Biochimica et Biophysica Acta, 775 (1984) 150–160
Elsevier

BBA 72191

ION SELECTIVITY OF AQUEOUS LEAKS INDUCED IN THE ERYTHROCYTE MEMBRANE BY CROSSLINKING OF MEMBRANE PROTEINS

B. DEUTICKE, P. LÜTKEMEIER and M. SISTEMICH

Abt. Physiologie, Medizinische Fakultät der Rheinisch-Westfälischen Technischen Hochschule, Pauwelstrasse, D-5100 Aachen (F.R.G.)

(Received February, 29th, 1984)

Key words: Ion channel; Ion selectivity; Protein crosslinking; SH group; (Erythrocyte membrane)

The aqueous leak induced in the human erythrocyte membrane by crosslinking of spectrin via disulfide bridges formed in the presence of diamide (Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210) was further characterized with respect to its ion selectivity by means of (a) measurements of cell volume changes or hemolysis, (b) determination of membrane potentials and (c) analysis of potential-driven ion fluxes. The leak turned out to be slightly cation-selective (P_K:$P_{Cl} \approx 4:1$). It discriminates mono- from divalent ions ($P_{Na}:P_{Mg} > 100:1$, $P_{Cl}:P_{SO_4} > 10:1$) and to a much lesser extent monovalent ions among each other. The selectivities for monovalent ions follow the sequence of free solution mobilities, increasing in the order $Li^+ \leqslant Na^+ < K^+ \leqslant Rb^+ < Cs^+$ and $F^- < Cl^- < Br^- < I^-$. Polyatomic anions also fit into that order. Quantitatively, the ratios of permeabilities of the leak are larger than those of the ion mobilities in free solution. The ion permeability of the leak is concentration-independent up to at least 150 mM. The ion milieu, however, has marked effects on leak permeability, most pronounced for chaotropic ions (guanidinium, nitrate, thiocyanate), which increase leak fluxes of charged and uncharged solutes. The results support the view that, besides geometric constraints, weak coulombic or dipolar interactions between penetrating ions and structural elements of the leak determine permselectivity.

Introduction

The barrier function of plasma membranes, which prevents unspecific leakage of nonelectrolytes and uncontrolled movements of ions between cells and their environment can be severed by exogenous agents forming transmembrane channels, e.g., toxins [1,2], ionophorous antibiotics [3–5], by endogenous peptides [6], but also by chemical modification of native membrane components [7–10]. In the case of the erythrocyte, such perturbations of the barrier function are easily detectable by the hemolysis ensuing from the formation of leaks for small ions or even larger constituents.

The lytic effect of many group-specific agents, in particular SH-reagents, has long been recognized [7–10]. The size, number and selectivity of the underlying leaks, however, was never characterized to any greater detail. We have recently reported such characteristics for the leak induced by diamide and tetrathionate. These mild oxidants of membrane SH-groups promote formation of disulfide bonds and intermolecular crosslinking of the membrane skeletal protein, spectrin [11]. The leaks exhibit the properties of aqueous pores with equivalent radii less than 0.65 nm, pervious to

Abbreviation: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene.

nonelectrolytes and ions such as Cl^- and K^+. They are reversible upon reduction of the induced disulfide bonds.

In order to characterize these experimentally induced leaks further, we have now studied their ion selectivity by several techniques.

Materials

Human blood from healthy donors was obtained from the local blood bank and stored no longer than 3 days. Standard chemicals and compounds used as test permeants were from Merck, Darmstadt; Fluka, Neu-Ulm; or Sigma, Munich. Diamide (diazenedicarboxylic acid bis(dimethylamide)) was from Calbiochem, Munich, or Sigma, Munich; valinomycin was from Sigma, FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) from Boehringer, Mannheim. DIDS (diisothiocyanostilbenedisulfonate) was a generous gift from Professor K.F. Schnell, University of Regensburg.

Labelled test permeants [*methyl*-^{14}C]choline [^{36}Cl]chloride, [U-^{14}C]erythritol, D-[1-^{14}C]mannitol were from Amersham-Buchler (Braunschweig).

Methods

Modification of SH-groups

Freshly obtained human erythrocytes were treated with SH-reagents as described in the first paper of this series [11]. All media used during the modifying treatment contained 44 mM sucrose or 30 mM poly(ethyleneglycol) 1500 (Serva, Heidelberg) to balance the colloid-osmotic pressure of intracellular impermeant constituents (hemoglobin, organic phosphates, glutathione).

Measurement of the time-course of cell volume changes or hemolysis

(A) Rationale. Cells, made leaky to ions, swell and hemolyse when suspended in isotonic solutions of salts capable to permeate the leaks. The uptake of salt and water is driven by the, now unbalanced, colloid-osmotic pressure of intracellular impermeable solutes. The time required for lysis is a function of the membrane's permeability to the salt, usually consisting of two ions. When one of the two ions is replaced by another one, while the counterion is kept constant, resulting differences in the time for hemolysis will indicate differences of the permeabilities of these two ions. The permeabilities can be characterized in relative terms by the inverse of the half-time of hemolysis.

In a modification of this approach, changes of cell volume can be followed by determinations of hematocrit after different times. This procedure has the advantage that cell shrinkage due to salt efflux can also be studied.

(B) Experimental procedure. Cells treated with diamide were washed three times in medium A (100 mM KCl/50 mM NaCl/12 mM Na_2HPO_4/NaH_2PO_4/44 mM sucrose), suspended in 12 vol. of an isotonic (300 mosmol/l) solution of the salt to be tested and incubated at 22–23°C under gentle shaking. After appropriate periods of time, 300 μl samples were collected and centrifuged (12000 × *g*, 30 s). The hemoglobin content in the supernatant, as a measure of lysis, was determined photometrically as cyanomethemoglobin.

