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Variations of Intracellular pH in Human Erythrocytes via $K^+(Na^+)/H^+$ Exchange Under Low Ionic Strength Conditions

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Abstract. The change of intracellular pH of erythrocytes under different experimental conditions was investigated using the pH-sensitive fluorescent dye BCECF and correlated with (ouabain + bumetanide + EGTA)-insensitive K^+ efflux and Cl^- loss. When human erythrocytes were suspended in a physiological NaCl solution ($pH_o = 7.4$), the measured pH_i was 7.19 ± 0.04 and remained constant for 30 min. When erythrocytes were transferred into a low ionic strength (LIS) solution, an immediate alkalinization increased the pH_i to 7.70 ± 0.15, which was followed by a slower cell acidification. The alkalinization of cells in LIS media was ascribed to a band 3 mediated effect since a rapid loss of approximately 80% of intracellular Cl^- content was observed, which was sensitive to known anion transport inhibitors. In the case of cellular acidification, a comparison of the calculated H^+ influx with the measured unidirectional K^+ efflux at different extracellular ionic strengths showed a correlation with a nearly 1:1 stoichiometry. Both fluxes were enhanced by decreasing the ionic strength of the solution resulting in a H^+ influx and a K^+ efflux in LIS solution of 108.2 ± 20.4 mmol $(l_{cells}\ hr)^{-1}$ and 98.7 ± 19.3 mmol $(l_{cells}\ hr)^{-1}$, respectively. For bovine and porcine erythrocytes, in LIS media, H^+ influx and K^+ efflux were of comparable magnitude, but only about 10% of the fluxes observed in human erythrocytes under LIS conditions. Quinacrine, a known inhibitor of the mitochondrial $K^+(Na^+)/H^+$ exchanger, inhibited the K^+ efflux in LIS solution by about 80%. Our results provide evidence for the existence of a $K^+(Na^+)/H^+$ exchanger in the human erythrocyte membrane.

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Key words: Ion transport — Cotransport — Erythrocytes — Ionic strength — Intracellular pH

Introduction

Recently, a $K^+(Na^+)/H^+$ exchanger was postulated to exist in the human red blood cell membrane (Richter et al., 1997) accounting for a major fraction of the "ground state permeability" (Lew & Beaugé, 1979) of the membrane to monovalent cations. This ground state permeability (also referred to as "residual" or "leak" transport) persists when all known transport pathways are inactive or inhibited. In the presence of ouabain, bumetanide, and EGTA the Na^+/K^+ pump, the $Na^+/K^+/2Cl^-$ cotransport, and the Ca^{2+}-activated K^+-channel, respectively, are quiescent (*see* e.g., Bernhardt, Hall & Ellory, 1988). The volume-sensitive K^+/Cl^- cotransport is silent in mature human erythrocytes and can only be activated under specific conditions (Hall & Ellory, 1986). Thus, the residual transport of K^+ is relatively easy to measure. The study of residual Na^+ transport across the red blood cell membrane is more complicated because of the greater variety of specific transport systems for Na^+ compared to K^+ (Bernhardt et al., 1988).

Under certain conditions, including exposure to an extracellular solution of low ionic strength (LIS), the residual transport of K^+ and Na^+ ions (both unidirectional fluxes and net effluxes) are markedly enhanced in human erythrocytes (Denner, Heinrich & Bernhardt, 1993). On the basis of measured K^+ and Na^+ fluxes, as well as theoretical predictions, it is possible to ascribe this effect to a $K^+(Na^+)/H^+$ exchanger, taking into account the influence of the ionic strength on the outer surface potential according to the Gouy-Chapman-theory

(i.e., the ion concentration near the membrane surface) (Richter et al., 1997). The fact that the residual K^+ transport does not depend on the transmembrane potential (Bernhardt, Hall & Ellory, 1991), provides further support for the proposal that it is mediated by an electroneutral carrier mechanism.

The aim of the present investigations was to provide further experimental evidence for the existence of the $K^+(Na^+)/H^+$ exchanger in the human red blood cell membrane. Changes of intracellular pH of erythrocytes suspended in solutions of different ionic strength were measured using the pH-sensitive fluorescent dye 2,7-biscarboxyl-5(6)-carboxyfluorescein (BCECF). Our results provide a direct demonstration of changes of intracellular pH induced by low ionic strength media. The calculated H^+ fluxes have been compared to the K^+ fluxes measured under equivalent conditions to confirm a coupling of these fluxes. Human erythrocyte membranes contain a powerful anion exchanger (band 3), which determines the transmembrane distribution of anions and proton equivalents (OH^-), necessitating that this anion transporter was also taken into consideration.

Materials and Methods

Blood and Solutions

Stored bank blood (3 to 6-days-old) from healthy donors was used for the experiments. Blood from cows and pigs was supplied by the School of Veterinary Medicine (Free University, Berlin). The blood was washed three times by centrifugation (1500 × *g*, 8 min) at room temperature in the physiological NaCl solution containing (mM): 145 NaCl, 10 glucose, 10 morpholinoethanesulphonic acid/Tris(hydroxymethyl)aminomethane (MES/Tris), pH 7.4. Plasma and buffy coat were removed by aspiration. The cells were then washed once in the appropriate medium used for the experiment. Experiments were carried out in the physiological NaCl solution or in solutions where 145 mM NaCl was replaced by 106 mM Na-tartrate or 145 mM N-methyl-D-glucamine-chloride (NMDG-Cl). For measurements in LIS media cells were resuspended in a solution of the following composition (mM): 200 sucrose, 10 glucose, 10 MES/Tris, pH 7.4. The solution that is commonly used to replace 145 mM NaCl contains 250 mM sucrose to maintain the osmolarity. However, our solution contained only 200 mM sucrose to avoid an initial shrinkage of the cells. (Cell water content was measured by a wet weight/dry weight method to monitor cell volume changes. Cells were dried for three days at 70°C and for one day at 150°C. In one series of experiments 145 mM NaCl was gradually replaced with sucrose. In all experiments ouabain (0.1 mM), bumetanide (0.1 mM) and EGTA (0.1 mM) were present in the solution during the experiment. In experiments with the anion transport inhibitors 4,4′-diisothiocyanato-2,2′-stilbene-disulphonic acid (DIDS), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) and niflumic acid these substances were either already present in the solution or added after transfer of the cells into the solution. Other inhibitors (quinacrine, amiloride) were present in the solution before the cells were added.

