumes of 5 mM Na_2HPO_4 (pH 8.0), 0 °C. The hemolysate was left for 10 min at 0 °C and then the membranes were washed three times in the phosphate medium until white. The ghosts were incubated for 45 min at 37 °C, 25% hematocrit, in a medium containing (mM): KCl (120), Tris-HCl (20) (pH 7.6) with the trypsin levels indicated in Table II. At the end of the incubation period the ghosts were centrifuged at 30000 × *g*, 0–1 °C, for 35 min to ensure pelleting of all membrane material.

(b) Polyacrylamide gel electrophoresis

For SDS-polyacrylamide gel electrophoresis, the resealed pink ghosts were washed once in a medium containing (mM): NaCl (130) and Tris (20), either with or without saponin (0.1%). After centrifugation, a sample of the supernatant was freeze dried, redissolved in 5% SDS and subjected to polyacrylamide electrophoresis. The membranes were washed three times in ice-cold saponin-free Tris-NaCl medium. They were then boiled for 1 min with 5% SDS, mixed after cooling with Bromophenol blue-glycerin-dithiothreitol medium and run in SDS on a gradient gel (7.5–20%) in a phosphate medium (0.05 M phosphate, pH 7.1).

The pellets of white ghosts were dissolved in 5% SDS. Aliquots were then taken for determination of protein by the method of Lowry et al. (1951) [13] and of phospholipids by the method of Bartlett (1959) [14]. A further aliquot was taken for polyacrylamide gel electrophoresis. After Coomassie blue staining and drying, the gels were scanned using a Sigma FTR 20 densitometer (Sigma, Berlin). The area under each peak was calculated by integration.

The addition of diisopropylfluorophosphate (final concentration 35 nM) at the end of the incubation period with trypsin and prior to washing in the absence of trypsin at 0 °C–1 °C had no significant effect on the band patterns seen on the gels.

Ca^{2+}-stimulated K^+ efflux from resealed ghosts

For studies of the Ca^{2+}-stimulated K^+ efflux from trypsinised ghosts ^{86}Rb was used as a marker for K^+. To incorporate the ^{86}Rb into the ghosts, after the 10 min period at 0 °C before resealing, the ghosts were centrifuged for 5 min at 9000 × *g* and resuspended, at a ratio ghosts: medium of 1 : 5. The medium contained in addition to (mM): KCl (120), Tris (20), $MgSO_4$, $^{86}RbCl$ (Amersham, U.K.), trypsin at the concentration desired. The ghosts were subsequently resealed as described earlier.

To measure ^{86}Rb efflux, the ^{86}Rb containing ghosts were incubated at 6% hematocrit at 37 °C in a medium containing (mM): KCl (120), Tris (20), citrate (10) (pH 7.6) with 0.05 mM $CaCl_2$, corresponding to a concentration of free Ca^{2+} of 1 μM (calculated according to [15] and 0.66 μM A23187). 200 μl samples of the incubation medium were taken at the times indicated in the figures and centrifuged. A 100 μl sample of the supernatant was mixed with 2 ml Supersolve scintillation fluid and counted for ^{86}Rb in a Packard scintillation counter. From the time course of ^{86}Rb release the rate constants were calculated.

Measurements of the Ca^{2+}-induced K^+-efflux from intact red cells were executed by suspending the red cells in media containing (mM): $NaNO_3$ (146), Tris (10), citrate (10) (pH 7.6) and either 2 or 4 μM A23187, and Ca^{2+} at the appropriate concentrations. Cell K^+ contents were determined flame photometrically, after hemolysis of the cells in 1 mM CsCl. From the time course of K^+ efflux, the rate constant was calculated.

Lateral mobility measurements

The lateral mobility of the band 3 protein in

the erythrocyte membrane was measured by fluorescence microphotolysis (for review, see Refs. 16–18). In a first step the band 3 protein was labeled either with fluorescein isothiocyanate (FITC) according to Fowler and Branton [19] or with dichlorotriazinylaminofluorescein (DTAF) according to Sheetz et al. [20]. In both cases intact erythrocytes were used and suspended at 10% hematocrit in a medium containing sucrose (30 mM), Hepes (20 mM), KCl (130 mM), (pH 8.5). To this suspension FITC (Sigma, Deisenhofen, F.R.G.) was added to a final amount of 3.8 mg per g of cells, the incubation time was 24 h at 0 °C. DTAF (Molecular Probes, Eugene, OR, U.S.A.) was employed at an amount of 15 mg per g of cells with an incubation time of 30 min at 0 °C. After labeling the cells were washed two times in medium plus 0.5% (w/v) bovine serum albumin, then three times in medium without bovine serum albumin. In order to study the selectivity of labeling, pink ghosts were prepared and subjected to SDS gel electrophoresis. Gels were scanned for fluorescence, then stained with Coomassie blue and scanned for absorption. In accordance with previous studies [19,20] it was found that 80–90% of total fluorescence was associated with the band 3 protein. Pink ghosts for mobility measurements were prepared from labeled erythrocytes. Preparation of ghosts, incorporation of trypsin, and the quantitation of digestion by SDS gel electrophoresis and gel scanning was performed in the same manner as described above for unlabeled cells.

In a second step the fluorescently labeled ghosts were positioned in the fluorescence microphotolysis apparatus described previously [21]. A flat glass capillary ('microslide', pathlength 50 μm, Camlab, Cambridge, U.K.) was filled with a ghost suspension. The capillary was sealed and fixed to a plate. Before mounting the plate with the attached capillary on the stage of the fluorescence microscope it was kept upside down for about 10 min. During this time some ghosts spontaneously adhered to the glass/water interface and became immobilized. A 100-fold magnifying oil-immersion objective was used to focus the laser beam on the surface of immobilized ghosts. All measurements were performed at room temperature (22 °C).

The third step consisted of the actual measurement of lateral mobility. This was complicated by the fact that erythrocytes are small cells. Certain approximations which are valid for large cells may not apply to erythrocytes. For example the measuring area may not be negligibly small as compared to the total cell surface area. In order to exclude the possibility that the mobility measurements are biased by such technical problems, two variants of the fluorescence microphotolysis methods were employed. The first variant [5,22], referred to in the following as 'single-area variant', employs a stationarily illuminated area for both fluorescence measurement and bleaching. In the present case a circular membrane area of 1.6 μm diameter was illuminated by the 488 nm line of an argon laser at low intensity. A uniform intensity profile was used. Fluorescence of the illuminated area was measured, then irreversibly bleached by increasing the excitation energy 100000-fold for 1/30–1/15 s, and measured again at the initial beam power. The recovery of fluorescence was recorded, the half-time of recovery was determined graphically and the effective lateral diffusion coefficient D calculated according to Eqn. 19 of Axelrod et al. [22].

