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Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. I. Impairment of resealing and formation of aqueous pores in the ghost membrane after modification of SH groups

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Resealed human erythrocyte ghosts prepared by a two-step procedure were shown to have small residual barrier defects with the properties of aqueous pores, such as size discrimination of hydrophilic nonelectrolytes (erythritol to sucrose), indicative of an apparent pore radius of about 0.7 nm, and a low activation energy (about 12–20 kJ/mol (mannitol, sucrose)) of the leak fluxes. As in other cases (Deuticke et al. (1991) Biochim. Biophys. Acta 1067, 111–122) these leak fluxes can be inhibited by phloretin. Treatment of such resealed ghosts with the mild SH oxidizing agent, diamide, induces additional membrane leaks to the same extent and with the same properties as in native erythrocytes (Deuticke et al. (1983) Biochim. Biophys. Acta 731, 196–210), including reversibility of the leak by SH reducing agents, inhibition by phloretin and stimulation by alkanols. In contrast, resealed ghosts prepared either from diamide-treated erythrocytes or by adding diamide to the 'open' membranes prior to reconstitution of high ionic strength and raising the temperature, exhibit a state of greater leakiness. This leakiness is somewhat different in its origin from the former class of leaks, since it can also be produced by *N*-ethylmaleimide, which is essentially ineffective when added to the membrane in its 'tight' state. The leaks induced in the 'open' state of the membrane, which can be regarded as a consequence of an impaired resealing, are nevertheless reversible by reducing agents added after resealing and are comparable in many, but not all their characteristics to leaks induced in the 'tight' state of the membrane. Resealing in the presence of the isothiocyanostilbenes DIDS or SITS mimicks the leak forming effect of diamide by modifying a small population of SH groups, while amino groups seem not to be involved. The findings indicate and substantiate an important role of the redox state of membrane skeletal protein sulfhydryls in the maintenance and the re-establishment of the barrier function of the erythrocyte membrane.

Introduction

Resealed erythrocyte membranes (ghosts) are valuable tools for membrane studies, since after hypotonic lysis and subsequent resealing the membrane can regain many of its native characteristics. The extent of the restoration of the original state depends on the conditions of ghost preparation [1–9]. Many authors have used ghosts to characterize transport properties of the membrane [10–15]. An important prerequisite underlying the evaluation of transport studies on resealed ghosts is the restoration of the original barrier function of the membrane after ghost preparation. This barrier function can conveniently be assessed by measuring the permeability to hydrophilic nonelectrolytes varying in size and polarity.

This report presents a study of the permeability of resealed ghosts to large hydrophilic nonelectrolytes, some of which are even impermeant in the native red blood cell membrane.

In particular we were interested to find out, whether: (1) chemically induced defects in resealed ghosts are comparable to those in erythrocytes, (2) leaks induced in intact erythrocytes are carried through the process of ghost preparation or disappear during resealing and (3) amino- and carboxyl groups like sulfhydryl groups are involved in the barrier function of the erythrocyte membrane.

Abbreviations: diamide, diazenedicarboxylic acid bis(dimethylamide); DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide; SDS, sodium dodecylsulfate; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbenedisulfonic acid.

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Materials and Methods

Materials

Fresh human blood from healthy donors was obtained from the local blood bank. Standard chemicals and compounds used as test permeants were from Merck (Darmstadt), Fluka (Neu Ulm) or Sigma (Munich).

^{14}C-labelled test permeants (erythritol, mannitol, sucrose, inulin) were from Amersham-Buchler, Braunschweig. EDC was from Merck (Darmstadt), phloretin from Carl Roth (Karlsruhe), DIDS, SITS and DTE from Sigma (Munich), diamide from Calbiochem (Frankfurt), NEM from Fluka (Neu Ulm).

Methods

1. Preparation of cells

Fresh human blood, anticoagulated with citrate, was stored at 4°C in a conventional storage medium containing glucose (20 mM) and adenine (25 μM) and used for experiments within the following 10 days. Erythrocytes were isolated by centrifugation (5750 × g, 5 min). After removing plasma and buffy coat, the erythrocytes were washed three times in an excess of isotonic NaCl and packed at 5750 × g for 5 min.

2. Preparation of resealed ghosts by a 'two step' procedure

In a first step one vol. washed cells was suspended in one vol. 51 mM ice-cold sodium phosphate buffer (pH 7.7–8.0) and rapidly mixed with 16.5 vols. ice-cold lysis buffer (5.1 mM sodium phosphate, pH 7.5–7.7) [2]. The lysate was stirred for 15 min at 0°C and the pH adjusted to 7.2–7.4 if necessary. After centrifugation (32000 × g, 10 min, 0°C) the supernatant was removed. In a second step 1 vol. of sedimented, unsealed ghosts was resuspended in 1 vol. 15 mM $MgSO_4$ [16] containing the choosen permeants, i.e. ^{14}C-labeled hydrophilic nonelectrolytes (erythritol, mannitol, sucrose, inulin; 160 nCi/ml) and 8 mM of the unlabeled nonelectrolyte (0.2 mM in case of inulin). The suspension was incubated for 15 min at 0°C. After this period of loading with the test permeant, isotonicity was restored by adding 0.5 vol. reconstitution medium containing KCl (700 mM), Hepes (100 mM) and MgCl (2.5 mM), (pH 7.4). By varying the pH of this reconstitution medium the pH of the ghost interior can be changed. The suspension with a final ghost volume fraction of 0.1 was stirred for 10 min (0°C, pH 7.4) and subsequently incubated for resealing at 37°C for 45 min. The resealed ghosts were washed twice in a medium containing KCl (140 mM), Hepes (20 mM) and $MgCl_2$ (0,5 mM) (medium A) at 0°C and pH 7.4 (centrifugation at 18000 × g, 10 min, 0°C) and immediately used for measurements of permeability.