Measurement of Intracellular pH (pH_i)

The fluorescent dye 2,7-biscarboxyl-5(6)-carboxyfluorescein (BCECF) was used to monitor intracellular pH (Tsien, 1989). Washed erythrocytes at a hematocrit of 1% were loaded at 37°C for 45 min by exposure to 10 μM of the cell-permeable acetoxymethyl ester of BCECF (BCECF-AM) in physiological NaCl solution. The cells were washed three times with the physiological NaCl solution (20 sec, 12.000 × *g*) and resuspended in the same medium (hematocrit 10%). pH measurements were initiated by the addition of small aliquots of this cell suspension to 4 ml of experimental solution in a well stirred fluorimeter cuvette.

The fluorescence of BCECF in the cells was measured with a SLM 8000 fluorimeter (SLM Instruments) at a hematocrit of 0.01% and at 37°C. The emission wavelength was set at 530 nm, cells and dye were alternately excited with 439 and 505 nm light. The ratio of the two fluorescence intensities (505/439) is a direct reflection of pH_i, that is independent of dye leakage, photobleaching and cellular volume changes (Tsien, 1989).

To convert the fluorescence ratio of BCECF-loaded erythrocytes into intracellular pH values, a calibration was carried out by equalizing pH_i and pH_o using the K^+/H^+ ionophore nigericin (5 μM) (Thomas et al., 1979). Cells were exposed to high K^+ solution (135 mM KCl, 10 mM NaCl, 10 mM glucose, 10 mM MES/Tris) and titrated to different pH_o (determined with a pH electrode). In all experiments the fluorescence at 439 nm did not change significantly during the time of measurements.

Measurement of K^+ Efflux

For measurements of unidirectional K^+ efflux the cells were loaded at least for 2 hr at a 50% hematocrit in the physiological NaCl solution in the presence of ^{86}Rb (0.5 MBq/ml). After this incubation at 37°C, cells were washed (10 sec, 12,000 × *g*) five times at 4°C immediately before the experiment with the flux solution. K^+ fluxes were started by adding the cells to the prewarmed flux medium at 5% hematocrit at 37°C. Aliquots were taken at several times, spun down through 0.5 ml $MgCl_2$ solution (106 mM) layered on 0.25 ml dibutylphthalate. The radioactivity of ^{86}Rb in the supernatant was determined by Cerenkov counting using a liquid scintillation analyzer (TRI-CARB 1600 TR, Packard). At the end of the experiment, an aliquot of the suspension was removed and cells lysed with 0.1 ml 50% (w/v) trichloroacetic acid in 0.5 ml $MgCl_2$ solution. After centrifugation the total radioactivity in the supernatant was determined. The rate constant was calculated as the negative slope of the linear regression line obtained by semilogarithmic plot of normalized counts against time. Taking into account the intracellular K^+ concentration of human and porcine erythrocytes on average of 90 mmol/l_{cells} (Marongiu, Holtmeier & von Klein-Wiesenberg, 1966) and 88 mmol/l_{cells} (Miseta et al., 1993), respectively, the efflux rate constants were used to calculate values of K^+ efflux (although such an approach excludes the small changes in intracellular K^+ concentration which will occur during the flux measuring period). The same procedure of calculating K^+ efflux was carried out with bovine erythrocytes. However, in this case the intracellular K^+ concentrations were measured by flame photometry giving values between 18 and 21 mmol/l_{cells}.

Measurement of Cl^- Efflux

The efflux of $^{36}Cl^-$ was only determined in LIS solution, e.g., in the absence of any extracellular Cl^-. For measurements of $^{36}Cl^-$ efflux, washed erythrocytes were loaded with $^{36}Cl^-$ (10 kBq/ml) for 15 min at 50% hematocrit and 37°C in the physiological NaCl solution. The procedure for the Cl^- efflux measurement was the same as for the K^+ efflux measurements, except that aliquots of the cell suspension were spun down through the flux solution layered on dibutylphthalate, and

Ultima Gold XR (Packard) was used for scintillation counting. In addition, if the effect of anion transport inhibitors on the Cl^- efflux was studied, the inhibitors were present both in the flux solution and during the washing procedure.

Reagents

Inorganic salts, sucrose and glucose were of analytical grade. NMDG-Cl, Na-tartrate, EGTA, DIDS, niflumic acid, quinacrine, and amiloride were obtained from Sigma (St. Louis, MO). DNDS was purchased from Pfalz and Bauer (Waterbury, CT), MES from SERVA GmbH (Heidelberg, Germany) and Tris from Fluka Chemic AG (Buchs, Switzerland). Na-methylsulfate was obtained from Merck (Darmstadt, Germany). ^{86}Rb (in RbCl) was produced by Amersham International (Amersham, U.K.) and ^{36}Cl (in NaCl) by Isocommerz (Berlin, Germany). BCECF-AM and BCECF (Molecular Probes, Leiden, The Netherlands) were dissolved in DMSO.