The second variant, introduced by Koppel [23,24] and referred to below as 'scanning variant', also bleaches fluorescence in a single membrane area but measures membrane fluorescence in multiple membrane areas. In our version of Koppel's method the laser beam was focussed to a diameter of approx. 0.5 μm. With a fast scanning stage (Zeiss, Oberkochen, F.R.G.) a ghost was moved through the beam on a linear pathway in steps of 0.25 μm. The pathway had a length of 15 μm and was recorded in 8 s. After an initial scan at low beam power the ghost was relocated and its central part bleached at high beam power for a fraction of a second. Scanning was then resumed at the initial beam power. Evaluation followed the procedure outlined by Koppel [24]. The second momentum of the concentration distribution was calculated. The relaxation time of the second momentum was used to calculate the effective diffusion coefficient whereas the mobile fraction was estimated from the time-independent residual of the second momentum. It may be noticed that Koppel's method is particularly suited for small cells be-

cause data evaluation is independent of the concentration distribution induced by photobleaching.

Measurement of membrane viscosity and elasticity of intact cells

Red cells were washed three times in a medium containing (mM): KCl (100), NaCl (46), Na citrate (10), Tris (10) (pH 7.6) with $CaCl_2$ added to give the concentrations of free Ca^{2+} indicated in the text and figures. Cells were suspended at 25% hematocrit, A23187 was then added in ethanolic solution. The final concentration of the ionophore was 1 μM. Cells suspended in the absence of Ca^{2+} received an equal volume of ethanol as the cells suspended in the presence of Ca^{2+}. After 10 min incubation at 37 °C, samples of the cells were taken for measurement of membrane elasticity (μ) and viscosity (η). μ was calculated from measurements of the deformation of the red cells in a parallel plate flow chamber as described by Hochmuth et al. (1973) [25]. η was determined by measuring, after elastic deformation of the cell, the rate of return of the cell to its undeformed shape and multiplying the relaxation time (t_c) with μ (see also Ref. 4).

Viscosity and elasticity measurements of trypsinized ghosts were made as described for the intact cells.

Results

(1) Effects of intracellular Ca^{2+} on membrane viscosity and elasticity and on the activity of the K^+-selective channels

Fig. 1 shows that both elasticity and viscosity of the red cell membrane increase upon increasing the Ca^{2+} concentration at the inner membrane surface. The effect on elasticity is just significant and remains small (some 20–30%) up to an internal concentration of Ca^{2+} of 10 μM. The increase of viscosity is larger (about 70%) and does not seem to reach a plateau at 10 μM. It is known that over this concentration range, virtually no change of the membrane skeletal proteins can be demonstrated by SDS gel electrophoresis [26]. Thus, the changes of elasticity described seem to indicate a slight increase of the rigidity of the membrane skeletal protiens in response to Ca^{2+}. Moreover,

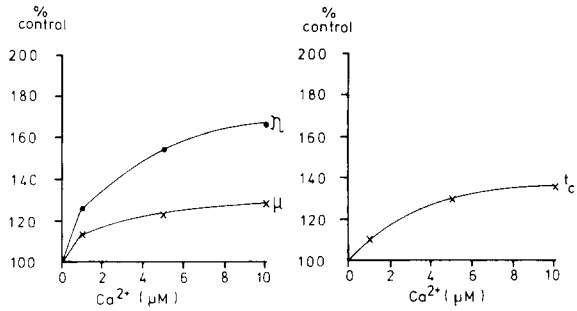

Fig. 1. Effect of internal Ca^{2+} on shear modulus of surface elasticity (μ) and surface viscosity (η) of human red cells (left panel). Red cells were prepared and measurements of μ and t_c made as described in the method section. η is the product of the multiplication of μ by t_c, the recovery time constant (right panel). The values of μ, η and t_c are expressed as a percent relative to control values measured in cells incubated in the absence of Ca^{2+}. Each value is the mean of 2–6 experiments. The mean values of 11 controls ($\pm$S.D.) were $\mu = (5.64 \pm 0.96) \cdot 10^{-3}$ dyn·cm^{-1}, $t_c = 0.110 \pm 0.01$ s, $\eta = (0.66 \pm 0.18) \cdot 10^{-3}$ dyn·s·cm^{-1}.

the increase in surface viscosity most likely reflects a change of a proteinaceous matrix underneath the lipid bilayer. This may be inferred from a comparison of the surface viscosity of the red cell's lipid bilayer as derived from measurements of lateral diffusion of membrane lipids with the surface viscosity obtained in the present work. The rate of lateral diffusion depends on the viscosity of the environment in which the diffusion takes place. Hence, it is possible to infer from the diffusion coefficient D the surface viscosity η. Actual calculations can be performed using the equation of Saffman (1976) [27]:

$$D = \frac{kT}{4\pi\eta}\left[\ln\frac{\eta}{\bar{\eta}\cdot a} + \gamma\right]$$

where k = Boltzmann's constant, T = absolute temperature, η = viscosity of adjacent aqueous medium (0.01 poise), a = radius of diffusing particle, $\gamma = 0.5772$ (Euler's constant).

A graphical representation of the relationship between D and η is found in an article by Hochmuth (1980) (Ref. 28, Fig. 5).

According to Bloom and Webb (1983) [29], the coefficient for lateral diffusion of lipid molecules in the intact red cell membrane and in red cell ghosts amounts to $8.2 \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, and $7.8 \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1}, respectively (25 °C). Insertion into

the Saffman equation yields a surface viscosity of about $5 \cdot 10^{-6}$ dyn · s · cm^{-1}. If one assumes that lipid mobility is independent of the state of the membrane skeleton, this value would represent the surface viscosity of the lipid bilayer of the red cell. It should be compared with the surface viscosity measured by the method employed in this paper, which leads to values in the range $(4–9) \cdot 10^{-4}$ dyn · s · cm^{-1} [28,30]. Thus, the contribution of the lipid bilayer to the overall surface viscosity is negligible. The increment of surface viscosity observed after incorporation of Ca^{2+} into the red cells is most likely to reflect, therefore, an increase of the contribution to the surface viscosity of membrane protein rather than membrane lipid.