3. Measurements of permeability

Efflux of the trapped labeled test permeant was initiated by resuspending 1 vol. tracer-loaded ghosts in 12 vols. tracer-free medium A. At various times, samples of 280 μl were taken. After centrifugation (6500 × g, 5 min, 0°C) the radioactivity in the supernatant was measured by liquid scintillation counting. Rate coefficients k of tracer exfflux were derived by linear regression analysis from the slope of a plot of $\ln((dpm_\infty - dpm_t)/(dpm_\infty - dpm_0))$ versus time, subscripts 0, t and ∞ referring to radioactivities (disintegrations per min per ml) in the supernatant at the beginning, at any time and after attainment of equilibrium. The lines derived by linear regression analysis should cut the origin of the coordinates. After extensive membrane damage this is, however, not the case; this indicates the presence of a small population with higher permeability, which can not be determined exactly, because of the duration of separation of medium from ghosts.

The release of erythritol, which uses the glucose transport system [17] in addition to unspecific pathways, was measured in the presence of cytochalasin B (10 μM) (Sigma, Munich).

For measurements of K^+ release ghosts were prepared as usual, whereas in medium A KCl was substituted by NaCl. K^+ release of the ghosts was measured by taking 500 μl samples after appropriate intervals (0, t, ∞), which were centrifuged and analysed for K^+ concentration in the supernatant by flame photometry (Instrumentation Laboratory Type 543) using an internal lithium standard.

In case of results derived from three or more experiments ($n \geq 3$) mean values ± S.D are given, if $n < 3$ only the arithmetic mean values are given.

4. Determination of hemoglobin release

Tests for release of hemoglobin were initiated by resuspending 1 vol. of resealed ghosts in 12 vol. hemoglobin-free medium A. After appropriate periods of time 800-μl samples were collected and centrifuged. The hemoglobin contents in the supernatant, as a measure of ghosts fragmentation or 'lysis' was determined photometrically at 405 nm (Soret band). It was compared to the total hemoglobin contents of the ghost suspension, which was measured after complete lysis with 0.1% Triton X-100.

5. Determination of retention of test solute

After several modifications a part of ghosts was damaged to such an extent, that they lost the trapped test solute during the washing process. This reflects the problem of different populations in the ghost preparation.

For estimation of the fraction of these damaged ghosts, we made use of the fact, that each ghost preparation, respectively modification, starts with the same volume of erythrocytes. All ghosts obtained from this volume were washed two times and subsequently suspended in equal quantums of medium A. In this ghost suspension the total radioactivity, trapped during the ghost preparation and retained during the washing process, was measured. The fraction of the radioactive test solute retained by modified ghosts, termed the 'retention' of test solute, is expressed as percentage of the total amount of test solute found in simultaneously prepared untreated ghosts.

6. Treatment with covalently modifying reagents

During the preparation of resealed ghosts there are three possibilities to modify the membrane:

A. Treatment of resealed ghosts (termed 'post' treatment). The reagents were dissolved in medium A and added to the ghosts, loaded with the labeled test permeant, immediately after the resealing. At the end of the exposure to the modifier at 37°C the ghosts were washed and used for permeability measurements as usual.

B. Pretreatment of intact erythrocytes before ghost preparation (termed 'pre' treatment). 1 vol. erythrocytes was mixed with 8 vols. solution containing KCl (100 mM), NaCl (50 mM), Na_2HPO_4/NaH_2PO_4 (12.5 mM) and sucrose (44 mM) (medium B), at desired pH at 37°C. After adjusting the pH one vol. of 10-fold concentrated stock solution of the modifying agent under study in medium B was added to 9 vols. erythrocyte suspension. After 45 min incubation at 37°C the cells were washed three times with medium B. Ghosts were prepared from the washed erythrocytes as usual. After preincubation of the cells at pH 8 it is important to adjust pH for lysis to 7.2–7.4.

C. Addition of modifying reagents during ghost preparation (termed 'para' treatment). 1 vol. unsealed ghosts, prepared as described above, was mixed with 1 vol. 15 mM $MgSO_4$ containing the radioactively labeled and unlabeled form of the test solute as well as the reagents at the desired concentration. After 15 min equilibration at low ionic strength at 0°C isotonicity was restored and ghosts were resealed at 37°C as usual. All subsequent procedures including efflux measurements were carried out at 0°C. Permeable reagents were removed from the ghosts by the washing process. In these cases efflux measurements could be carried out at higher temperatures.

7. Determination of thiols and protein content of the membrane

Membrane SH groups were quantified in membranes isolated from modified ghosts as described earlier [18]. Protein contents was measured according to Lowry et al. [19].

Results and Discussion

Characterisation of the resealed ghosts

Resealed pink ghosts, prepared by the two-step-procedure decribed in Methods appear as spheres with fine, homogeneously distributed spicules and an approximate radius of 2.3 ± 0.2 μm ($n = 80$) when examined by dark-field microscopy. The mean volume calculated from these radii, 51 ± 13 μm^3, is about 60% of the mean volume of the original erythrocytes. This value was also confirmed by measurements of osmotic fragility [20]. The true surface areas of ghosts and original erythrocytes are probably the same, since during ghost preparation no vesiculation could be observed. The difference between the original erythrocyte surface area of 140 μm^2 and the apparent ghost surface area of 66 μm^2, calculated from the spherical radius, reflects the total area of the spicules.