Statistical Treatment of Results

Experimental results represent mean values of at least four independent experiments carried out on blood of different donors or animals ± SD. Where errors are not shown they were smaller than the symbols. When necessary, a paired *t*-test was used to determine whether there is a significant difference between flux values obtained. The values were taken as significantly different when $P < 0.05$.

Results

Measurement of Intracellular pH

The intracellular pH of erythrocytes under different experimental conditions was determined with intracellular BCECF, and nigericin to equilibrate the intra- and extracellular pH. We confirmed that, in the presence of nigericin, the response of the dye is a linear function of pH_o (and hence also of pH_i) over the range investigated. Based on this calibration a pH_i of 7.19 ± 0.04 ($n = 8$) was obtained, when erythrocytes were suspended in a physiological NaCl solution ($pH_o = 7.4$). The measured intracellular pH which did not change significantly during a 30 min measuring period is in agreement with the pH_i value reported in the literature for comparable conditions (Dill, 1931; Fitzsimons & Sendroy, 1961; Funder & Wieth, 1966). In addition, pH electrode measurements of cell lysates confirmed the validity of the fluorescent calibration. Red blood cells suspended in physiological NaCl solution at a hematocrit of 1% were centrifuged and resuspended in deionized water. The pH of the lysate was 7.2 ± 0.1 ($n = 4$).

Our measurements were carried out at a very low hematocrit. To check whether the quenching of the fluorescence emission by hemoglobin or self quenching of the dye has to be taken into consideration, experiments were carried out at different hematocrits (0.001–0.1%). Although at higher red blood cell concentration a quenching of the fluorescence signal occurred, no significant change of the intracellular pH could be detected. A hematocrit of 0.01% was chosen for an optimal signal.

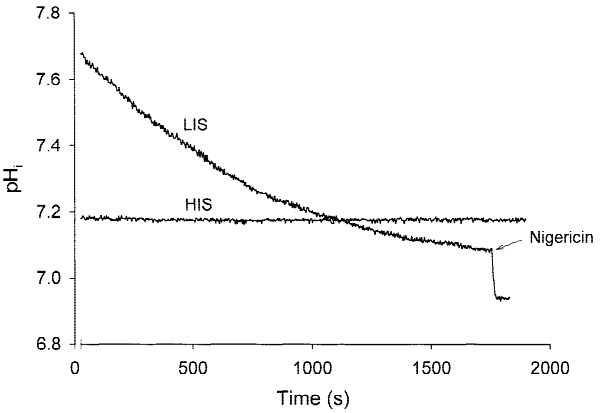

Fig. 1. Records of pH_i changes in human red blood cells in a physiological (*see* Materials and Methods, HIS) and low ionic strength (LIS) solution. Nigericin (10 μM) was added after 30 min inducing a K^+ and H^+ equilibrium. Results shown are one representative experiment of twelve.

The temperature sensitivity of the method was investigated over the range from 20° to 37°C. With increasing temperature a variation of the pH_i of the erythrocytes was determined to be −0.015 ± 0.010 ΔpH/°C, in agreement with data reported by Rosenthal (1948) and Dalmark (1975).

Effect of the Ionic Strength of the Extracellular Media on the Intracellular pH

If BCECF-loaded erythrocytes were transferred from a physiological NaCl solution into the LIS solution there was an immediate alkalinization of the intracellular medium to 7.70 ± 0.15 ($n = 16$) followed by a slower cell acidification. A representative record is shown in Fig. 1. Moreover, under these conditions the pH_i decreased after 30 min to values lower than the pH_i in the physiological NaCl solution.

Addition of nigericin during the 30 min measuring period resulted in a rapid reduction of the pH_i to a final value of 6.87 ± 0.07 ($n = 12$) (Fig. 1). This value of about 6.9 was also obtained after a time period of 40–50 min without nigericin (*not shown*). On the other hand, the addition of KCl (30 mM) to the LIS suspension after the pH_i already decreased (>20 min) led to an alkaline shift in pH_i (*not shown*) showing that the cell acidification induced by a reduction of the ionic strength of the solution is reversible.

For human erythrocytes the effect of lowering the ionic strength of the extracellular solution (gradual replacement of NaCl by sucrose) on H^+ influx over the range 145 to 0.3 mM NaCl is illustrated in Fig. 2. With

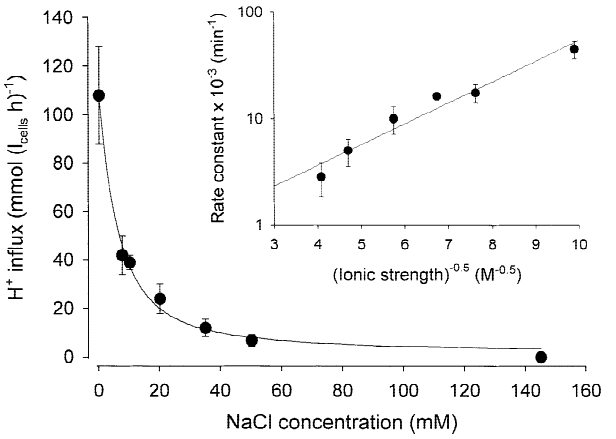

Fig. 2. The H^+ influx of human red blood cells as a function of the NaCl concentration of the extracellular solution (NaCl replaced by sucrose). H^+ fluxes were calculated from cell acidification taking into account the buffer capacity of hemoglobin (*cf.* Fig. 1). The insert shows a replot of the data, logarithm of the rate constant of H^+ influx against the inverse of the square root of the ionic strength of the extracellular solution. Results (mean ± SD) are pooled data from at least 4 independent experiments. Where not shown, the error bar is smaller than the symbol.