Over the Ca^{2+} concentration range covered in the experiments presented in Fig. 1, K^+-selective channels are opened (Table I). The effect depends, however, not only on the Ca^{2+} concentration but also on the concentration of the ionophore A23187 used to equilibrate Ca^{2+} between the medium and the cell interior.

(2) Effects of intracellular trypsin on viscoelastic properties of the red cell membrane and on the red cell's membrane skeleton

Trypsin can be sealed into red cell ghosts (see Materials and Methods). In the course of the enzymatic digestion of the membrane skeleton, the ghosts tend to assume a spherical shape. They become fragile and, under the influence of shearing forces, they disintegrate into vesicles. In spite of these changes, anion transport remains nearly unaltered. It can still be inhibited by the same specific inhibitors that act in untreated red cells and ghosts, and the typical differences of the sidedness of action seen with different non-penetrating inhibitors can still be observed. This indicates that the enzyme does not produce a generalized leak. Nevertheless, at sufficiently high trypsin concentrations, the membrane becomes permeable to K^+ and Na^+ [31]. This has been attributed to the modification of Ca^{2+}-activatable K^+ channels. It is believed that by proteolysis, the selectivity filter of the channels is removed. The channels no longer discriminate between Na^+ and K^+ and are now open without activation by Ca^{2+} [32].

When red cell ghosts treated with internal trypsin are handled without much mechanical agitation, they survive without vesiculation. In these ghosts, membrane viscosity and elasticity can be measured as described in Materials and Methods. The results are summarized in Table II. In spite of easily demonstrable changes of the major membrane proteins seen on gel electrophoretograms (see Table II, columns 2 and 3) neither elasticity nor viscosity change much. If anything, there is an increase of the two parameters, as compared to the control. The increase is maximal at 5 ng/ml trypsin, the lowest concentration used in our work.

At the highest trypsin concentrations used to measure μ and η (20 ng/ml), the ghosts become quite fragile and at trypsin concentration exceeding this limit, the fragility becomes too large to permit measurements of elasticity. The increased susceptibility to fragmentation under the influence of mechanical forces had been previously demonstrated by Lepke and Passow [31] who observed the onset of vesiculation under the influence of centrifugical forces after treatment with incorporated trypsin at concentration in the range between 10 and 500 ng/ml (Table III). These observations were fully confirmed in the course of the present work, using the capacity of tether formation with micropipettes as a measure of

TABLE I

RATE CONSTANT FOR K^+ EFFLUX FROM INTACT RED CELLS INCUBATED WITH Ca^{2+} AND A23187

Intact cells were washed in a medium containing (mM): $NaNO_3$ (146), Tris nitrate (10), sodium citrate (10) (pH 7.6) and then suspended at 25% hematocrit in the same medium at 37 °C, to which was added sufficient $CaCl_2$ to give the free Ca^{2+} indicated (calculated according to Ref. 15). A23187, 2 or 4 μM, was added and the K^+ content of the cells measured after timed periods of incubation at 37 °C. From the maximal slopes of the curves relating K^+ content with time K^+ efflux was calculated.

$CaCl_2$ (μM)	K^+-efflux (mmol/l cells per min) A23187	
	2 μM	4 μM
0	0.13	0.15
1.25	0.50	2.4
2.5	1.80	4.0
5.0	2.0	4.6
10.0	3.6	6.0
20.0	3.3	6.0

TABLE II

EFFECT OF INTERNAL TRYPSIN ON PROTEIN CONTENT, MECHANICAL PROPERTIES OF THE RED CELL GHOST MEMBRANE AND Ca^{2+}-STIMULATED K^+ EFFLUX

Pink ghosts were resealed with the trypsin concentrations indicated for the study of the mechanical properties (columns 7, 8 and 9) and the Ca^{2+}-stimulated K^+-efflux (column 4). For the study of spectrin and band 3 content (columns 2 and 3), white ghosts were used. For experimental details see Materials and Methods. Measurements of elastic shear modulus (μ) and relaxation time (t_c) were made at room temperature (about 24° C). The data are the means from 4–9 experiments except at 20 ng/ml trypsin which is from one experiment. The Ca^{2+}-stimulated K^+-efflux was elicited by the addition of 1 μM Ca^{2+} and 0.66 μM A23187 and the rate constant of K^+ efflux was calculated from the time course of ^{86}Rb release at 37° C. The results presented are from one experiment. The band 3 and spectrin were estimated from the density of Coomassie blue staining of polyacrylamide gels of the isolated membranes. Data are taken from graphs (see Fig. 4) relating spectrin or band 3 content, to the trypsin concentration (average of three experiments). The lateral diffusion coefficient D and the mobile fraction R were determined by fluorescence microphotolysis as described in Materials and Methods.

Trypsin ($ng \cdot ml^{-1}$)	Band 3 (%)	Spectrin (%)	Rate constant of K^+ efflux (min^{-1})	D (10^{-10} $cm^2 \cdot s^{-1}$)	R (%)	μ (10^{-3} dyn $\cdot cm^{-1}$)	t_c (s)	η (10^{-3} dyn $\cdot s \cdot cm^{-1}$)
0	100	100	0.053	< 0.1		2.12 ± 0.006	0.085 ± 0.002	0.18
5	84	76	0.058			2.50 ± 0.13	0.100 ± 0.003	0.25
10	70	60	0.053	1.0 ± 0.4		2.52 ± 0.12	0.102 ± 0.007	0.26
20	56	45	0.053	4.2 ± 1.4	80–100	2.40	0.110	0.26
40	42	28	0.041	4.8 ± 1.7	80–100			
50	38	23	–	–				
100	24	7	–	5.7 ± 2.1	≈ 100			

mechanical stability. The ghosts could no longer be deformed without beaking after a treatment with more than 10–20 ng/ml trypsin. Thus the experiments described demonstrate that the viscoelastic properties of the trypsinized ghosts remain virtually unaltered up to the point were the stability of the membrane becomes too small to allow further measurements. This indicates that the viscoelastic properties and the stability of the membrane are essentially independently controlled properties.