Ground permeability of resealed ghosts

In order to characterize the barrier properties of the resealed ghosts, ground permeabilities for a number of nonelectrolytes were obtained from efflux measurements. Moreover, in order to characterize the pathway(s) involved we determined the activation energies of the fluxes and their sensitivity to phloretin [21]. Test permeants were (Stokes-Einstein radius in nm [22,23]): erythritol (0.36), mannitol (0.42), sucrose (0.52) and inulin (1.4).

Erythritol fluxes in erythrocytes are based on two processes: mediated transfer via the glucose transporter [17] and simple diffusion, assumed to involve the lipid domain. Distinction between the two pathways is based on inhibition of the specific transporter by cytochalasin B. Erythritol fluxes measured in the presence of this inhibitor can be regarded as simple diffusion, based on the ground permeability [24]. As shown in Fig. 1A, the release of ^{14}C-labeled erythritol from resealed ghosts in presence of cytochalasin B at temperatures between 0°C and 30°C can be fitted by single exponentials up to a released fraction of more than 90%. Mean rate coefficients derived from a series of experiments are listed in Table I.

The release of entrapped [^{14}C]mannitol and [^{14}C] sucrose (Fig. 1B, 1C, Table I) is only based on simple diffusion, since cytochalasin B had no inhibitory effect on the fluxes (data not shown). The permeability of ghosts to inulin is extremely low ($k_0 = (0.10 \pm 0.02) \cdot 10^{-3}$ min^{-1} ($n = 6$) at 0°C).

Since mannitol in the native erythrocyte has an extremely low permeability [25] and sucrose and inulin do not penetrate to any measurable extent [25], the permeability observed in resealed ghosts must – con-

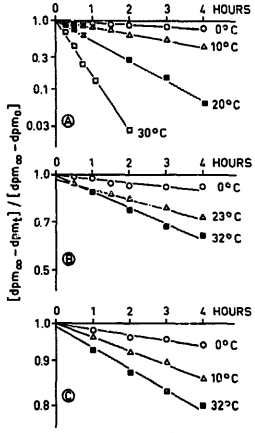

Fig. 1. Release of hydrophilic nonelectrolytes from resealed ghosts at different temperatures. Time course of efflux measured as described in Methods. (Panel A) Erythritol (in presence of cytochalasin B, 10 μM), (Panel B) mannitol, (Panel C) sucrose. (Note the different ordinate scales.)

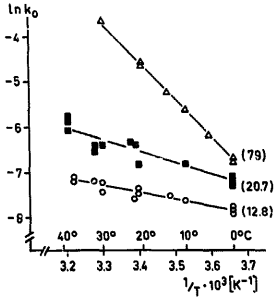

Fig. 2. Arrhenius diagram for ground permeabilities of resealed ghosts to various hydrophilic nonelectrolytes (erythritol (△), mannitol (■), sucrose (○)). Fluxes were measured as described in Methods. Numbers in brackets are activation energies (kJ/mol) calculated from the slopes of the lines (regression analysis).

ceptually – reflect a leak. This leak is not based on an insufficiently long resealing period, since it was the same, when ghosts were resealed for 90 instead of 45 min (data not shown).

In order to further evaluate the quality of our two-step ghost preparation method, we also measured potassium leakage from resealed ghosts. The rate coefficients ($k_0 \times 10^3$ (min^{-1}) ranging from 1.72 ± 0.5 ($n = 3$) at 0°C to 1.92 ($n = 2$) at 37°C (resulting activation energy about 2 kJ/mol)) compare well with values obtained for ghosts prepared by a one-step method in presence of magnesium at the time of lysis ($k = 1.2 \cdot 10^{-3}$ min^{-1}, 37°C, [26]). The presence of magnesium during lysis is assumed to be important for a restoration of the K^+ barrier [26]. Our results indicate that ghosts, lysed in the absence of magnesium, will aquire an acceptably low potassium permeability if magnesium is added before reconstitution and resealing.

The activation energies for the release of the three test permeants, derived from Arrhenius diagrams (ln k versus $1/T$ (K^{-1})), are quite different (Fig. 2). In terms of established concepts, the value for erythritol (about 79 kJ/mol) indicates diffusion through the lipid domain of the ghost membrane as in native cells [27], while the lower activation energies for mannitol and sucrose permeability (20.7 kJ/mol and 12.8 kJ/mol between 0°C and 37°C) indicate diffusion through aqueous pores [17,28], since they correspond to values for free diffusion in bulk water. This interpretation raises the problem, however, why erythritol should not pass pores accessible to mannitol and sucrose. Further studies will thus have to check alternative hypotheses. Based on observations by Lieber and Steck [5], temper-

TABLE I

Summary of ground permeabilities of resealed ghosts

Permeabilities were calculated as described in the text. Data for erythrocytes given for comparison [17]. Number of experiments in brackets.

Test solute	Membrane system	T_{efflux} (°C)	$k \times 10^3$ (min^{-1})	$t_{1/2}$ (h)	$P \times 10^{10}$ (cm/s)	E_a (kJ/mol)
Erythritol	Erythrocyte	0	0.34	33	2.41	90
		30	25.6	0.45	182	
	Ghost	0	1.16 ± 0.03 (12)	9.9	6.96	78 (2)
		30	27.3	0.42	164	
Mannitol	Ghost	0	0.79 ± 0.15 (5)	14.8	4.68	20.7 (2)
Sucrose	Ghost	0	0.34 ± 0.1 (30)	35.0	1.98	12.7 (2)

ature induced changes of hole size might be assumed to affect the observed temperature dependences of permeation. According to their results hole radii might decrease slightly (0.8-fold per 10°C) with temperature. Even such an effect can, however, not account for the large difference in activation energy between erythritol on the one and mannitol on the other hand.