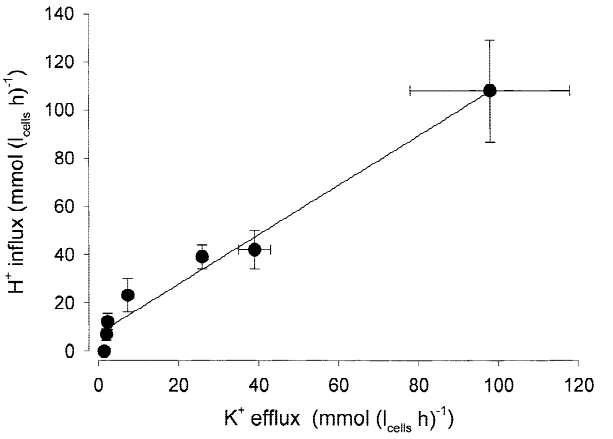

Fig. 3. Correlation of the H^+ influx and the K^+ efflux of human red blood cells obtained at different NaCl concentrations of the extracellular solution. NaCl concentration was reduced from 145 mM to 0.3 mM (sucrose replacement). Results (mean ± SD) are pooled data from at least 4 independent experiments. Where not shown, the error bar is smaller than the symbol.

decreasing NaCl concentration of the medium the H^+ flux into the cells increased. One has to take into consideration that the deviation of the H^+ flux values at very low NaCl concentration is relatively high since under such conditions small alterations of the salt concentration will lead to very significant changes of the flux. The proton fluxes (Fig. 2) have been calculated from the cell acidification over 10 min taking into account the buffer capacity of hemoglobin (10 mmol (mmol hemoglobin pH)$^{-1}$ that is constant over 2–3 pH units (Dalmark, 1975).

One important consideration is whether the pH_i measurements are influenced by the cell volume affecting the buffer capacity of hemoglobin. Usually, in LIS solution sucrose was present at a concentration of 200 mM since under this condition the cell volume measured immediately after transferring the cells into this solution was the same as in the physiological NaCl solution (relative cell volumes 1.03 ± 0.02 (NaCl) *vs.* 1.04 ± 0.04 (LIS), $n = 3$), although the time resolution of our measurement would not detect immediate and rapid fluctuations of volume. However, the cell volume was decreased after 30 min exposure of the cells to LIS solution. Therefore, for comparison, experiments were carried out with a reduced sucrose concentration (170 mM) in the LIS solution. Under this condition the cell volume was increased after transferring the cells but had the same volume after 30 min as cells in physiological NaCl solution (measured with dry weight analysis). No significant difference of the measured change of pH_i in the LIS solution and in the solution with reduced sucrose content was observed (*not shown*).

Correlation of the H^+ Influx with the K^+ Efflux

It is known from the literature (e.g., Davson, 1939; Donlon & Rothstein, 1969; Jones & Knauf, 1985) and our own investigations (Bernhardt et al., 1991; Denner et al., 1995; Richter et al., 1997) that in LIS solution there is a significant increase in the net (K^+ and Na^+) efflux. To compare the H^+ influx and K^+ efflux a correlation of both fluxes measured in solutions of varying ionic strengths is shown in Fig. 3. In fact we calculated the net H^+ influx from pH_i measurements and buffering power, and this flux was correlated with the net K^+ efflux. Under our experimental conditions, i.e., at zero extracellular K^+ concentration, there is no significant K^+ influx and thus only the K^+ efflux has to be considered. As one can see from Fig. 3 there is close to a 1:1 coupling of the H^+ influx and the K^+ efflux.

For the K^+ and Na^+ fluxes mediated by the postulated $K^+(Na^+)/H^+$ exchanger it was shown that the fluxes depend on the ion concentration near the membrane surface, i.e., near the ion binding site of the carrier. In support of such an effect, Richter et al. (1997) derived an equation combining the Gouy-Chapman theory, the Boltzmann distribution and the flux equation and plotted the logarithm of the rate constant of the K^+ fluxes against the inverse of the square root of the ionic strength of the solution. From the slope of the curves (straight lines), the ion permeability and the surface charge density of the erythrocytes were determined (*see* Richter et al., 1997). For comparison, the same plot for the rate constant of the H^+ fluxes is shown in the insert of Fig. 2. From the slope of the curve the surface charge density and the proton permeability of -0.024 C m^{-2} and 1.3×10^{-5} M · sec^{-1}, respectively, were determined for the human erythrocyte

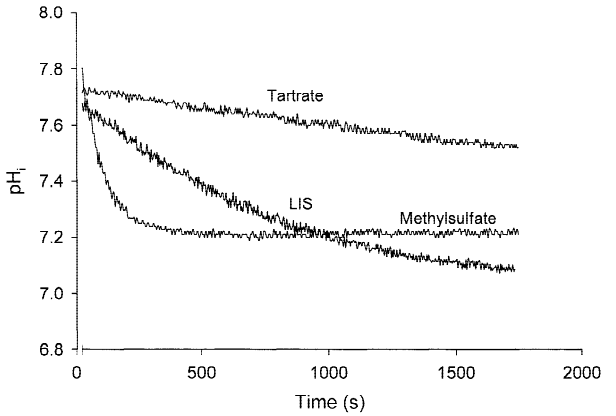

Fig. 4. Records of pH_i changes in human red blood cells in low ionic strength (LIS), tartrate, and methylsulfate solution. Results shown are one representative experiment of at least four.