When volume changes served as a measure of solute transport, cells pretreated with diamide (or other modifying agents) at pH 8.0 in medium A were washed and suspended in 2 vol. of an isotonic solution of the permeant under investigation, buffered by 2.5 mM phosphate. Immediately after mixing, and after appropriate incubation times at 37°C, hematocrit values were determined by 5 min centrifugation at 12000 × *g*.

Measurements of electrodiffusive fluxes

(A) Rationale. When colloid-osmotically protected cells with an ion-selective leak are suspended in a salt solution consisting of one ion also dominating on the inside of the cell (e.g., K^+ or Cl^-) and one 'foreign' ion with a permeability different from the permeability of its co-ion on the opposite side of the membrane, a bi-ionic potential may be set up [12]. Depending on its sign and magnitude, this potential difference will either enhance or retard the egress, by electrodiffusion, of a labelled anion (chloride) or cation (choline) from the cells via the leak. On a comparative scale, the rate coefficient of Cl^--flux may provide some indications for the relative permeability of the foreign ions.

(B) Experimental procedure. Cells were treated with diamide (for concentrations and exposure

times see Results), washed three times in medium A, loaded with $^{36}Cl^-$ in medium A at a hematocrit of 50% for 10 min at 25 °C and then exposed to DIDS (0.16 μmol/ml cells) at 37 °C for 30 min. Subsequently, cells were spun down (5 min, 6000 × *g*) and $^{36}Cl^-$-efflux initiated by suspending the packed cells into 20 vol of media containing, in addition to the salt to be studied, 6.5 mM phosphate buffer/44 mM sucrose/50 μmol/l DIDS (pH 7.4), 0 °C.

Rate coefficients of efflux at 0 °C were derived from a semilogarithmic plot of $(1 - {}^{36}Cl_t/{}^{36}Cl_\infty)$ vs. time, where $^{36}Cl_\infty$, the labelled chloride in the external medium after attainment of equilibrium was set equal to the ^{36}Cl content of the total suspension.

Measurements of the membrane potential

(A) Rationale. As shown by Macey et al. [13], changes of membrane potential in erythrocytes can be monitored by following changes of the extracellular H^+ activity (i.e., the pH) in cell suspensions in which H^+ distributes between cells and medium according to the transmembrane potential. Changes of pH_e are a reliable measure for changes of membrane potential under the chosen conditions, since H^+ is essentially not buffered in the extracellular phase while being strongly buffered intracellularly by hemoglobin, etc. Thus, changes of

$$V_m = \frac{RT}{F} \cdot \ln \frac{H_e^+}{H_i^+}$$

are fully reflected by changes of pH_e.

Attainment of H^+ equilibrium is facilitated by addition of a protonophore (e.g., FCCP). This allows changes of the transmembrane pH difference independent of the anion exchange system. For further details see Refs. 13 and 14. Moreover, blockage of the anion exchange system by DIDs prevented dynamic reequilibrations of OH^- and consequently of pH_e due to coupled OH/Cl-exchange processes governed by the kinetic properties of the anion exchange system and the concentration ratio of anions [14,15].

(B) Experimental procedure. Cells were treated with diamide (5 mM, 45 min, hematocrit 10%, pH 8.0, 37 °C) and washed twice with medium A. They were then resuspended in medium A and exposed to DIDS (0.16 μmol/ml cells, corresponding to 10^7 molecules per cell) for 30 min at 37 °C. After a further washing, samples of 0.1 ml packed cells were pipetted into 20 ml of iso-osmotic solutions containing KCl at different concentrations, sucrose to maintain isotonicity and 15 μmol/l DIDS. The pH and the voltage, as sensed by a combined glass electrode (METROHM EA 125) immersed into the suspensions, were registered on a recorder. 5 μl of a solution of 10 mg FCCP/2 ml ethanol were then added to give a final concentration of 5 μmol/l and the changes of pH and voltage readings followed at room temperature.

The stable voltage readings attained were plotted against the log of the KCl concentration, The linear relationship obtained could be used to calculate the ratio $P_K : P_{Cl}$ by the Goldman equation assuming that only these two anions contribute to the membrane potential. For testing and calibrating the system, normal erythrocytes were suspended in media of different KCl concentrations, and valinomycin (10^{-6} mol/l) added prior to FCCP.

Further techniques

K^+ efflux from diamide-treated cells into choline chloride media (pH 7.4) was followed at 0 °C by a K^+-sensitive combined glass electrode (INGOLD Type PH-401-90-88-NS-K7), coupled to a voltmeter and a recorder. The signals were converted into K^+ concentrations and first-order rate coefficients of K^+-efflux derived from semilogarithmic plots.

Tracer fluxes at transmembrane concentration equilibrium for the test permeants tested ([^{14}C]choline, [^{14}C]erythritol, [^{14}C]mannitol) were measured as described elsewhere [11].

Results and Interpretation

Human erythrocytes, loaded with labelled chloride and treated exhaustively with DIDS, release the tracer into phosphate-buffered NaCl/KCl media at a rate, at 0 °C, pH 7.4, of $3 \cdot 10^{-4}$ min^{-1}, equivalent to a permeability of $2 \cdot 10^{-10}$ cm · s^{-1}. The activation enthalpy of this DIDS-insensitive chloride flux amounts to 16.3 kcal/mol [11] resulting in permeabilities, at 25 and 37 °C, of $2.7 \cdot 10^{-9}$ and $8 \cdot 10^{-9}$ cm · s^{-1}, respectively.