The transport properties of our ghosts are compared with those of original cells in Table I. Permeabilities were calculated as usual: P (cm/s) = k (min^{-1}) · V_g/(A_g · 60), where V_g = ghost volume (51 μm^3) and A_g = surface area (142 μm^2) of the ghosts *.

The presence of residual 'holes' in resealed ghosts is also supported by our finding that the fluxes of both, mannitol and sucrose, are inhibited by 40% in the presence of 200 μM phloretin (data not shown, for details see Ref. 20). Phloretin (3-(*p*-hydroxyphenyl)-2,4,6-trihydroxypropiophenone) has long been known to inhibit the carrier-mediated transport of hydrophilic nonelectrolytes, anions and other solutes [27,28]. Recently it was shown that phloretin also inhibits the diffusion of nonelectrolytes and ions through aqueous leaks induced in erythrocyte membrane by treatment with oxidants [21,29].

Calculations of the size of these aqueous holes in the membrane according to a procedure introduced by Lieber and Steck [4] yield an apparent mean radius of about 0.8 nm. Some defects may be greater then 1.4 nm, since inulin is also slightly permeant. The number of holes per ghost, which was obtained according to Ref. 4, is very low (0.02). As discussed earlier [17], such a low number indicates a dynamic character of the holes, which probably arise from the formation of statistical defects in the barrier opening and closing in random distribution over the whole membrane surface. Lieber and Steck [4,5] reported a radius of 0.7 nm for the leak in resealed ghosts prepared under experimental conditions comparable to ours.

Permeability of ghosts after covalent membrane modifications

Modification of resealed ghosts ('post'-procedure). Treatment of intact human erythrocytes with diamide markedly enhances the efflux of nonelectrolytes [17]. According to the data shown in Table II, treatment of resealed ghosts with diamide has the same effect. As expected, its magnitude depends on the pH during

* The true surface area was supposed not to change during the preparation of ghosts and thus to be identical with that of the original cell. This assumption is supported by the lack of a detectable shedding of vesicles during ghost preparation and by the satisfactory agreement between ghost volumes calculated from direct measurements of spherical radii, and volumes obtained by osmotic fragility measurements [20].

TABLE II

Formation of membrane leaks by diamide in resealed ghosts ('post'-procedure)

Leaks quantified by the increase of rate coefficients ($\Delta k \times 10^3$, min^{-1}) of efflux of various test solutes on top of the ground flux of these test solutes ($k_0 \times 10^3$, min^{-1}). Ghosts were treated with 5 mM diamide (pH 7.4 or 8.0, 45 min, 37°C) after resealing. Efflux measured at 0°C. Further experimental details as described in Methods. number of experiments in brackets.

Test solute	$k_0 \times 10^3$ (min^{-1})	$\Delta k \times 10^3$ (min^{-1})		
		pH 8		pH 7.4
Erythritol	1.16 ± 0.03 (12)	19.8	(2)	6.0 (2)
Mannitol	0.79 ± 0.15 (5)	7.1	(2)	1.2 (1)
Sucrose	0.34 ± 0.1 (30)	0.81 ± 0.32 (5)		0.0 (6)

treatment. The extent of leakiness induced in ghosts by diamide after resealing is quantitatively similar to the leakiness induced in intact erythrocytes. In ghosts, the diamide-induced (5 mM, pH 8, 45 min, 37°C, 'post'-procedure) erythritol permeability amounts to $2.2 \cdot 10^{-8}$ cm/s (n = 2) at 35°C, to be compared with $(1.5–2.1) \cdot 10^{-8}$ cm/s in erythrocytes treated under comparable conditions, i.e. after removal of glutathione (by pretreatment with iodoacetate or chlorodinitrobenzene), (Deuticke, B. unpublished results). The leak fluxes induced in ghosts by diamide have low activation energies (11 and 12.8 kJ/mol (Fig. 3)) indicating the involvement of aqueous 'holes'.

In Fig. 4 the solute size selectivities of the diamide-induced holes are compared for ghosts and erythro-

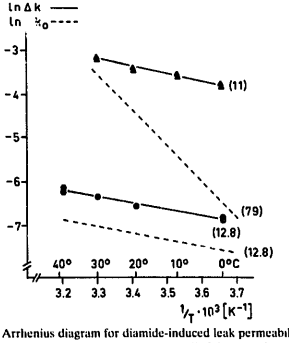

Fig. 3. Arrhenius diagram for diamide-induced leak permeabilities of resealed ghosts (erythritol (▲), sucrose (●)). Diagrams for unmodified ghosts (from Fig. 2) are given for comparison. Fluxes were measured as described in Methods. Numbers in brackets are activation energies (kJ/mol).

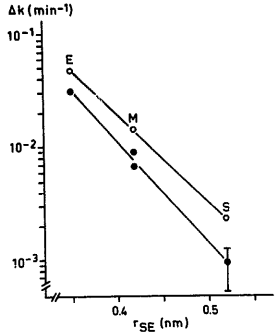

Fig. 4. Relationship between the Stokes-Einstein radii (r_{SE}) of various test solutes (erythritol (E), mannitol (M), sucrose (S)) and their diamide-induced leak permeabilities in resealed ghosts. Ghosts (●) were treated with diamide (5 mM, pH 8, 45 min, 37°C) after resealing and fluxes measured as described in Methods. Data for erythrocytes (○, [17]) are given for comparison.

cytes (data from Ref. 17). The slopes suggest apparent radii in ghosts, as in erythrocytes, of at least 0.5–0.6 nm.