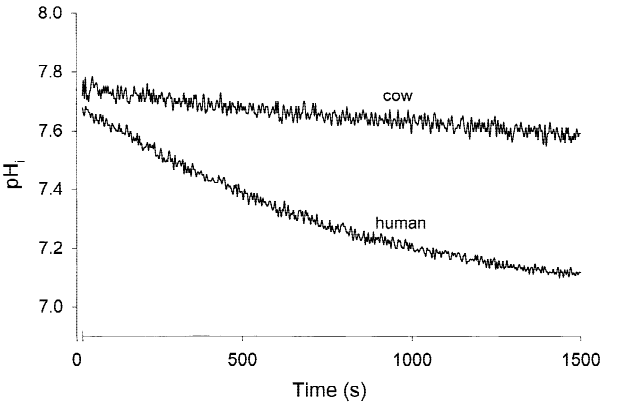

Fig. 5. Records of pH_i changes in human and bovine red blood cells in low ionic strength (LIS) solution. Results shown are one representative experiment of at least four.

membrane. The obtained value of the surface charge density in the present study is similar to that which we have previously calculated from K^+ fluxes (Richter et al., 1997) and is in agreement with biochemical data (−0.019 C m^{-2}, *see* e.g., Donath et al., 1996). Given the magnitude of the proton flux (*see above*) and the low concentration of H^+ relative to K^+ ions, the H^+ permeability must be far higher than that for K^+ if both fluxes are to be equal.

Effect of the Transmembrane Potential and the Composition of the Extracellular Solution

To decide whether the H^+ flux across the red blood cell membrane depends on transmembrane potential, experiments were carried out in a solution in which Cl^- was replaced by the impermeable anion tartrate, shifting the transmembrane potential to substantial positive values (*cf.* Lew & Bookchin, 1986). As one can see from Fig. 4 the replacement of Cl^- by tartrate led to nearly the same alkaline shift of pH_i (corresponding to the Cl^-/OH^- exchange) as under conditions where the cells were transferred into LIS medium. However, the subsequent acidification was much slower in tartrate compared to sucrose (LIS) medium, since the tartrate solution is 212 mM, i.e., high ionic strength. Since the transmembrane potential after the rapid alkalinization of the cells is similar in both LIS and tartrate solutions, it is evident that the observed H^+ influx was not due to the altered transmembrane potential.

If Cl^- is replaced by methylsulfate (a less permeable band 3 substrate than Cl^-, but still transported at a significant rate), a rapid initial pH increase occurred towards values observed for LIS and tartrate media, but followed by an immediate decrease of pH_i in the first 3 min of the experiment and consistent with methylsulfate/hydroxyl exchange re-establishing a pH_i of 7.2.

To investigate further the effect of the cation composition of the external solution on pH_i, NaCl was replaced by NMDG-Cl, $NMDG^+$ representing an impermeable cation. In NMDG-Cl containing medium, as in physiological NaCl solution, there is no significant change of pH_i in the time interval of 30 min (pH_i = 7.23 ± 0.09 (n = 8), *not shown*).

Comparison of Human Erythrocytes with Bovine and Porcine Erythrocytes

Since it has been shown previously that bovine and porcine erythrocytes do not exhibit the normal LIS effect (Erdmann et al., 1990), i.e., an enhanced K^+ efflux in sucrose media, pH_i measurements were carried out on cells from these species. As for human erythrocytes, in bovine and porcine erythrocytes a rapid alkalinization via band 3 was found if the cells were transferred from a physiological NaCl solution into a LIS solution. The mean pH_i value was 7.58 ± 0.14 (n = 7) and 7.63 ± 0.18 (n = 4) for bovine and porcine erythrocytes, respectively. As expected, in erythrocytes of both species the subsequent acidification in LIS media was found to be much less than in human erythrocytes. The H^+ influx in LIS solution was only 9.1 ± 1.5 mmol $(l_{cells}\ hr)^{-1}$ and 12.1 ± 2.2 mmol $(l_{cells}\ hr)^{-1}$ for bovine and porcine erythrocytes, respectively. For the calculation of these flux values it was assumed that the buffer capacity of bovine and porcine hemoglobin is the same as for human hemoglobin. One representative record of bovine erythrocytes is shown in Fig. 5. Similar records were obtained for porcine erythrocytes (*not shown*). The corresponding values for the K^+ effluxes of bovine and porcine erythrocytes in LIS solution were 3.9 ± 1.0 mmol $(l_{cells}\ hr)^{-1}$ (n = 4) and 9.5 ± 0.7 mmol $(l_{cells}\ hr)^{-1}$ (n = 4), respectively.

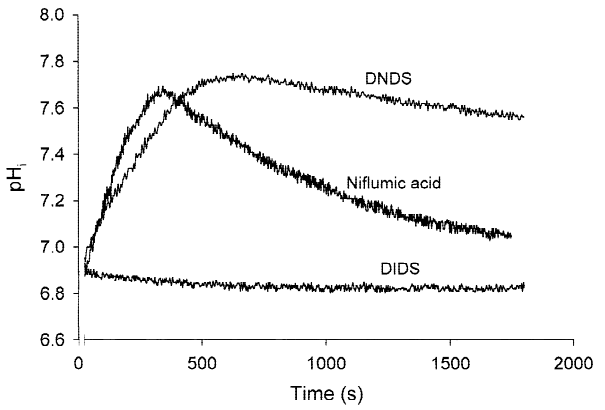

Fig. 6. Effect of anion transport inhibitors DIDS, DNDS, and niflumic acid on pH_i changes of human red blood cells in low ionic strength (LIS) media. The inhibitors (100 μM) were present in the solution before the cells were added. Results shown are one representative experiment of six.