It may be noted that the elasticity and surface viscosity of the untreated ghosts used in these experiments (Table II, 1st row) are lower than observed in the red cells (legend to Fig. 1) from which they were derived. This is different from the observations of Nash and Meiselman (1983) [30], who found the surface viscosity of the ghost membrane to be indistinguishable from that of the intact red cells. It should be noted that the latter authors took special precautions to prevent a loss of spectrin from the ghost membrane, while our ghost preparation was incubated during resealing for one hour at 37° C prior to the measurements of elasticity, viscosity and diffusion coefficient. This incubation period was necessary to provide sufficient time for the digestive action of trypsin and also preceded the measurements in the control ghosts. During this time some release or reorganization of membrane skeletal constituents may have taken place, which did not occur in the work of Nash and Meiselman [30].

A possible objection against the results described could be visualized. The tryptic digestion of different ghosts of the same population may differ to some extent. More heavily affected ghosts can be expected to be more fragile than the less heavily affected ones. This could lead to a bias of the micro-pipette experiments in favor of the less fragile ghosts with less roughly digested membrane skeletons. Although we have no evidence for individual variations of the degree of tryptic degradation of the membrane skeleton, the possibility of a bias should be kept in mind. Nevertheless, it should be recalled that after exhaustive digestion at high concentrations of trypsin (≥ 1 μg/ml), on SDS-polyacrylamide gel electrophoretograms, no undegraded peptides can be seen. This indicates that there exists no subpopulation in the ghost suspension in which the mem-

TABLE III

INCREASED FRAGILITY OF RED BLOOD CELL GHOSTS AFTER TRYPSINATION

Resealed ghosts containing trypsin at the concentrations indicated were incubated for 45 min at 37 °C. They were subsequently subjected to mechanical stresses by three centrifugations (10 min each) in a Sorvall SS 34 rotor, at 20000 rpm. Microscopical examination shows the appearance of vesicles at trypsin concentrations > 10 μg/ml. The table represents a compilation of data taken from the work of Lepke and Passow [31], where the experimental conditions are described in detail.

Trypsin ($ng \cdot ml^{-1}$)	Total membrane protein (%) [a]	Vesicle volume (%) [b]	SO_4^{2-} transport (%) [c]
0	100	100	100
10	78	100	96
50	50	44	103
100	44	32	110
10000	39	38	not measured
50000	40	35	not measured
100000	40	37	not measured

[a] Determined by the method of Lowry et al. [13] in the vesicle suspensions derived from equal numbers of ghosts, after lysis by 0.1% saponin and subsequent washes to remove liberated protein.

[b] The aqueous volume entrapped by the vesicles as a percent of the volume entrapped by the ghosts from which the vesicles were derived. The volume decreases since the same amount of membrane material that forms the intact ghost has to suffice for the formation of the smaller vesicles that originate after tryptic digestion of the membrane skeleton.

[c] Rate of equilibrium exchange measured by means of $^{35}SO_4$.

brane skeleton is inaccessible to trypsin.

The alterations in the membrane that take place over the trypsin concentration range used (up to 40 ng/ml) are not accompanied by an increase of K^+ efflux (Table II), while addition of Ca^{2+} + A23187 elicits the Gárdos effect. This indicates that the selectivity filter of the Ca^{2+}-activatable K^+ channels remained unaffected.

(3) Effects of intracellular trypsin on lateral diffusion of band 3 in the lipid bilayer of the red cell ghost

A fairly selective fluorescence labeling of the band 3 protein can be achieved by exposure of the intact red cell to either FITC or DTAF (see Materials and Methods). In the ghosts made from labeled erythrocytes the lateral mobility of band 3 was measured by fluorescence microphotolysis.

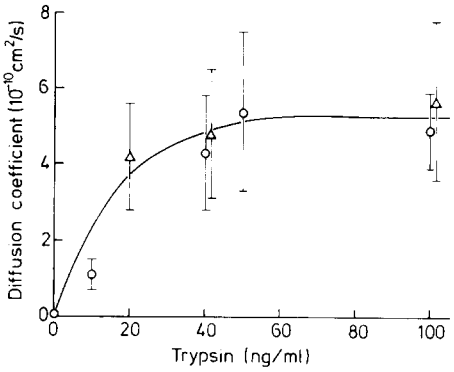

Fig. 2. Effective lateral diffusion coefficient D of the band 3 protein in the membrane of human erythrocyte ghosts as function of internal trypsin concentration. Resealed, trypsin-containing ghosts were prepared from labeled erythrocytes. Diffusion coefficients were measured at 22 °C either by the single-area variant (circles) or the scanning variant (triangles) of fluorescence microphotolysis (see Materials and Methods). Each entry represents mean ± S.D. of 5–20 measurements on different ghosts. The mobile fraction was 80–100% at all trypsin concentrations larger than zero. In the absence of trypsin, mobility was very small and mobile and immobile fractions were not resolved. The curve was drawn by eye. A sigmoidal shape cannot be excluded.

Band 3 mobility was found to be extremely small with an effective diffusion coefficient below $0.1 \cdot 10^{-10} cm^2 \cdot s^{-1}$ at 22 °C (Table II and Fig. 2). This agrees with previous results obtained by us and others. Earlier measurements by fluorescence microphotolysis [5] which did not discriminate between a mobile and an immobile fraction yielded an average diffusion coefficient of $0.03 \cdot 10^{-10} cm^2 \cdot s^{-1}$ at 20 °C. A fusion method [19] which does not involve photobleaching, allowed minimum estimates of the diffusion coefficient in fresh and aged red cells of $0.06 \cdot 10^{-10} cm^2 \cdot s^{-1}$ and $0.02 \cdot 10^{-10} cm^2 \cdot s^{-1}$, respectively. Golan and Veatch [6] employed the single area variant of fluorescence microphotolysis (see Materials and Methods) and observed that about 70% of band 3 was mobile; this mobile fraction had a diffusion coefficient of $0.5 \cdot 10^{-10} cm^2$ at 21 °C. Schindler et al. [33], used cell fusion in combination with microfluorometry, and reported a mobile fraction of 60%; the diffusion coefficient as measured at 30 °C was $0.9 \cdot 10^{-10} cm^2 \cdot s^{-1}$ in fresh ghosts and $0.2 \cdot 10^{-10} cm^2 \cdot s^{-1}$ in ATP-depleted ghosts.