The further characteristics of the diamide-induced leak in resealed ghosts, summarized in Table III, also underline the similarity with leaks in diamide-treated erythrocytes. Both are: (a) reversible by treatment with the disulfide reducing agent DTE to a large extent [17,30], (b) inhibited by phloretin and (c) stimulated by addition of hexanol (25 mM, 0°C) to the efflux medium. Aliphatic alcohols (hexanol, butanol) have previously been shown [21] to enhance leak permeabilities induced in native erythrocytes by oxidative damage.

TABLE III

Characteristics of the leaks produced by diamide in resealed ghosts ('post'-procedure)

Leaks characterized by the increase of rate coefficients ($\Delta k \times 10^3$, min^{-1}) of sucrose efflux on top of the ground flux ($k_0 = (0.34 \pm 0.1) \cdot 10^{-3}$ min^{-1} ($n = 30$)). Ghosts were treated with diamide (5 mM, pH 8) at 37°C for 45 min after resealing. Reversibility was established by quenching unreacted diamide with glutathione (10 mM) which itself has no effect on leak flux and subsequent exposure (45 min) of the ghosts to dithioerythritol (10 mM). Hexanol (25 mM) and phloretin (200 μM) were added to the efflux media (for details see Methods). Efflux at 0°C, number of experiments in brackets.

	$\Delta k \times 10^3$ (min^{-1})
Diamide	0.81 ± 0.32 (5)
Diamide, then glutathione	0.75 (2)
Diamide, then glutathione + dithioerythritol	0.12 (2)
Diamide, phloretin	0.25 ± 0.1 (3)
Diamide, hexanol	3.97 (2)

Modification of intact erythrocytes prior to ghost preparation ('pre'-procedure). In a further set of experiments we tested whether the diamide-induced leak survives the procedure of ghost preparation or closes during the resealing of ghosts. To this end we prepared resealed ghosts from erythrocytes treated with diamide and measured the release of test permeants. Such ghosts proved to be even more leaky than ghosts modified only after resealing (Table IV). The leak surviving ghost preparation also discriminates permeants according to their size. The retention values (see Table IV) agree with this discrimination. The modified ghosts are impermeable to hemoglobin (data not shown).

The leak produced by the pre-procedure shares certain features such as reversibility by DTE and inhibition by phloretin with the leak formed by diamide in the native cell (Table V). Furthermore, preliminary measurements of the activation energy of the leak flux indicate a value of about 12 kJ/mol.

TABLE IV

Effect of a pretreatment of erythrocytes with diamide or N-ethylmaleimide on the permeability of resealed ghosts prepared from these cells ('pre'-procedure)

Leaks characterized by the increase of rate coefficients of leak flux ($\Delta k \times 10^3$, min^{-1}) and retention of entrapped test solutes (in % of retention by control ghosts) for ghosts, prepared from erythrocytes treated with diamide or *N*-ethylmaleimide (each 2 mM, 45 min, 37°C, pH 8, Hct 10%, medium B) (for details see Methods). Efflux at 0°C. Number of experiments in brackets. k_0 = ground flux of ghosts prepared from erythrocytes not pretreated with modifying agents (from Table II).

Test solute	$k_0 \times 10^3$ (min^{-1})	Diamide		*N*-Ethylmaleimide	
		$\Delta k \times 10^3$ (min^{-1})	retention (%)	$\Delta k \times 10^3$ (min^{-1})	retention (%)
Erythritol	1.16 ± 0.03 (12)	24.97 * (2)	60 (2)	4.75 (2)	80 (2)
Sucrose	0.34 ± 0.1 (30)	4.34 ** ± 1.1 (4)	100 (4)	1.08 ± 0.3 (3)	106 (3)

* To be compared with 19.8 for 5 mM diamide 'post'-procedure (see Table II).
** To be compared with 0.81 for 5 mM diamide 'post'-procedure (see Table II).

TABLE V

Characteristics of the leak permeability for sucrose of ghosts, prepared from erythrocytes pretreated with diamide or N ethylmaleimide ('pre'-procedure)

Leaks defined by the increase of flux rate coefficients ($\Delta k \times 10^3$, min^{-1}) over the ground flux for sucrose ($k_0 = (0.34 \pm 0.1) \cdot 10^{-3}$ min^{-1} ($n = 30$)). Erythrocytes treated with diamide or *N*-ethylmaleimide, each 2 mM, 45 min, 37°C, pH 8, medium B, 10% Hct. Efflux at 0°C. Number of experiments in brackets.

Second treatment (of resealed ghosts)	$\Delta k \times 10^3$ (min^{-1}), first treatment (of erythrocytes)			
	diamide		*N*-ethylmaleimide	
None	4.34 ± 1.1 (4)		1.08 ± 0.3 (3)	
Dithioerythritol 5 mM, 45 min, 37°C pH 7.4	1.59	(2)	0.92	(1)
Phloretin 260 μM during efflux	1.56	(2)	0.61	(2)
Hexanol 25 mM	3.25	(2)	1.02	(1)

In order to check whether the leak in ghosts prepared from diamide-treated cells is the one already present prior to ghost preparation or rather a consequence of a faulty resealing, we measured the permeability of resealed ghosts prepared from cells treated with NEM, which does not produce leakiness in the native erythrocyte membrane [17]. According to Table IV, the flux rates in such ghosts are in fact enhanced, although to a lesser extent than in ghosts prepared from diamide-treated cells. Fluxes in such ghosts are also inhibited by phloretin (Table V). On the other hand, hexanol had no effect on leaks in ghosts prepared from cells pretreated with NEM or diamide, in contrast to leaks induced by diamide treatment of resealed ghosts (see above). Since the defect produced by NEM cannot be the relic of a leak induced during the NEM pretreatment, modification of SH groups by NEM must have caused an incomplete resealing.