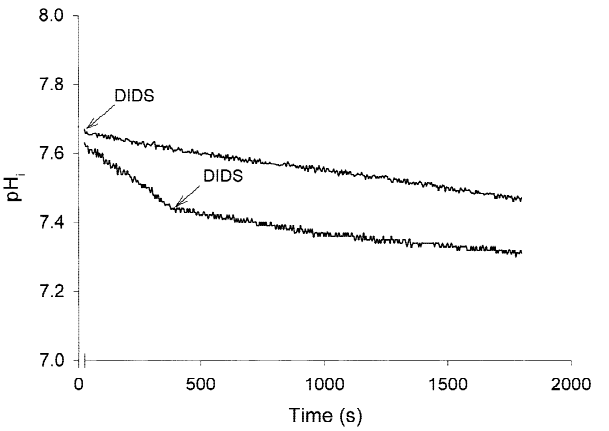

Fig. 7. Effect of DIDS (100 μM) on pH_i changes of human red blood cells in low ionic strength (LIS) solution. DIDS was added after the cells were transferred into the LIS medium (immediately after transfer — upper curve, 7 min after transfer — lower curve). Results shown are one representative experiment of four.

Effect of Anion Transport Inhibitors on pH_i

To determine whether the anion exchanger (band 3) is involved in the process of initial alkalinization in LIS media and the subsequent acidification (*see above*), pH_i changes were investigated in the presence of the anion transport inhibitors, DIDS, DNDS and niflumic acid. When the erythrocytes were added to the physiological NaCl solution containing one of these inhibitors no significant effect on pH_i was observed (*not shown*). If, however, the cells were transferred from the physiological NaCl solution into LIS solution containing the inhibitors, inhibitory effects on the time course of pH_i were found (Fig. 6). In the presence of DIDS the pH_i was 6.84 ± 0.05 ($n = 5$) and constant over 30 min. If DNDS was present in the LIS solution, one can see the alkalinization process which, however, was slower than in the absence of the inhibitors (*cf.* Figs. 1 and 6). The subsequent acidification of the cells in the solution containing DNDS was significantly smaller than in LIS solution without the inhibitor resulting in H^+ fluxes of 8.8 ± 0.9 mmol $(l_{cells}\ hr)^{-1}$ ($n = 5$) as compared to 108.2 ± 20.4 mmol $(l_{cells}\ hr)^{-1}$ ($n = 16$) in the absence of inhibitor. The same deceleration of the pH_i increase at the beginning of the time course was found when the experiments were carried out in the presence of niflumic acid (Fig. 6). However, the subsequent decrease of pH_i in the presence of niflumic acid is faster than in the presence of DNDS and comparable to the acidification in the absence of any anion transport inhibitor. The corresponding H^+ flux in the presence of niflumic acid was 90.4 ± 10.2 mmol $(l_{cells}\ hr)^{-1}$ ($n = 5$). In addition, the effect of the anion transport inhibitors on the process of alkalinization of the cells in tartrate-containing media was the same as in LIS media (*not shown*).

If, on the other hand, DIDS was added to the cell suspension after the erythrocytes were transferred to the low ionic strength media, resulting in an initial increase of pH_i, an inhibition of the acidification process was observed (Fig. 7). The H^+ flux under such conditions was 10.7 ± 1.7 mmol $(l_{cells}\ hr)^{-1}$ ($n = 5$). For DNDS and niflumic acid no significant difference in the pH_i decrease, i.e., the H^+ flux, was observed if the inhibitors were present either before or after transferring the cells to the LIS solution (*not shown*).

Effect of Anion Transport Inhibitors on K^+ Efflux

To demonstrate that the H^+ influx is coupled to the K^+ efflux of the cells, for comparison the inhibitory potency of different anion transport inhibitors on the K^+ efflux in LIS media was investigated. DIDS and DNDS reduced the K^+ efflux to about 8.1 ± 1.2 mmol $(l_{cells}\ hr)^{-1}$ ($n = 4$) and 7.7 ± 1.5 mmol $(l_{cells}\ hr)^{-1}$ ($n = 4$), respectively. In contrast, niflumic acid did not block the K^+ efflux significantly (Fig. 8). However, niflumic acid induced a delay of the K^+ efflux of the erythrocytes, comparable with the deceleration induced for the H^+ influx (*cf.* Figs. 6 and 8). As for H^+ flux measurements, the K^+ efflux was measured not only under conditions where the anion transport inhibitors were present in the flux medium before the erythrocytes were added but also under conditions, where the inhibitors were added after the cells were transferred into the LIS media. However, no significant difference of the K^+ efflux in either case was found (*not shown*).

Effect of Anion Transport Inhibitors on Cl^- Efflux

The question arises as to what extent the immediate pH_i increase in LIS solution is mediated by anion exchange.