When trypsin is sealed into fluorescently labeled ghosts the lateral mobility of band 3 increases

dramatically. Measurements by the scanning variant of fluroescence microphotolysis show that 80–100% of the band 3 molecules become mobile whenever trypsin is present (Table II). The effective lateral diffusion coefficient of the mobile fraction, measured by both the single-area and the scanning variant of fluorescence microphotolysis (Fig. 2 and Table II), increases with increasing trypsin concentration until at about 40 ng/ml a plateau at about $5 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1} is reached. At this concentration about 70% of the spectrin is degraded (see below and Figs. 3 and 4). The hydrophilic inward-directed domain of the band 3 protein is also susceptible to proteolytic digestion. However, the transmembrane domain of 55 kDa stays intact and the fluorescent label remains attached to this protein. Thus the increase of lateral mobility is the result of either one of two factors, or a combination thereof: the destruction of the spectrin meshwork underneath the lipid bilayer, and/or the digestion of the cytoplasmic domain of the band 3 protein. When the trypsin concentration inside the ghosts is raised from 40 ng/ml to 100 ng/ml the degradation of spectrin increases from 70% to 90% and the degradation of band 3 from 60% to 75%. However, progress of digestion does not result in much further increase of lateral mobility.

The proteolytic digestion of the cytoskeleton raises the lateral diffusion coefficient of band 3 up to a maximum of about $5 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1}. This value is compatible with the measured surface viscosity but not with unimpeded diffusion in the lipoprotein bilayer of the red cell membrane: From the viscosity values given in Table II one can calculate by means of the Saffman equation that the lateral diffusion coefficient of band 3 in the trypsinized erythrocyte membrane should be $1.65 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1}, i.e. similar to the experimentally determined value. However, when the same calculation is made using the lipid diffusion coefficients of Bloom and Webb [29] cited above, a diffusion coefficient for the band 3 protein is obtained which is more than one order of magnitude larger than the experimental value of $5 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1}. Diffusion coefficients of proteins in lipoprotein bilayers have also been determined experimentally. The lateral diffusion coefficient of bacteriorhodopsin in reconstituted dimyristoylphosphatidylcholine bilayers is $70 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1} at a molar ratio lipid: protein of 90 (23° C) [34]. Membrane blebs of isolated muscle cells retain integral proteins which, apparently, are detached from the membrane skeleton. In such blebbed membranes the lateral diffusion coefficients of acetylcholine receptors and concanavalin A receptors was about $30 \cdot 10^{-10}$ cm$^2 \cdot$ s^{-1} [35]. The comparison of these data with our results suggests that even after treatment with high trypsin concentrations the lateral diffusion of band 3 remains partially restricted. This may be brought about either by the survival of a small fraction of the membrane skeleton or by degradation products of spectrin that are still adsorbed to the bilayer. Percolation [36] effects due to the high concentration of band 3 in the lipid bilayer may also contribute to reduce the rate of lateral diffusion. In addition, disruption of the cytoskeleton might induce the aggregation of band 3 and thus reduce its lateral mobility. However, patching or capping of band 3 was not noticed in the fluorescence microscope even at high trypsin concentrations.

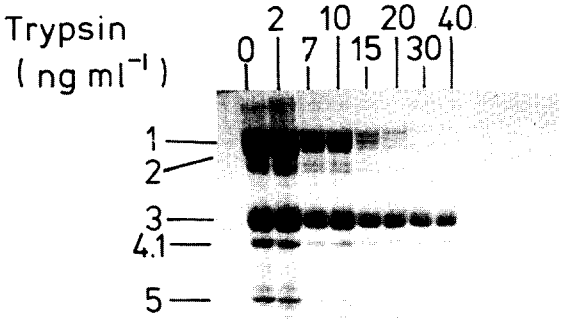

Fig. 3. SDS-polyacrylamide gel electrophoretograms of human red blood cell ghosts resealed to contain trypsin at the concentrations indicated. Ghosts were sampled after an incubation period of 1 h, washed in a saponin-containing medium, solubilized with SDS and subjected to electrophoresis as described in Materials and Methods. In gels showing greater resolution it could be seen that band 4.1 but not 4.2 disappeared at trypsin concentrations above 10 ng/ml. Thus, the decreased density of the band designated 4.1 in the figure reflects an essentially complete disappearance of band 4.1 and survival of 4.2.

(4) Effect of intracellular trypsin on the major proteins on the red cell membrane

In the preceding sections, correlations between the alterations of membrane elasticity and viscosity or of lateral mobility of band 3 with a degradation of the major membrane protiens, band 3 and spectrin, have already been described. It would seem desirable, however, to provide a more detailed account of the changes of the band patterns seen on SDS-polyacrylamide gel electrophoretograms in trypsin-treated red cell ghosts.

The most conspicuous findings that are relevant in the present context are the following: Scans of the Coomassie blue stain of the gels presented in Fig. 3 and of additional gels not represented show that trypsin concentrations below 2 ng/ml produce no detectable degradation of spectrin during the time of exposure chosen in these experiments (1 h). Above this concentration, both α and β chains of spectrin are nearly equally degraded. The decrease of the sum of the two chains follows a straight line when plotted against the log of the trypsin concentration (Fig. 4a). At about 80–100 ng/ml, more than 90% of the spectrin is degraded. It is unclear whether the remaining 10% is less sensitive to digestion than the bulk of spectrin. The uncertainties of the base line of the densitometric scans do not permit a sufficiently accurate evaluation of the data at these high degrees of degradation to allow a definitive