In analogy, the leak in resealed ghosts prepared from diamide-treated cells is conceptually not necessarily identical with the original leak in the native cell but may – at least in part – stem from an impaired resealing due to SH group modification prior to lysis. It is interesting to note that such 'resealing defects' are quite similar in their properties to 'primary' leaks.

Membrane modification before reconstitution of the ghosts ('para'-procedure). In order to further characterize the relevance of SH groups in the membrane for ghost resealing, diamide and NEM were added to open ghosts during the 'loading period' at 0°C and low ionic strength. The reagents are thus present on both sides of the ghost membrane during the resealing process (45 min, 37°C) and only removed from the cells during subsequent washing in the cold.

For diamide (1.25 mM) the consequences of this 'para'-treatment are shown in Table VI. A marked leak is produced with a lesser size discrimination than the leak induced by the reagent in resealed ghosts ('post'-treatment, see Table II). The ghosts are, however, completely sealed to hemoglobin (data not shown). As expected, the effect is also more pronounced at alkaline pH (Table VI). Vesicles were not released from the ghosts during flux measurements as evident from microscopic examination. Leakiness induced by membrane modification with diamide by the 'para'-procedure shares a number of properties with leakiness induced by 'pre'-treatment of native cells [17]: (a) reversibility by dithioerythritol (Table VII), (b) considerable suppression by pretreatment of the erythrocytes with NEM (data not shown) and (c) inhibition by phloretin (Table VII). In contrast, alcohols do not stimulate this leak.

For reasons of comparison NEM was added in some experiments instead of diamide to open ghosts prior to resealing. This modification also induced leakiness in the resealed ghosts, though again to a much lesser extent than diamide (Table VII). Phloretin inhibited NEM-induced leak fluxes, while addition of alcohols had no effect, as in the case of diamide. Obviously alcohols do not generally stimulate leakiness in modi-

TABLE VI

Leak formation in resealed ghosts by diamide added before reconstitution ('para'-procedure)

Leaks quantified by the increase of flux rate coefficients ($\Delta k \times 10^3$, min^{-1}) of various test solutes on top of corresponding k_0 values ($k_0 \times 10^3$, min^{-1}) of untreated ghosts. Diamide was added before reconstitution of isotonicity of the ghost suspension (1.25 mM, pH 7.4 or 8.0) (for details see Methods). Efflux at 0°C. Number of experiments in brackets.

Test solute	$k_0 \times 10^3$ (min^{-1})	pH during diamide treatment			
		pH 8		pH 7.4	
		$\Delta k_0 \times 10^3$ (min^{-1})	retention (%)	$\Delta k \times 10^3$ (min^{-1})	retention (%)
Erythritol	1.16 ± 0.3 (12)	40.2 ± 3 (3)	69 (3)	13.3 ± 0.6 (3)	66 (3)
Sucrose	0.34 ± 0.1 (30)	8.0 ± 3.2 (3)	84 (3)	3.9 ± 1.3 (6)	100 (3)
Inulin	0.1 ± 0.02 (6)	1.0 ± 0.17 (3)	100 (3)	0.7 ± 0.13 (3)	100 (3)

TABLE VII

Characteristics of the leaks induced by diamide, N-ethylmaleimide and DIDS, when added before reconstitution ('para'-procedure)

Leaks quantified by the increase of the rate coefficients of sucrose flux ($\Delta k \times 10^3$, min^{-1}) over the ground flux ($k_0 = (0.34 \pm 0.1) \cdot 10^{-3}$ min^{-1} ($n = 30$)). Reversibility established by exposure to dithioerythritol (DTE) after the treatment with reagent. Phloretin and alcohols were present during the efflux period (for details see Methods and Results). Efflux at 0°C. Number of experiments in brackets. n.d., not determined.

Second treatment (of resealed ghosts)	$\Delta k \times 10^3$ (min^{-1}), first treatment (during ghost preparation)					
	diamide 1.25 mM, pH 7.4		*N*-ethylmaleimide 10 mM, pH 7.4		DIDS 0.07 mM, pH 7.4	
None	3.9 ± 1.3	(6)	1.35 ± 0.19	(5)	1.93 ± 0.45	(4)
DTE 10 mM 37°C						
45 min	1.64	(2)	1.2	(2)	n.d.	
75 min	n.d.		n.d.		0.75	(2)
90 min	0.57	(1)	n.d.		n.d.	
Phloretin						
200 μM	4.2	(2)	1.13	(1)	1.72 ± 0.2	(3)
600 μM	2.1	(2)	0.26	(2)	0.45	(2)
Hexanol						
25 mM	3.8	(2)	1.17	(1)	1.8	(1)
Butanol						
200 mM	3.5	(1)	n.d.		1.9	(1)

fied membranes. Only defects induced by modification of the resealed ghosts ('post') are sensitive to alcohols, while defects induced by treatment under 'pre' or 'para' conditions can not be stimulated.

Dependence of the leak-inducing effect of permeable sulfhydryl reagents (diamide, NEM) on the time regime of reagent addition

When ghosts are treated with diamide by the 'para'-procedure, membrane damage is much more pronounced than when the agent is added only after resealing ('post'-procedure) (compare Tables II and VI). This discrepancy could result from a change, during the resealing procedure, in the reactivity or the accessibility of the SH groups reacting with diamide and might provide a possibility to characterize the time course of resealing in terms of the diamide sensitivity of the membrane. Parameters, which promote resealing, comprise increase of temperature and of ionic strength. Both might be responsible for changes in the sensitivity of membrane proteins for diamide. To test this assumption diamide was added to ghost suspensions, after reconstitution of isotonicity, at various times after the onset of the resealing procedure (i.e. exposure of the cells to 37°C). The experiments were carried out at pH 7.4, where diamide has no detectable effect on the sucrose permeability of fully resealed ghosts (see Table III). As becomes evident from Table VIII, the effect of diamide on sucrose leak flux rapidly decreases with increasing latency between starting the resealing and addition of diamide. The $t_{1/2}$ of this decrease of sensitivity is lower than 30 s and thus much shorter than the $t_{1/2}$ of resealing itself (about 6 min at 37°C [6,7,31]). In the case of treatment with NEM, a similar loss of sensitivity during resealing can be observed.