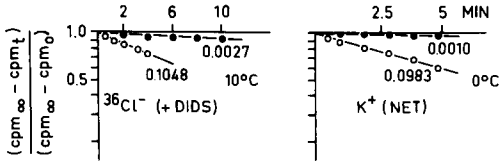

Fig. 1. Time-course of the efflux of ^{36}Cl and of K^+ from human erythrocytes treated with diamide (5 mM, 37 °C, 45 min, pH 8). ^{36}Cl fluxes measured at ion equilibrium, K^+ net efflux in isotonic choline chloride medium. For further details see Methods. Numbers at the regression lines are rate coefficients (min^{-1}) of efflux calculated for a two-compartment system. ●——●, controls; ○——○, diamide-treated cells.

A 45-min treatment of erythrocytes with diamide produces a marked enhancement of the efflux of labelled Cl^-, and also of K^+ as measured by an ion-sensitive electrode (Fig. 1). The characteristics of the induced permeabilities suggest the formation of an aqueous pore [11]. The almost linear concentration dependence of the flux shown in Fig. 2 for a nonelectrolyte and for Cl^- is in line with this assumption.

Ion selectivities of the leak established by volume changes and rates of the hemolysis

Properties of the induced leak can also be studied by more indirect techniques circumventing labelled permeants or specific analytical techniques. Erythrocytes pretreated with diamide under colloid-osmotic protection will swell or shrink

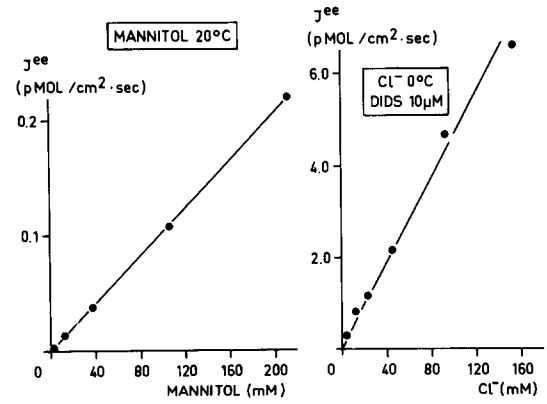

Fig. 2. Linear concentration dependence of ion and nonelectrolyte fluxes through the diamide-induced leak. Cells treated with diamide (5 mM, 90 min, pH 8, 37 °C). Cl^- fluxes measured in isotonic media composed of 0.15 mM NaCl and 0.1 mM sodium oxalate, respectively. For further details see Methods.

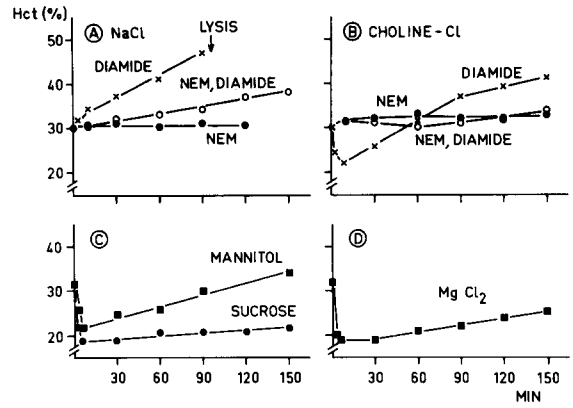

Fig. 3. Volume changes of diamide-treated cells in various isotonic media as indicators of solute net fluxes. Cells were treated, at a hematocrit of 10%, with diamide (5 mM, pH 8, 45 min, 37 °C) or with *N*-ethylmaleimide (NEM, 1 mM, 15 min, 37 °C, pH 8) and diamide. Subsequently, the cells were washed and resuspended in isotonic solutions of the solutes indicated, which contained 2.5 mM phosphate buffer (pH 7.4). For details see Methods.

when subsequently suspended in isotonic salt or nonelectrolyte media (Fig. 3). The leak responsible for the salt fluxes underlying these volume changes is most certainly the one described previously [11], since the volume changes can be suppressed by pretreatment of the cells with *N*-ethylmaleimide (Fig. 3). Immediate swelling of the leaky cells in NaCl medium indicates that Na^+ is at least as permeable through the induced leak as intracellular K^+. Choline$^+$ is obviously less permeable than K^+, since the cells shrink initially, due to a loss of K^+, Cl^- and H_2O, before beginning to swell secondary to the uptake of choline, Cl^- and H_2O. From the volume changes in Fig. 3C and D, it can further be concluded that mannitol permeates through the induced leak, while sucrose just seems to have a limiting size for permeation, as indicated previously by tracer fluxes [11]. The induced leak is also slightly permeable to a divalent cation, Mg^{2+}.

For monitoring higher permeabilities, measurements of colloid-osmotic hemolysis, induced by salt influx via induced leaks, were an adequate procedure. Time courses of lysis, in 150 mM NaCl, of diamide-treated cells are shown in Fig. 4. The inverse of the half-time of hemolysis is a measure of the rate of uptake of the external salt. These rates increased overproportionally with the time of

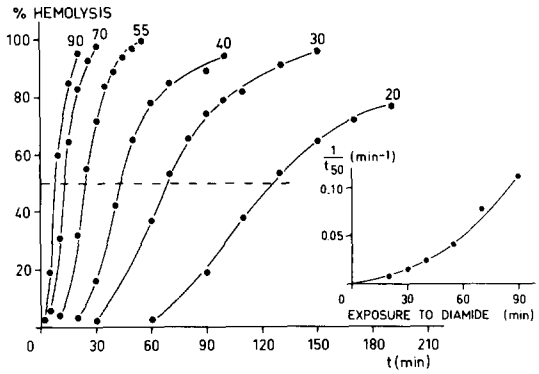

Fig. 4. Time-course of hemolysis, in isotonic NaCl solutions, of cells treated with diamide (5 mM, pH 8, 37 °C) for the time periods (min) noted at each lysis curve. Inset: Reciprocal half-times of hemolysis, equivalent to a rate coefficient of hemolysis, as a function of exposure time to diamide.

exposure to diamide (inset Fig. 4), a finding consistently observed with different external salts but at variance with the linear time-dependence of the diamide-induced increase of permeability as measured by tracer techniques [11].