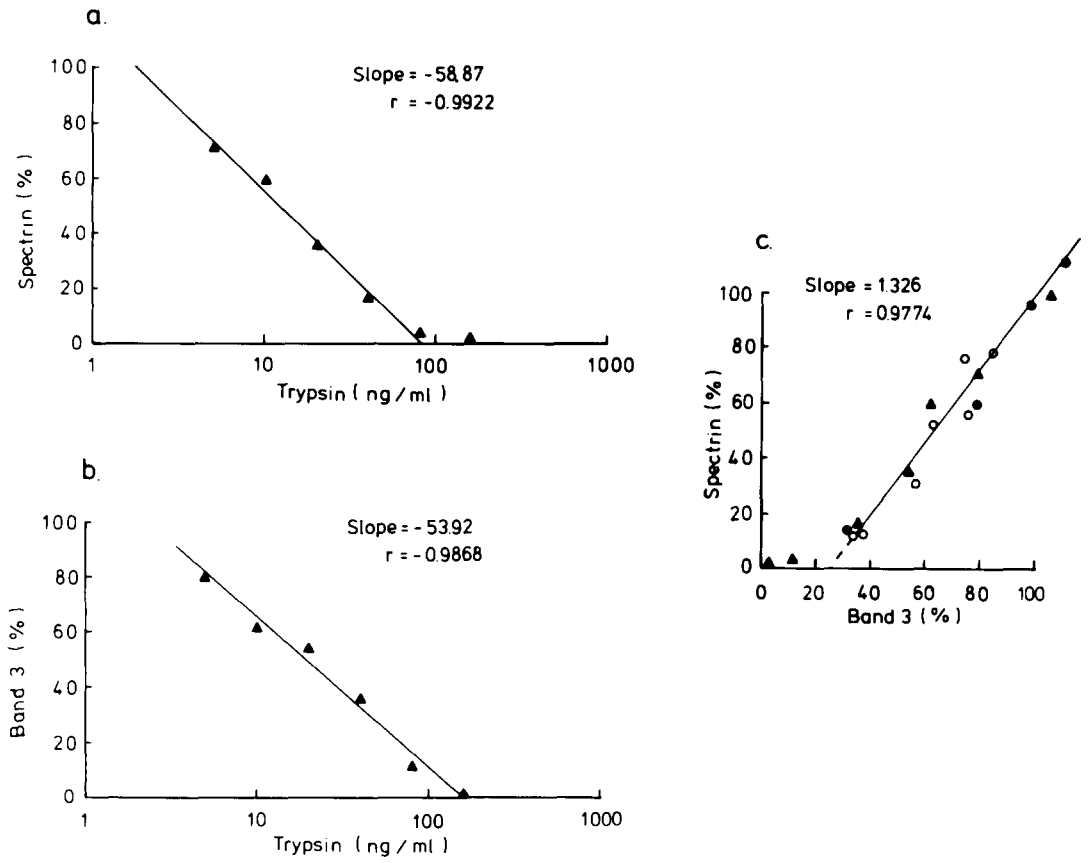

Fig. 4. Effect of internal trypsin on spectrin and band 3 in white ghosts. White ghosts were trypsinized at the concentrations indicated on the abscissa for 45 min at 37° C. Samples of the membranes, dissolved in SDS, were run on a polyacrylamide gradient gel (7.5–20%). After Coomassie blue staining and drying, the gel was scanned on a densitometer (Sigma, Berlin). The data from three different experiments were normalized to the same amount of membrane phospholipid. The areas under the spectrin and band 3 peaks are expressed as percentages of the controls incubated in the absence of trypsin (ordinate). Spectrin (a) or band 3 (b) plotted against the log of the trypsin concentration. The straight lines represent least-squares fits of the data. (c) Spectrin versus band 3. The data from three independent experiments are represented by different symbols. The line was drawn according to least-squares fit.

answer. Extrapolation of the straight line relationship yields 100% digestion of spectrin at 90 ng/ml trypsin. Experimentally we find that at trypsin concentrations ≥ 1 μg/ml no surviving spectrin can be detected.

The band 3 also disappears upon digestion. Again, up to about 2 ng/ml trypsin, little if any effect is seen. Above this concentration, the plot of band 3 vs. the log of the trypsin concentration is linear (Fig. 4b). By extrapolation of the straight line one finds 100% degradation at about 135 ng/ml trypsin. A plot of spectrin vs. band 3 yields a straight line until about 80% of band 3 and 90% of spectrin has become degraded (Fig. 4c). Extrapolation of the straight line shows that at 100% digestion of spectrin about 20% of the band 3 molecules are still intact.

Ankyrin (band 2.1) is, perhaps, somewhat more resistant to proteolysis than spectrin, but the results are not quite clear since the rather faint band 2.1 becomes obscured by the appearance of a degradation product of one or both of the spectrin chains. Both the band 4.1 protein and actin (band 5) disappear. This is only seen, however, when the trypsin-containing ghosts, which had originally been resealed, are rendered permeable to peptides by washing in saponin-containing medium as in the ghosts used for the gel electrophrogram shown in Fig. 3. If the washes are performed in isotonic salt solutions without added saponin, the ghosts remain impermeable for these peptides and the bands 4.1 and 5 can still be demonstrated on the gel at their original locations (not documented). Thus several of the proteins that are important for the formation of the membrane skeletal meshwork and its attachment to the lipid bilayer become extractable by saponin without detectable reduction of their molecular weights.

Discussion

(a) The role of the membrane skeleton in the maintenance of the mechanical properties of the red cell membrane

There is little doubt that the mechanical properties of the red cell membrane are largely determined by the membrane skeleton. Most interpretations of the elastic and viscous properties of the membrane take this into account. Nevertheless, experimental data that could serve as an empirical basis for the establishment of relationships between the specifics of the composition and structural organization of the membrane skeleton and the mechanical properties have begun to accumulate only recently (e.g., Waugh, 1987 [37], Chassis and Mohandas 1986 [6a]).

The systematic degradation of the membrane skeleton by trypsin described above yielded the surprising result that about 40% of the membrane skeletal protein and possibly more can be degraded without a decrease of either membrane elasticity or viscosity. This degradation does not only encompass the spectrin filaments but is accompanied by the loss of at least one protein involved in interconnecting spectrin meshes (actin) and another involved in attaching the meshes to the lipid bilayer (the band 4.1 protein). Thus the survival of a fraction of the protein meshwork and few if any of the anchoring proteins seems to be required to account for the maintenance of the mechanical properties.

Upon trypsinization, a dramatic increase of the lateral diffusion of band 3 takes place. This increase is not unexpected on the basis of previous findings of Golan and Veatch (1980) [6] who showed a similar increase at low ionic strength, which tends to reduce the stability of spectrin binding to the lipid bilayer. After trypsination the lateral diffusion coefficient reaches a plateau before all of the spectrin is degraded and further degradation of the spectrin (up to 90%) has no further influence on lateral diffusion. This again resembles the findings of Golan and Veatch who observed the maximal increase of lateral diffusion at ionic strengths higher than those required to actually detach the 'loosened' spectrin from the bilayer.