Surprisingly, the extent of membrane SH group oxidation by diamide does not change with increasing latency between onset of resealing and time point of diamide addition. In all cases 1.25 mM diamide oxidizes 36%.

Ionic strength has been claimed to affect membrane resealing [3,4,6–8]. In order to study its effect on diamide sensitivity we added the reagent before resealing but after reconstitution of isotonicity by KCl. Under these conditions diamide induced a less pronounced damage of ghost membranes than under the

TABLE VIII

Changes in the effectivity of diamide as a function of the time regime of diamide addition

Leaks quantified by the increase of rate coefficients ($\Delta k \times 10^3$, min^{-1}) of sucrose efflux on top of the ground flux ($k_0 = (0.34 \pm 0.1) \cdot 10^{-3}$ min^{-1} ($n = 30$)). Diamide (1.25 mM, pH 7.4) was either added at 0°C 15 min before or 10 min after reconstitution of high ionic strength or at different time points after starting the resealing process by putting the suspension into a 37°C water-bath. Efflux at 0°C. Numbers of experiments in brackets.

Time point of diamide addition	$\Delta k \times 10^3$ (min^{-1})
Before reconstitution ('para', 37°C)	3.35 ± 0.6 (3)
After reconstitution (0°C)	1.1 ± 0.32 (3)
After a period of resealing of	
0.5 min (17°C)	0.58 (2)
2 min (32°C)	0.57 ± 0.2 (3)
4 min (37°C)	0.30 (3)
15 min (37°C)	0.15 (2)
45 min ('post', 37°C)	0.05 (2)

conditions of the usual 'para'-procedure, where the modifiers are added prior to reconstitution (Table VIII). Nevertheless, the induced leakiness was still greater than that induced by addition of diamide during or after complete resealing.

Earlier investigators [6,7,31] have proposed a causal relationship between the resealing of ghosts and a reassociation of skeletal protein elements. A reorganisation within the cytoskeletal meshwork might also be claimed.

The present results support such concepts by demonstrating incomplete resealing after membrane modifications known to affect SH groups in peripheral proteins with some preference [17,32]. The extent of this disturbance seems to depend on the state of the membrane, as a function of temperature and ionic strength, at the time of SH group modification. Since differences in the total number of modified SH groups do not seem to account for these differences, it has to be postulated that either different populations of SH groups react with the modifiers in dependence of temperature and ionic strength, or that structural alterations, induced by modification of the same SH groups, vary with temperature and ionic strength.

The conformation of spectrin is known to be influenced by these two parameters [33,34]. If one assumes that such conformational transitions are involved in resealing, the effects of SH group modification might be claimed to arise from a perturbation of these transitions, most strongly at low temperature and low ionic strength. Irrespective of the time point of SH modification (before, during or after ghost preparation) diamide-induced membrane defects can always be annealed at least partly by treating the final resealed ghosts with the disulfide reducing agent, DTE. The conformation of spectrin (or other skeletal protein targets for the effect of diamide) can thus always return to a normal or more nearly normal state.

Effect of DIDS on the barrier function of the membrane

The present study and many other data [35,36] clearly indicate the crucial involvement of SH groups in the barrier function of the erythrocyte membrane. Equally reliable evidence is not available for amino groups. In an attempt to deal with this problem we studied the effect of DIDS on the permeability of resealed ghosts. DIDS, well known as an inhibitor of the anion exchanger (band 3) [37,38], is a potentially bifunctional amino reagent. The covalent reaction involves the two isothiocyano groups, which exhibit high specifity for lysine residues (NH_2-groups), but can also react with cysteine residues (SH groups) [37,39].

As expected, DIDS induced no leakiness when added to resealed ghosts, since essentially only one exofacial amino-group of band 3 is modified under these conditions [38]. Effects of the agent on the cytoplasmic side of the membrane can only be studied by entrapment of DIDS ('para'), since it is impermeable. As shown in Fig. 5, DIDS in fact produces leaks in resealed ghosts and is about 6-times more effective than diamide. Modification by DIDS also induced a decrease of the retention of test solute to an extent which was never seen after modification with diamide (Table VI, Fig. 5). The DIDS-induced leak discriminates hydrophilic nonelectrolytes according to size (data not shown). In its further characteristics the DIDS induced leak flux is comparable to the leakiness induced by 'para'-treatment with diamide (Table VII).

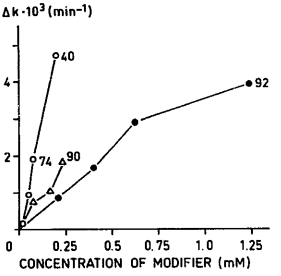

Fig. 5. Concentration dependence of the effect of diamide (●), SITS (▲) and DIDS (○) on the sucrose permeability of resealed ghosts. Modification at pH 7.4, 'para'-procedure (see Methods). The effect is defined as the increase of rate coefficients (Δk) on top of the control ($k_0 = (0.34 \pm 0.1) \cdot 10^{-3}$ min^{-1} ($n = 30$)). Numbers at curves refer to the retention (% of controls) of the test solute by the modified ghosts at different concentrations of the modifying reagents. Points without numbers refer to 100% retention. Efflux at 0°C. Fluxes and retention of sucrose measured as described in Methods. Mean values from 2–6 experiments.