Different ions can be compared with respect to their passage through the diamide-induced leaks by varying the cation of a salt while keeping the anion constant, or vice versa. Although the ratios between the reciprocal half-times may not reflect the true permeability ratios (see Discussion), the sequence of these numbers is likely to be the same as that of the leak permeabilities. Two characteristic sets of such curves are given in Fig. 5 for alkali chlorides and sodium halides. Marked differences between the anions become evident and lesser but still consistent ones between the cations.

Since the absolute time-course varied to some extent from experiment to experiment, the curves were normalized to those for lysis in NaCl in the same experiment. Relative rates of uptake obtained for a number of salts are compiled in Table I. Besides inorganic cations, only strongly basic organic cations with a quaternary nitrogen were used as probes. Analogous, less basic, nitrogen compounds with lower pK_s values (NH_4^+, mono-, di- and trialkylammonium chlorides) rapidly penetrate the erythrocyte membrane by nonionic diffusion as is well established for NH_4^+ [16] and was demonstrated in the present study for the other ions mentioned (data not shown). The organic anions included do not penetrate the membrane by nonionic diffusion to any extent relevant under our experimental conditions [17].

Considerable differences become obvious within the various families of ions, indicating that the induced leaks discriminate ions of the same charge. Monovalent cations up to the size of tetramethylammonium or arginine, and monovalent anions at least up to the size of isethionate penetrate across

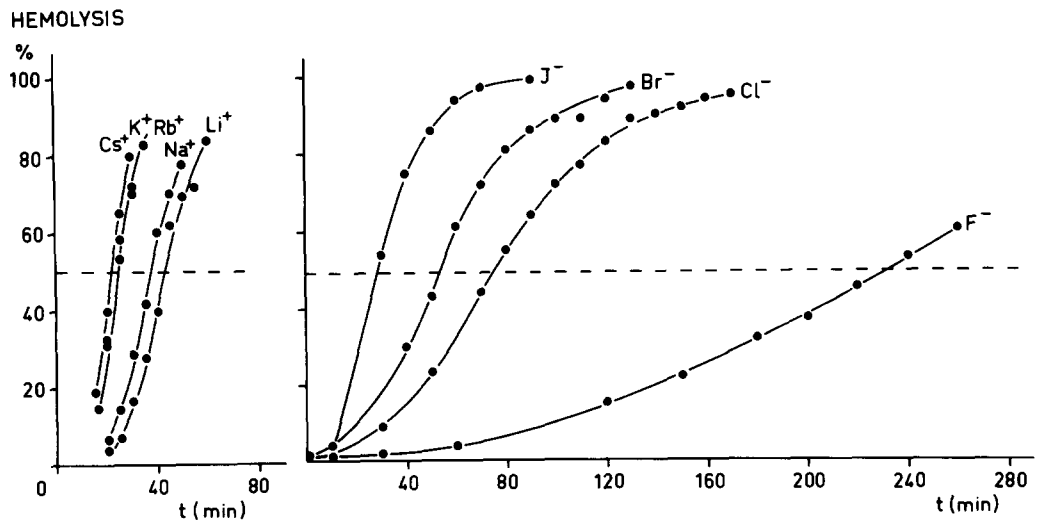

Fig. 5. Time-courses of hemolysis of diamide-treated erythrocytes (5 mM, pH 8, 37 °C, 45 min), suspended in isotonic solutions of alkali chlorides and sodium halides. Reciprocal half-times are a relative measure of the rate of entry of the respective electrolytes.

TABLE I

RELATIVE RATES OF LYSIS AS INDICATORS OF LEAK PERMEABILITIES FOR SALTS IN DIAMIDE-TREATED ERYTHROCYTES

Cells were pretreated with diamide (5 mM at pH 8, 37 °C, for 45 min) under protection by sucrose, against colloid-osmotic lysis. Subsequent rates of lysis were determined in salt solutions as described in Methods. Numbers below the ions indicate relative rates.

Inorganic cations									
Mg^{2+}	$\ll Na^+ = Li^+ <$	$K^+ = Rb^+ <$	Cs^+						
0.01	1.0 1.06	1.79	2.1						
Organic cations									
Arginine	$\ll$ choline, tetraethylammonium	$<$ tetramethylammonium	$< Na^+$						
0.01	0.38	0.47	1.0						
Inorganic anions									
SO_4^{2-}	$\ll F^- <$	$IO_3^- <$	$BrO_3^- <$	$Cl^- <$	$Br^- <$	$ClO_3^- <$	$NO_3^- <$	$I^- <$	SCN^-
< 0.1	0.45	0.6	0.8	1.0	1.5	1.6	2.0	2.5	4.1
Organic anions									
Oxalate	$<$ (glyoxylate, lactate, isethionate)	$<$ (glycolate, pyruvate, oxamate)	$< Cl^-$						
< 0.1	0.13–0.15	0.35–0.40	1.0						

the induced leak. The ratios of the uptake rates are essentially independent of their absolute values. Rates of uptake of halide and alkali ions increase in the order of their crystalline radii (Ref. 18, p. 70). Discrimination is more pronounced for anions than for cations. A problem arising in the interpretation of these data concerns possible ion effects on the diamide-induced leak pathway. This question will be addressed in a later section.

Ion selectivity indicated by the membrane potential

To establish anion/cation discrimination by the leak, estimates of the membrane potential were used. To measure membrane potentials in human erythrocytes, an approach involving the potential-controlled distribution of H^+ [13] has proven successful. The rationale behind this approach has been outlined in the Methods.