The increase of the protein diffusion coefficient up to the plateau value is less than anticipated for a complete removal of the membrane skeletal protein from the bilayer. Hence, some viscous matrix seems to survive. This resembles the findings of Sheetz et al. (1981) [20] with spectrin-deficient, spherocytotic mouse red cells where the coefficient of lateral diffusion of band 3 is also about one order of magnitude lower than expected for diffusion in the lipid bilayer without an underlying protein matrix.

These conclusions are fully corroborated by our measurements of elastic shear modulus and surface viscosity. At the trypsin concentrations required to increase lateral diffusion to 70% of the plateau value, the elastic modulus is still about the same as in the absence of a tryptic digestion of the membrane skeleton. The surface viscosity is also much higher than anticipated for a protein-free lipid bilayer and is of the order of magnitude predicted on the basis of the Saffman equation from the rate of lateral diffusion of band 3 in the exhaustively trypsinized membranes (see pp. 185 and 189).

Why does the extensive disruption of the membrane skeleton result in little change of the mechanical properties of the red cell membrane right up to the point where the mechanical stability gets lost? Two possible explanations come to mind: (1) a fraction of surviving membrane skeleton involving less than half of the spectrin, which does not suffice to restrict lateral diffusion of band 3, suffices to maintain the mechanical properties; (2) neither the integrity of the skeletal meshwork nor the fixation of its elements at specific locations by 'binding proteins' is necessary for the maintenance of the membrane elasticity and surface viscosity.

Neither the data available in the literature nor our own data lend support in favor for or against the idea that the membrane skeleton may exist as two functionally different subsets. The results presented in Fig. 4 indicate that spectrin and about 80% of the band 3 molecules are equally accessible to tryptic digestion. Some 20% of the band 3 molecules seem to be considerably more resistant. They could possibly be protected against proteolysis by association with a small fraction of spectrin, but there is no experimental evidence to support such a claim. The higher resistance to proteolysis could also be conveyed by other factors, e.g. binding to ankyrin, which would involve some 20% of the band 3 molecules in the membrane. For the time being, it seems more reasonable to consider the possibility that the maintenance of the mechanical properties does not require the complete structural integrity of the membrane skeleton and its major constituent, spectrin.

In this context, studies of the temperature dependence of the elastic behavior of the red cell membrane by Evans and Waugh (1980) [39] are most relevant. They derived from their measurements the conclusion that the configurational entropy of the membrane is minimal in the undeformed state and increases with extension. Stretching causes an increase in the degrees of freedom of the spectrin molecules, which is largely represented by a positive heat of extension. At mechanical equilibrium, this is opposed by a contribution of the internal enthalpy, which depends on ionic, dipolar, etc. interactions that stabilize molecular conformation. The energies involved in membrane shear deformation are very small. Evans and Waugh [39] estimate that one half percent increase of exposure of the hydrophobic portion of spectrin to the aqueous phase could completely account for the energy stored in shear deformation. Thus the elastic forces that resist deformation or lead to a return of deformed cells to the undeformed state are most likely due to changes of the enthalpy of the system. This would comprise the enthalpy changes associated with a reorientation of the skeletal proteins with respect to the lipid bilayer. If this were true, one could expect that the elastic forces change little if at all after the hydrolysis of a number of peptide bonds, provided the peptide fragments remain adsorbed to the bilayer and retain much of their tertiary structure. At the same time, the disruption of the associations between integral proteins and the meshwork would allow the lateral diffusion of the integral membrane proteins, whereby the rate of diffusion is limited by percolation effects and possibly by interactions with absorbed fragments of skeletal proteins.

The adsorption of isolated spectrin to lipid monolayers has been demonstrated. The adsorption requires no binding proteins and is solely due to weak hydrophobic interactions. It seems to involve primarily the β-chain, the isolated α-chain does not become adsorbed. The α,β-heterodimer does associate with the monolayers, suggesting that in the dimer the β-chain is also responsible for the adsorption process [40]. It has further been shown that proteolytic fragments of spectrin and other membrane skeletal proteins remain bound to lipid vesicles [41].

These pieces of evidence support the view that in the untreated red cell membrane and after

proteolytic degradation of spectrin and other skeletal elements, the critical skeletal elements are adsorbed to the bilayer, and the thermodynamical considerations discussed above indicate why this adsorption conveys to the membrane the essential mechanical properties.

If the maintenance of the mechanical properties does not require an intact membrane skeleton, what then is the physiological function of the membrane skeleton? The discussion of this question requires a differentiation between viscoelastic properties and the stability of the membrane. The present experiments clearly show that upon proteolytic digestion of the membrane skeleton, the stability of the membrane is lost prior to measurable changes of elasticity or viscosity of the membrane.

The red cell ghosts used in the experiments described above were not subjected to the recurrent deformations that take place under natural conditions in the circulation. Consequently, the arrangement of the membrane skeletal elements was unlikely to change in the course of our experimental work. However, the endlessly repeated deformations of the red cell membrane in the circulation are most likely to lead to a fragmentation when the membrane skeleton is disrupted. This disruption may either be a direct consequence of the occurrence of breaks in the meshwork, or the indirect result of an aggregation of the skeletal proteins under the influence of the continually occurring deformations, which would leave the lipid bilayer uncoated with protein and thus convey to the membrane an extreme fragility.

(b) The membrane skeleton and the Ca^{2+}-activatable K^+ channels of the red cell membrane

The original aim of the present work was the study of the relationship between the membrane skeleton and the mechanical properties of the red cell membrane. However, the data obtained are also pertinent to deal with a question related to the Ca^{2+}-activatable K^+ channels that are responsible for the so-called Gárdos effect.

Determinations of the number of these channels per cell by a comparison of single channel conductance with isotopically measured Ca^{2+}-induced K^+ permeability yielded an estimate of maximally 50 channels per red cell. This number is significantly lower than an estimate by Lew, Muallem and Seymour (1982) [42]. Using inside-out vesicles, these authors found that only a fraction responded to Ca^{2+} with an increase in K^+ permeability. On the assumption that the number of channels available in one cell is smaller than the number of vesicles formed per cell, they inferred that there are about 100–200 channels per cell. This number is significantly higher than the estimate from our laboratory. The estimate of Lew et al. (1982) [42] depends on the assumption that the vesiculation procedure does not affect the transport system. While we determine the number of channels that are actually activated in the intact cell, the vesicles could contain channels that become activatable by the vesiculation procedure. This raised the question whether or not in the intact red cell membrane some of the channels could exist in a 'dormant' form. Such channels could become activatable by Ca^{2+} after disruption of the membrane skeleton that could conceivably occur during the formation of the inside-out vesicles.