In preliminary experiments it could also be shown, that the formation of leaks by DIDS can be suppressed by a short (2 min) preincubation of the erythrocytes with low levels of NEM (0.6 mM, for details see Ref. 18). This suppression by NEM, an agent rather specific for spectrin-SH groups under these conditions [32], and a partial reversal of the leak formation by the reducing agent DTE (Table VII) provide indications for a major involvement of SH groups in the enhancement of permeability by DIDS. Surprisingly, determination of SH groups revealed a decrease in SH groups by only 5% after 0.07 mM DIDS ($n = 2$) as compared to 28% after 0.4 mM diamide ($n = 2$). Only very few endofacial SH groups are thus modified by DIDS, although we added an excess of the reagent. These few groups seem to be essential for the resealing process. In contrast, the modification of amino groups by DIDS, which must

have occurred in parallel to SH modification [37,39], does not seem to have a great influence on the resealing process.

In order to study the relevance of the bifunctionality of DIDS for the formation of leakiness, we used the structurally homologous but monofunctional stilbene derivative, SITS. As shown in Fig. 5, the effect of SITS on the sucrose leak flux of ghosts is somewhat lesser than that of DIDS. Some selective crosslinking by DIDS may thus be involved in the effect, although we could not detect crosslinked proteins in gels from ghosts treated with DIDS under our experimental conditions (data not shown). In any case, effects of DIDS mediated by modification of SH groups may deserve general interest.

Carbodiimide protection of the membrane from diamide-induced damage

The carboxyl group modifying carbodiimide, EDC has recently been shown to induce extensive crosslinking of erythrocyte membrane proteins by formation of amide bonds [40]. As one consequence of this crosslinking the membrane is markedly rigidified and cell deformability decreased [40]. In order to investigate whether this crosslinking stabilizes the membrane against damage by diamide, we used EDC-treated erythrocytes for experiments.

Pretreatment of intact erythrocytes with low levels of EDC (5 mM, 45 min, 37°C, pH 7.4, medium B) produces only minor leakiness by itself. The rate coefficients ($k_0 \times 10^3$ (min^{-1}), 0°C) of sucrose increased from 0.34 ± 0.1 ($n = 30$) to 0.54 ± 0.1 ($n = 5$). Interestingly, EDC-pretreatment diminishes the enhancement of sucrose leak permeability ($\Delta k \times 10^3$ (min^{-1})) produced by diamide treatment during the resealing procedure ('para'-treatment) from 3.9 ± 1.3 ($n = 6$) to 0.56 ± 0.24 ($n = 4$). A similar prevention of diamide-induced leakiness has been shown in EDC-pretreated intact erythrocytes (Deuticke, B., unpublished results). Preliminary results indicate a similar suppression of leakiness for DIDS-treated cells.

This suppressive effect might be caused by a blockage of SH groups by EDC, which would prevent their reaction with diamide, in analogy to the effect of NEM [17]. This assumption could not be tested, since the EDC-crosslinked membrane can no longer be solubilized, which obviates reliable quantification of SH groups by DTNB.

On the other hand, one might postulate that crosslinking by EDC stabilizes the native conformation of skeletal proteins against modification by diamide.

Concluding remarks

The data presented in this study demonstrate that not only the barrier function of the native membrane itself but also its recovery after lysis depend on the state of membrane SH groups. According to our results two classes of leakiness induced by SH modification may occur in the erythrocyte membrane: (1) leaks produced by modifying the membrane in its 'tight' state (intact cells and resealed ghosts ('post'-procedure)) and (2) leaks persisting after resealing of ghosts already containing SH modified proteins ('pre'-, 'para'-procedure). These two classes differ in some properties but are similar in others. The major differences consist in the extent of leakiness, the size selectivity and in the sensitivity to alcohols, the major agreement in reversibility by reducing agents and inhibition by phloretin.

Leak formation in the native erythrocyte following diamide treatment has been assigned [17] to oxidation of skeletal proteins SH groups in view of its suppression by mild selective alkylation of SH groups in these proteins. Since a similar mild alkylation with NEM also suppresses the impairment of resealing by diamide ('para'-procedure, see above) modification of skeletal proteins is probably involved in this defect, too.

Altered interactions of membrane skeletal elements with themselves and with the anchor proteins (ankyrin, band 4.1) after modification of SH groups are well documented [41–45]. Perturbations in the association of skeletal elements with the bilayer domain are assumed to induce changes of mechanical properties of erythrocytes such as cell deformability and membrane stability [46,47]. A destabilisation of the lipid bilayer after SH group modification by diamide is indicated by shedding of membrane vesicles [48], a loss of phospholipid asymmetry [32], an enhancement of non-mediated transbilayer mobility of phospholipids [49] and an enhancement of the membrane permeability to hydrophobic ions which precedes leak formation (Deuticke, B., unpublished results).

A close correlation has been established in native erythrocytes between the loss of barrier properties and changes in the transbilayer mobility of phospholipids after SH modification, at least for the 'tight state' of the membrane [21]. A similar correlation between the patterns of effects of both, NEM and diamide, on membrane stability [46] and on membrane barrier function (this work) can presently not be established. Diamide, for instance, increases and NEM decreases membrane stability [46], while both impair resealing. It would thus appear, that different SH groups, possibly on different membrane skeletal proteins, control membrane ground permeability and membrane stability.

Our new data are in full agreement with our working hypothesis that leak formation following SH group modification results from an extensive modification of the sub-bilayer skeletal protein meshwork, which by secondary mechanism leads to dynamic fluctuating perturbations of the integrity of the bilayer domain (lipid plus integral proteins) [17,36,50].

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