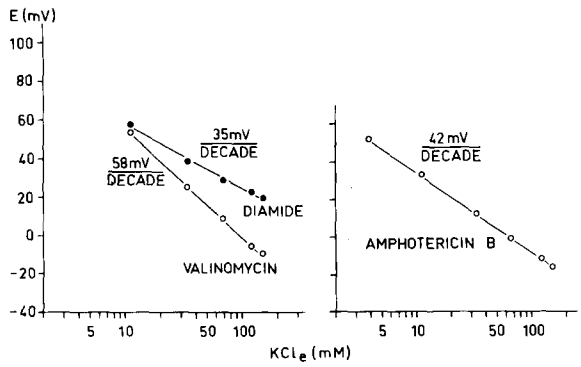

Fig. 6. Membrane potentials determined according to Refs. 13 and 14 by measurement of steady-state extracellular pH in unbuffered suspensions of erythrocytes treated with diamide (5 mM, pH 8, 37 °C, 45 min), valinomycin (1 μM) or amphotericin B (2.5 μM). Suspension media contained the KCl concentrations given on the abscissa; isotonicity was maintained by sucrose. For further details see Methods. The slopes obtained were converted into ratios P_K/P_{Cl} by the curve given in the inset.

In Fig. 6, voltages given by an H^+-sensitive glass electrode immersed into a valinomycin-containing red cell suspension are plotted for different extracellular KCl concentrations. The expected slope of -58 mV per 10-fold change of extracellular KCl indicates the validity of the approach, since an erythrocyte membrane doped with high concentrations of valinomycin behaves as a K^+ selective electrode [19–21].

Cells could be used for measuring such 'dilution potentials' when exposed to diamide for less than 45 min. Otherwise, stable voltage readings were not obtainable, presumably due to the equilibration of the KCl gradient. Within this time regime, the voltage readings were linearly related to the logarithm of the external KCl concentration (Fig. 6a), the slope being 35 mV/decade. The sign of the voltage is the same as for the valinomycin-treated cells, indicating K^+ to be more permeable than Cl^-. Interestingly, a very similar slope was obtained in erythrocytes exposed to the channel-forming polyene antibiotic amphotericin B (Fig. 6b).

The permeability ratios corresponding to these slopes were derived from a calibration curve obtained by solving the Goldman equation for the

intra- and extracellular concentrations of K^+ and Cl^- and variable values of the ratios P_K/P_{Cl}. The intracellular values of K^+ and Cl^- used for these calculations were taken from a study of Freedman et al. [22]. The slope of 35 mV/decade for diamide-treated cells corresponds to a permeability ratio P_K/P_{Cl} of about 4 : 1. Obviously, the induced leak discriminates between ions of opposite charge but equal size to a low but significant extent. In the case of amphotericin B, a ratio P_K/P_{Cl} of 6 : 1 was obtained.

Selectivities derived from ion-flux measurements

In a third approach used to characterize the selectivity of the diamide-induced pores, rate coefficients of the unidirectional electrodiffusive chloride efflux were measured under the modifying influence of bi-ionic potentials established by suspending the leaky cells in solutions of various salts. These salts usually consisted of one ion also present intracellularly and one foreign ion. Mediated anion fluxes were abolished by DIDS treatment. The extracellular presence of a 'foreign' cation less permeable than the intracellular K^+ should enhance $^{36}Cl^-$-efflux due to the hyperpolarization (outside positive) of the membrane potential. A more permeable cation should decrease $^{36}Cl^-$-efflux. Foreign anions should produce the reverse situation. Since the duration of the flux measurement (up to 6 min) was brief, relative to any salt net movement occurring in these situations, electrical driving forces for chloride efflux should be constant. The linear regressions obtained in the usual log/linear plot of the efflux kinetics (data not shown) indicate that this is indeed the case.

This qualitative approach is well-suited to demonstrate low permeability of an external cation relative to its intracellular co-ion, but not very useful when the permeabilities are fairly similar. Thus, it can clearly be shown (Table II), that the diamide-induced leak discriminates large monovalent organic cations and divalent cations from small monovalent inorganic cations (Na^+,K^+). Different K_{rel} values within each group indicate discrimination between the ions, e.g., $P_{Mg} > P_{Sr} > P_{Ba} = P_{Mn}$. Surprisingly, k_{rel} is enhanced in guanidinium chloride media and not diminished, as might be predicted from the greatly accelerated rate of hemolysis of diamide-treated cells in such media (Table I).

The influence of bi-ionic potentials on Cl^--fluxes through the diamide leak could also be demonstrated in experiments in which addition of gramicidin A ($2.5 \cdot 10^{-5}$ mol $\cdot$ l^{-1}) to suspensions of diamide-treated cells in $MgCl_2$ solutions further enhanced the rate of Cl^--efflux. This can be explained by increased hyperpolarization of the cells due to the selectivity of the gramicidin-induced channel for monovalent cations. In KCl medium, no enhancement of Cl^--efflux was observed.

In agreement with the very similar rates of

TABLE II

INFLUENCE OF EXTERNAL IONS ON RELATIVE RATES k_{rel} OF ^{35}Cl EFFLUX FROM DIAMIDE-TREATED CELLS

Cells were treated with diamide (5 mM, pH 8, 37 °C, 45 min) washed and loaded with ^{36}Cl. Tracer efflux was initiated by suspending the cells in isotonic media containing besides sucrose (44 mM), phosphate buffer (6.5 mM) and DIDS (10 μM), the chloride salts of the cations and the sodium salts of the anions studied. Cells were treated with DIDS (10^7 molecules/cell, 37 °C, 30 min), after ^{36}Cl-loading to block band 3-mediated anion transfer.