Our results show that the channels are activated at Ca^{2+} concentration in the same range where the viscoelastic properties of the membrane are affected (compare data in Table I and Fig. 1).

Nevertheless, it seems that the effects on the channels and the mechanical properties are not directly related. We infer this from the observation that extensive destruction of the membrane skeleton (up to 20% degradation of spectrin and nearly maximal increase of lateral diffusion of band 3, see Table II, column 5) does not change significantly the responsiveness of the K^+-selective channels to the action of Ca^{2+}.

In conclusion it may be recalled that the activity of the band 3 protein to transport anions across the red cell membrane also remains unaltered after exhaustive degradation of the membrane skeleton by incorporated trypsin (up to several $\mu g/ml$, see Ref. 31). Thus, if there exists a control of transport functions by membrane skeletal elements, it is certainly not a general phenomenon which applies to all transport systems.

Acknowledgments

We are grateful to Mrs. S. Lepke for the execution of the efflux measurements presented in Table I. We thank Mrs. Hanne Müller for her efforts during the concluding stages of this work and Drs. V. Rudloff, D. Schubert and W. Schwarz for their comments on the manuscript. Dr. P. La Celle made his contributions to this work during his stay at Frankfurt as a recipient of a Humboldt Award. Dr. Melanie Shields was a post-doctoral fellow of the Max-Planck-Gesellschaft.

References

1 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233–248
2 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
3 Bennett, V. (1984) in Cell Membranes Methods and Reviews, Vol. 2 (Elson, E., Frazier, W. and Glaser, L., eds.), pp. 149–195, Plenum Press, New York
4 Hochmuth, R.M. (1982) Annu. Rev. Biophys. Acta 11, 43–55
5 Peters, R., Peter, J., Tews, K.H. and Bähr, W. (1974) Biochim. Biophys. Acta 367, 282–294
6 Golan, D.E. and Veatch, W. (1980) Proc. Natl. Acad. Sci. USA 77, 2537–2541
6a Chasis, J.A. and Mohandas, N. (1986) J. Cell Biol. 103, 343–350
7 Grygorczyk, R., Schwarz, W. and Passow, H. (1984) Biophys. J. 45, 693–698
8 Shields, M., Grygorczyk, R., Fuhrmann, G.F., Schwarz, W. and Passow, H. (1985) Biochim. Biophys. Acta 815, 223–232
9 Gárdos, G. (1958) Biochim. Biophys. Acta 30, 653–654
10 Ørskov, S.L. (1935) Biochem. J. 297, 250–261
11 Passow, H., Shields, M., La Celle, P., Grygorczyk, R., Schwarz, W. and Peters, R. (1986) in The Cytoskeleton (Clarkson, T.W., Sager, P.R. and Syversen, T.L.M., eds.), pp. 177–186, Plenum Press, New York
12 Schwoch, G. and Passow, H. (1973) Mol. Cell Biochem. 2, 197–218
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
14 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
15 Perrin, D.D., and Dempsey, B. (1974) in Buffers for pH and Metal Ion Control (Perrin, D.D. and Dempsey, B., eds.), pp. 94–108, Champan and Hall, London
16 Cherry, R.J. (1979) Biochim. Biophys Acta 599, 289–327
17 Axelrod, D. (1983) J. Membr. Biol. 57, 1–10
18 Peters, R. (1986) Biochim. Biophys. Acta 864, 305–359
19 Fowler ,V. and Branton, D. (1977) Nature (Lond.) 268, 23–26
20 Sheetz, M.P., Schindler, M. and Koppel, D.E. (1980) Nature (Lond.) 285, 510–512
21 Peters, R. and Richter, H.P. (1981) Dev. Biol. 86, 285–293
22 Axelrod, D., Koppel, D.E., Schelssinger, J., Elson, E. and Webb, W.W. (1976) Biophys. J. 16, 1055–1069
23 Koppel, D.E. (1979) Biophys. J. 28, 281–291
24 Koppel, D.E. (1985) Biophys. J. 47, 337–347
25 Hochmuth, R.M., Mohandas, N. and Blackshear, Jr., P.L. (1973) Biophys. J. 13, 747–762
26 Lorand, L., Weissmann, L.B., Epel, D.L. and Bruner-Lorand, J. (1976) Proc. Natl. Acad. Sci. USA 73, 4479–4481
27 Saffman, P.G. (1976) J. Fluid mech. 73, 593–606
28 Hochmuth, R.M. (1980) in Erythrocyte Mechanics and Blood Flow (Cokelet, G.R., Meiselman, H.J. and Brooks, D.E., eds.), pp. 57–73, Alan R. Liss Inc., New York
29 Bloom, J.A. and Webb, W.W. (1983) Biophys. J. 42, 295–305
30 Nash, G.B. and Meiselman, H.J. (1983) Biophys. J. 43, 63–73
31 Lepke, S. and Passow, H. (1976) Biochim. Biophys. Acta 455, 453–370
32 Wood, P.G. and Müller, H. (1984) Eur. J. Biochem. 141, 91–95
33 Schindler, M., Koppel, D.E. and Sheetz, M. (1980) Proc. Natl. Acad. Sci. USA 77, 1457–1461
34 Peters, R. and Cherry, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 4317–4321
35 Tank, D.W., Wu, E.-S. and Webb, W.W. (1982) J. Cell Biol. 92, 207–212
36 Saxton, M.J. (1982) Biophys. J. 39, 165–173
37 Waugh, R.E. (1987) Biophys. J. 51, 363–369
39 Evans, E.A. and Waugh, R.E. (1980) in Eyrthrocyte Mechanics and Blood Flow (Cokelet, G.R., Meiselman, H.J. and Brooks, D.E., eds.), pp. 31–56, Alan R. Liss Inc., New York
40 Rudloff, V., Herbst, F., Passing, R. and Schubert, D. (1982) in Membranes in Growth and Development (Hoffman, J.F., Giebisch, G.H. and Bolis, L., eds.), pp. 107–119, Alan R. Liss Inc., New York
41 Schubert, D. (1973) Hoppe Seyler's Z. Physiol. Chem .354, 781–790
42 Lew, V.L., Muallem, S. and Seymour, C.A. (1982) Nature 296, 742–744