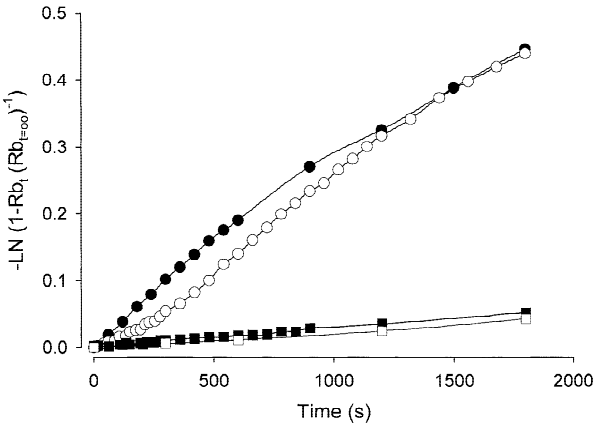

Fig. 8. Effect of anion transport inhibitors (100 μM) on the K^+ efflux of human red blood cells in low ionic strength (LIS) solution. Symbols represent: ●, control; ○, niflumic acid; ■, DIDS; □, DNDS. Results shown are one representative experiment of four.

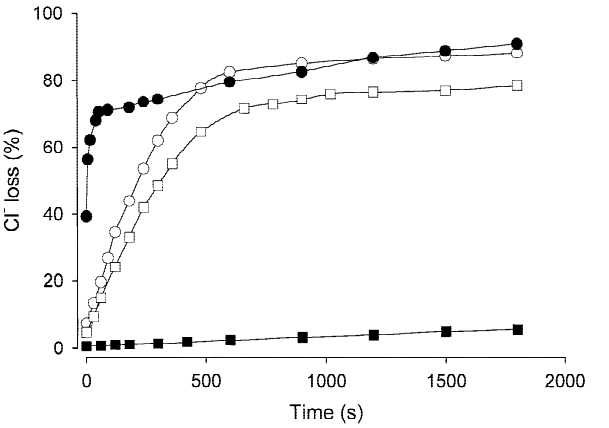

Fig. 9. Effect of anion transport inhibitors (100 μM) on Cl^- loss (in % of the internal chloride concentration) of human red blood cells in low ionic strength (LIS) solution. Symbols represent: ●, control; ○, niflumic acid; ■, DIDS; □, DNDS. Results shown are one representative experiment of four.

In addition, the possibility of a KCl loss, e.g., via the K^+/Cl^- cotransporter has to be ruled out. Therefore, we studied Cl^- effluxes and compared them with the H^+ and K^+ fluxes. A rapid Cl^- loss from the red blood cells in LIS solution in the absence of any extracellular chloride was observed. The intracellular Cl^- concentration was reduced to about 20% within seconds (Fig. 9).

The Cl^- efflux in LIS media was also investigated in the presence of anion transport inhibitors. Representative measurements of the effect of DIDS, DNDS and niflumic acid on the Cl^- flux are shown in Fig. 9. The most effective inhibition was found in the presence of DIDS, reducing the Cl^- flux to 7.2 ± 1.0 mmol $(l_{cells}\ hr)^{-1}$ ($n = 4$). In the presence of DNDS or niflumic acid a deceleration of the dramatic Cl^- loss occurred but the internal Cl^- concentration reached the same level as in the absence of inhibitors and was consistent with the delayed effect observed for the increase of pH_i induced by these substances. A comparable Cl^- efflux with that in LIS solution was also observed if the cells were suspended in tartrate media (*not shown*). These results show that the first process of cell alkalinization is due to band 3-mediated $Cl^-/OH^-(HCO_3^-)$ exchange.

Effect of Quinacrine and Amiloride on the K^+ Efflux

The effect of quinacrine, previously shown to inhibit the mitochondrial $K^+(Na^+)/H^+$ exchanger (Garlid et al., 1986) was investigated. In human erythrocytes quinacrine at a concentration of 1 mM inhibits the K^+ efflux in LIS solution effectively (Fig. 10). Because of fluorescence interference by quinacrine it was not possible to measure the H^+ flux via BCECF in the presence of this substance. Nevertheless, additional experiments using

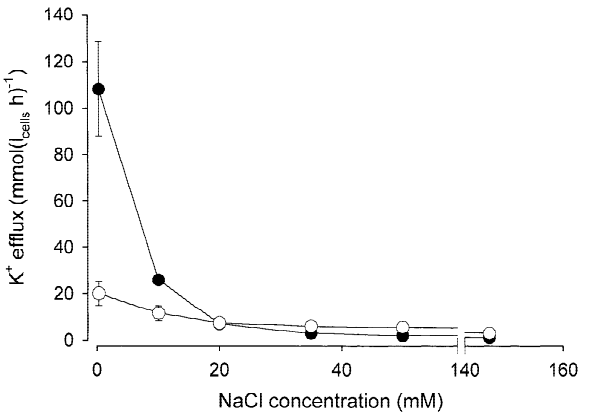

Fig. 10. Effect of quinacrine (1 mM) on the K^+ efflux of human red blood cells in solution of different NaCl concentrations of the extracellular solution (NaCl replaced by sucrose). Symbols represent: ●, control; ○, quinacrine. Results (mean ± SD) are pooled data from at least 4 independent experiments. Where not shown, the error bar is smaller than the symbol.

cell lysates (*see above*) showed that whereas the LIS-induced alkalinization persisted in quinacrine-containing solutions, the subsequent acidification was abolished. The pH immediately following suspension in LIS solution (hematocrit 1%) was 7.9 ± 0.1 ($n = 8$) and 7.9 ± 0.1 ($n = 4$) in the absence and presence of quinacrine, respectively. After 30 min incubation in LIS solution the pH was 7.4 ± 0.1 ($n = 8$) for untreated cells and 7.9 ± 0.1 ($n = 4$) for cells exposed to quinacrine.