External cation	k_{rel} [a]	External cation	k_{rel} [b]	External cation	k_{rel} [b,*]	External anion	k_{rel} [b]
Li^+	0.90	Guanidinium	2.58	Mg^{2+}	1.96	F^-	0.91
Na^+	1.15	Tetraethylammonium	1.42	Sr^{2+}	2.05	Cl^-	1.0
K^+	1.00	Tetramethylammonium	1.52	Ba^{2+}	2.21	Br^-	1.16
Cs^+	1.15	Choline	1.54	Mn^{2+}	2.18	NO_3^-	1.36
		Arginine	2.45	Mg^{2+} + 25 μM gramicidin	3.16	SCN^-	4.5

* Hepes buffered.

[a] Normalized to KCl.

[b] Normalized to NaCl.

hemolysis of diamide-treated cells in solutions of the different alkali chlorides, the rates of efflux of chloride were essentially not affected by substituting these cations for one another.

Variation of the external anions also changed the chloride fluxes through the diamide-induced leak (Table II). Although in agreement with the relative rates of hemolysis in such media (Table I), these data are rather surprising, in view of the cation selectivity (over anions) of the induced leak established above. In a cation-selective membrane, oppositely directed anion gradients should only produce very low bi-ionic potentials [23]. This observation raised the possibility that some of the ions used in these studies might more directly affect the properties of the diamide-induced pathway. To explore this conjecture, Cl^- tracer fluxes were measured under conditions under which the ion to be tested was at equilibrium.

Influence of the ion milieu on the properties of the leak

Unidirectional Cl^- fluxes measured in cells equilibrated with the sodium salts of various anions after diamide treatment were strongly dependent on the anion milieu (Table III). This is most probably a direct effect on the properties of the leak. The fluxes of a labelled cation, [^{14}C]choline, and a nonelectrolyte, [^{14}C]erythritol, respond to changes of the anion milieu in a way similar to the fluxes of Cl^-. The sequence of increasing permeabilities corresponds to the sequence of increasing chaotropic action but also of lipophilicity of the anions. In the light of this finding, the increase of Cl^- fluxes in guanidinium chloride media might also result from a change of the properties of the leak. This turned out to be true (Table III). In light of these results, the true anion selectivity of the diamide-induced leak is probably smaller than indicated by the data derived from hemolysis studies.

TABLE III

INFLUENCE OF THE ION MILIEU ON THE LEAK PERMEABILITY INDUCED BY TREATMENT WITH DIAMIDE

Cells treated with diamide (5 mM, pH 8.0, 37 °C, for 45 min) were loaded with the labelled test permeant by incubation in the salt media under investigation, containing 6 mM phosphate buffer and 44 mM sucrose (or 30 mM poly(ethylene glycol) 1550) as protectant against colloid-osmotic lysis. Leak fluxes into the same medium were measured at 0 °C as described in Methods. Cl^- fluxes were measured in DIDS-treated cells, erythritol fluxes in cytochalasin B-treated cells. Leak permeabilities were normalized to those in NaCl media.

Salt medium	P_{Cl}	$P_{choline}$	$P_{erythritol}$
NaF	0.97	0.76	1.10
NaCl	1.0	1.0	1.0
$NaNO_3$	1.43	2.06	1.54
NaSCN	1.55	7.53	3.54
Guanidinium chloride	2.93	1.81	2.77

Discussion

This paper further characterizes the properties of an aqueous pathway induced in the human erythrocyte membrane by diamide. We have demonstrated previously [11] that upon exposure of erythrocytes to 10 mM diamide at 37 °C, the DIDS-insensitive Cl^- leak permeability, at 0 °C, increases from its native value of $2 \cdot 10^{-10}$ cm · s^{-1} * at a rate of $13.3 \cdot 10^{-10}$ cm · s^{-1} per min of diamide treatment. This increase proceeds linearly for at least 2 h. The apparent activation enthalpy of the induced leak flux (2.0 kcal/mol) is consistent with simple diffusion across an aqueous pore. This assumption is also substantiated by the linear concentration dependence of the leak flux (Fig. 2) or else, the independence of the induced permeability of the concentration of the permeant.

Simple diffusion across an aqueous channel should be sensitive to the transmembrane potential. This expectation is borne out by the finding (Table II) that the DIDS-insensitive Cl^- efflux is

* This native, DIDS-insensitive chloride permeability is likely to result from a conductive chloride diffusion pathway. The nature of this pathway is a matter of present debate [20,21,44]. Our permeability coefficient is lower than the number derived from the total chloride conductance by other authors [19–21]. This is, however, not unexpected, since the chloride conductance measured by the usual net KCl flow techniques [19,20] comprises a DIDS-sensitive component (about 70% of the total net permeability at 150 mM Cl^- [20,21]), which is suppressed under our experimental conditions. Measurements of the net flow of KCl in DIDS-treated cells [20,21] have provided the same value for P_{Cl} as our tracer flux measurements.

markedly enhanced by membrane hyperpolarization. The diamide-induced pathway discriminates ions on the basis of their size and charge, as could be shown by a number of approaches. This feature will be discussed in the following sections.

Selectivity among cations

Alkali chlorides pass the leak more rapidly in the sequence of increasing ionic radii of the cations, which is also the sequence of increasing free solution mobility or decreasing hydrated radii [18]. It is, thus, likely that the ions move through the diamide-induced pore in the hydrated state. A quantitative evaluation of the hemolysis studies in terms of the permeability of a single ion is not possible, since rates of hemolysis arise from rates of salt entry, which in turn depend on the permeability for both ions. Nevertheless, the hemolysis studies indicate a low selectivity among alkali cations. Divalent cations, on the other hand, are discriminated sharply from monovalent ones (Tables I and II) probably due to their higher charge. In contrast, monovalent organic cations are probably discriminated from alkali cations on the basis of their larger size. A large cation such as tetraethylammonium ($r_{cryst} = 0.38$ mm [24]) is still half as permeable as Na^+, in agreement with a pore radius greater than 0.5 nm [11], while arginine ($r_{max} < 0.65$ nm) is essentially impermeable, limiting the apparent pore radius to a value below 0.7 nm. The ratios between the relative permeabilities of the leak, as indicated by the relative rates of hemolysis, differ much more from each other than those between the relative diffusion coefficients of the same salts (Fig. 7). This indicates that in penetrating across the diamide-induced pores, the cations interact with membrane constituents. The interaction is, however, probably rather weak, since the cation sequence corresponds to sequence 1 (or 2) of Eisenmann [25]: $Li^+ \leqslant Na^+ < K^+ \leqslant Rb^+ < Cs^+$, which results from very low interaction energies between ions and their binding sites. Sequence 1 has been reported, to our knowledge, only for a few biologically relevant systems, notably the channels formed by gramicidin [26] and by porins [24]. Moreover, we have demonstrated (Deuticke, B., unpublished results) that alkali chlorides penetrate across the channel induced in the erythrocyte membrane by the polyene antibiotic amphotericin B in the order of sequence 1.

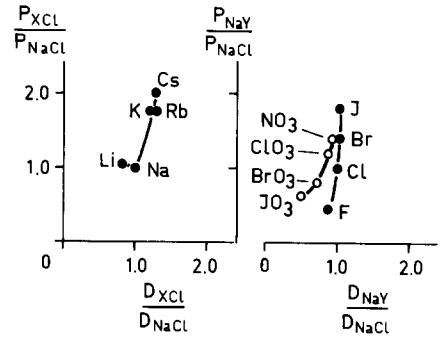

Fig. 7. Normalized permeabilities, P, of alkali chlorides and sodium halides in diamide-treated erythrocytes as a function of their normalized diffusion coefficients, D, in aqueous solution. D values were calculated from the limiting conductivities λ° (Ref. 18, p. 463) according to the equation (Ref. 18, p. 288):

$$D = RT/F^2 \cdot \left[\frac{(\nu_1 + \nu_2) \cdot (\lambda_1^\circ \cdot \lambda_2^\circ)}{\nu_1 Z_1 (\lambda_1^\circ + \lambda_2^\circ)} \right]$$

where ν_1 and ν_2 are the numbers of cations and anions formed from one 'molecule' of electrolyte and Z_1 is the cation valency.

Cation / anion discrimination

The discrimination of ions of different charge was characterized by evaluating dilution potentials for KCl in terms of the Goldman equation. A permeability ratio $P_K : P_{Cl}$ of about 4:1 was obtained for the diamide-treated erythrocyte. From this ratio and the chloride permeability measured with tracer, a P_K value, after 90 min treatment with diamide, of $6 \cdot 10^{-7}$ cm $\cdot$ s^{-1} (at 0 °C) can be calculated [11], to be compared with a native K^+ leak permeability of $5 \cdot 10^{-11}$ cm $\cdot$ s^{-1} [27]. A comparably low cation selectivity ($P_K : P_{Cl}$) has been reported for other channels, e.g., those formed by colicine K [28]; by porins [24] and by a toxin present in burned skin [29]. Amphotericin B also induces a cation-selective channel ($P_K : P_{Cl} \approx 7:1$) when inserted into lipid bilayers from one side, while forming anion-selective pores when inserted from both sides [5]. This difference is claimed to arise from 'half' and 'complete' pores (see Fig. 4 in Ref. 5). The cation-selective amphotericin B channels in erythrocytes, demonstrated in this work, indicate the polyene to form half pores in biomem-

branes presumably due to a lack of transbilayer mobility.

The low cation selectivity common to the different induced leak pathways excludes fixed coulombic charges within the pore as the mechanism underlying selectivity. Fixed charges near the entrance of the pore, or dipoles, could, however, account for a low selectivity.

Anion / anion discrimination and anion dependence of leak properties

A quantitative evaluation of the anion discrimination by the diamide-induced leak is complicated by the anion-induced changes of the leak properties (Table III). Fortunately, these effects are of a magnitude which does not affect the anion sequence.

The permeability sequence of the halides ($F^- < Cl^- < Br^- < I^-$) and of the oxy-anions (Table I) corresponds to sequence 1 of the Eisenmann series [30], also observed in case of the alkali cations. As outlined above, this sequence indicates weak interactions between penetrating ions and the pore-forming structures. The relevance of such interactions is indicated by the finding (Table III) that highly permeable anions increase the leak flux of ions otherwise less permeable. Sequences of anion permeabilities, conductive or mediated, in the native erythrocyte do not follow sequence 1 [22] with the exception of the conductance of the dog erythrocyte [34]. On the other hand, amphotericin B induces channels with sequence 1-type anion selectivity in erythrocytes (Deuticke, B., unpublished results). Artificial phospholipid membranes in the temperature range of their phase transition also discriminate halides according to this sequence [32].

Do other modifiers produce comparable leaks?

Cation permeable leaks, leading to hemolysis, have long been known to arise in erythrocytes treated with protein-modifying agents reacting covalently with SH- [7–10] or NH_2- [10,33–35] groups.

Protection against lysis by large solutes [36] was taken to indicate a defined size of the leaks. High rates of hemolysis indicate, furthermore, implicitly that the leaks are previous to anions such as chloride. The rates of native chloride net (conductive)m movements are too slow to allow for rapid salt fluxes [3].

It will be illustrative to compare the properties of these well-known but poorly characterized leaks with those of the leak induced by diamide. So far, results from a number of laboratories, including our own, indicate that *N*-ethylmaleimide induces leaks permeable to small ions (K^+ [7,8], Cl^-) but not to erythritol, although the agent blocks as many SH-groups in the erythrocyte membrane as diamide [37]. In contrast, Hg^{2+}, organic mercurials and fluorodinitrobenzene create leaks pervious to all types of small solutes [33,34,38,39]. Oxidants such as O_2^-, H_2O_2 or organic hydroperoxides are known to induce colloid-osmotic hemolysis [40–43]. In view of their pathologic relevance, the characteristics of the leaks underlying their action are presently under investigation.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160/C 3). The authors are indebted to Professor K.F. Schnell, University of Regensburg, for providing the DIDS, indispensable for these studies. We wish to thank Mrs. H. Thomas for secretarial help, and Mr. F.-J. Kaiser for preparing the figures.

References

1 Musso, G.F., Kaiser, E.T. and Kezdy, F.J. (1983) Biophys. J. 37, 329–338
2 Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. and Wellensiek, H.J. (1981). Cell Biol. 91, 83–94
3 Cass, A. and Dalmark, M. (1979). Acta Physiol. Scand. 107, 193–203
4 Deuticke, B., Kim, M. and Zöllner, C. (1973) Biochim. Biophys. Acta 318, 345–359
5 Marty, A. and Finkelstein, A. (1975). J. Gen. Physiol. 65, 515–526
6 Sims, P.J. and Lauf, P.K. (1980) J. Immunol. 125, 2617–2625
7 Jacob, H.S. and Jandl, J.H. (1962) J. Clin. Invest. 41, 779–792
8 Giebel, O. and Passow, H. (1961) Naturwissenschaften 23, 721–722
9 Sutherland, R.M., Rothstein, A. and Weed, R.I. (1966) J. Cell. Physiol. 69, 185–198
10 Knauf, P.A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190–210
11 Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210

12 Finkelstein, A. and Cass, A. (1968) J. Gen. Physiol. 52 (I, pt. 2), 145s
13 Macey, R.I., Adorante, J.S. and Orme, F.W. (1978) Biochim. Biophys. Acta 512, 284–295
14 Wieth, J.O. (1980) in Membrane Transport in Erythrocytes (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), Alfred Benzon Symposium 14, pp. 512–519, Munksgaard, Copenhagen
15 Knauf, P.A. (1979) Curr. Top. Membranes Transp. 12, 251–363
16 Jacobs, M.H. (1952) in Modern Trends in Physiology and Biochemistry (Barron, E.S.G., ed.), p. 149, Academic Press, New York
17 Deuticke, B. (1973) in Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds.), pp. 81–87, Georg Thieme Publishers, Stuttgart
18 Robinson, R.A. and Stokes, R.H. (1959) Electrolyte Solutions (2nd Edn.), Butterworth, London
19 Hunter, M.J. (1971) J. Physiol. (Lond.) 218, 49P–50P
20 Knauf, P.A., Law, F.-Y. and Marchant, P.J. (!973) J. Gen. Physiol. 81, 95–126
21 Fröhlich, O., Leibson, C. and Gunn, R.B. (1983) J. Gen Physiol. 81, 127–152
22 Freedman, J.C. and Hoffman, J.F. (197() J. Gen. Physiol. 74, 157–185
23 Lakshminarayanaiah, N. (1969) Transport Phenomena in Membranes, p. 168, Academic Press, New York
24 Benz, R., Janko, K. and Läuger, P. (!979) Biochim. Biophys. Acta 692, 52–60
25 Krasne, S. (1978) in Physiology of Membrane Disorders (Andreoli, T.E., Hoffman, J.F. and Fanestil, D.D., eds.), pp. 217–241, Plenum Medical, New York
26 Myers, V.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 313–322
27 Dalmark, M. (1974) J. Physiol. 250, 65–84
28 Schein, St. J., Kagan, B.L. and Finkelstein, A. (1978) Nature 276, 159–163
29 Schmidt, K. and Benz, R. (1982) Biochim. Biophys. Acta 692, 52–60
30 Wright, E.M. and Diamond, J.A. (1977) Physiol. Rev. 57, 109–156
31 Parker, J.C. (1983) Am. J. Physiol. 244, C313–C317
32 Block, M.C., Van der Neut-Kok, E.C.M., Van Deenen, L.L.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196
33 Poensgen, J. and Passow, H. (1971) J. Membrane Biol. 6, 210–232
34 Schwoch, G., Rudloff, V., Wood-Guth, I. and Passow, H. (1974) Biochim. Biophys. Acta 339, 126–138
35 Obaid, A.L., Rega, A.F. and Garrahan, P.J. (1972) J. Membrane Biol. 9, 385–401
36 Shapiro, B., Kollmann, G. and Martin, D. (1970) J. Cell. Physiol. 75, 281–292
37 Haest, C.W.M., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326
38 Castranova, V. and Miles, P.R. (1976) J. Gen. Physiol. 67, 563–578
39 La Celle, P. and Passow, H. (1971) J. Membrane Biol. 4, 270–283
40 Cohen, G. and Hochstein, P. (1964) Biochemistry 3, 895–900
41 Kellogg, E.W., III. and Fridovich, I. (1977) J. Biol. Chem. 252, 6721–6728
42 Maridonneau, I., Braquet, P. and Garay, R.P. (1983) J. Biol. Chem. 258, 3107–3113
43 Goldstein, B.D. and Harber, L.C. (1972) J. Clin. Invest. 51, 892–902
44 Kaplan, J.H., Pring, M. and Passow, H. (1983) FEBS Lett. 156, 175–179