Amiloride, an inhibitor of the Na^+/H^+ exchanger in many cell types (e.g., Escobales & Canessa, 1986) was also tested on K^+ efflux at a concentration of 1 mM. In physiological NaCl solution it was without a significant effect whereas in LIS solution it showed a small inhibi-

tory effect (inhibition of the K^+ efflux of about 25%, *not shown*). Although there is some interference of amiloride with BCECF fluorescence at 439 nm because it is used at a high concentration it was nevertheless possible to show that amiloride did not have a marked effect on the slow acidification in LIS media. However, neither quinacrine nor amiloride had an influence on the band 3 mediated Cl^- efflux in LIS media (*not shown*).

Discussion

The present study demonstrates that movement of protons or proton equivalents across the red blood cell membrane can be followed as an intracellular alkalinization or acidification using the pH fluoroprobe BCECF. After transfer of the erythrocytes into LIS solution, a rapid alkalinization of the cells is followed by a slower process of acidification (Fig. 1). These two events are affected by two distinct transport pathways. The pH_i increase within seconds is mediated by the anion exchanger band 3 and the second process of pH_i decrease is due to a cation transport pathway exchanging monovalent cations and protons. Similar effects on the extracellular pH (pH_o) have already been demonstrated by Funder & Wieth (1966). When erythrocytes were suspended in an unbuffered sucrose solution a continuous increase of the extracellular pH over 30 min was observed after an initial drop of pH_o. Furthermore, the addition of nigericin to a buffered 300 mM sucrose solution containing 10 mM NaCl led to a rapid K^+/H^+ exchange in human erythrocytes (Harris & Pressman, 1967). These results are confirmed in the present study, where the addition of nigericin to the cells in LIS solution resulted in their immediate acidification (Fig. 1).

We have demonstrated that for human erythrocytes suspended in LIS media or in solutions in which NaCl was replaced by Na-tartrate or Na-methylsulfate (Fig. 4), a rapid increase of pH_i and a loss of Cl^- from the cells occurred within seconds. That this effect occurs via the anion transport system (band 3) is supported by the following findings in LIS solutions: (i) an efflux of about 60 mM Cl^- occurred immediately, accompanied by an increase of pH_i of 0.51 units (Figs. 1 and 9) an observation consistent with 1:1 Cl^-/OH^- exchange when the buffer capacity of hemoglobin is taken into account; (ii) the Cl^- loss as well as the pH_i increase was strongly inhibited by DIDS and showed the same delayed time course as that observed in the presence of the anion transport inhibitors DNDS or niflumic acid (Figs. 6 and 9). For Na-methylsulfate media, the possibility that the relatively fast pH_i decrease (Fig. 4) is due to methylsulfate transport via band 3 which is nevertheless slower than that for Cl^- must be considered. This assumption is also supported by the fact that the Cl^- efflux in Na-methylsulfate solution is as fast as in LIS solution and that the K^+ efflux in Na-methylsulfate solution is not significantly different from the flux in physiological NaCl solution (*not shown*). In addition, in the seconds immediately following suspension in LIS solution the rapid loss of Cl^- is not accompanied by a significant loss of K^+ from the cells (Figs. 8 and 9).

The rapid loss of a significant amount of intracellular Cl^- from the cells is in agreement with the model of Lew & Bookchin (1986) and the results of Bookchin et al. (1984) and Gimsa et al. (1994). In the latter study, the authors measured a significant increase of the conductivity of a low ionic strength extracellular solution after cell suspension. This change was accompanied by a significant decrease of cell volume and of the intracellular conductivity measured respectively using a Coulter counter and by dielectric single cell spectroscopy.

The main focus in this study was the process of cell acidification after the initial pH_i increase. The movement of H^+ (or H^+ equivalents) was found to increase with decreasing ionic strength of the solution (Fig. 2). K^+ efflux and influx have been shown to be similarly affected when ionic strength is reduced (e.g., Davson, 1939; Donlon & Rothstein, 1969; Jones & Knauf, 1985; Bernhardt et al., 1991; Denner et al., 1993; Richter et al., 1997). The correlation of the calculated H^+ influx and the K^+ efflux (Fig. 3) showed a 1:1 stoichiometry. Therefore, we conclude that protons are exchanged for cations under LIS conditions and that H^+ and K^+ movements are mediated by the $K^+(Na^+)/H^+$ antiporter as suggested by Richter et al. (1997) (other possibilities are considered below). Under physiological conditions the (K^+ + Na^+) efflux nearly equals the (K^+ + Na^+) influx (Richter et al., 1997) such that no net movement of H^+ ions occurs (Fig. 1). The Na^+ efflux separate from that for K^+ has not been investigated in the present study, since the Na^+ concentration in human erythrocytes is small compared to the K^+ concentration.

If the K^+ efflux and the H^+ influx are mediated by the same transport pathway, i.e., the hypothetical $K^+(Na^+)/H^+$ exchanger, our earlier results (Richter et al., 1997) suggest that both fluxes should exhibit a similar dependence on the external surface potential of the cells. A comparison of the data reported in Fig. 2 with these earlier results shows that this is indeed the case. Interestingly, the apparent permeability for protons is in the same range as the OH^- net permeability measured by Knauf et al. (1977). This OH^- permeability is, however, much greater than the net permeabilities for Cl^- and for HCO_3^-.

The K^+ efflux as well as the H^+ influx was not dependent on the transmembrane potential. The replacement of Cl^- by the impermeable anion tartrate, which will result in an alkaline shift of pH_i and thereby establish the same positive transmembrane potential as arises

for cells suspended in LIS media, led only to a slight decrease of pH_i over 30 min (Fig. 4).

Further evidence for the existence of the $K^+(Na^+)/H^+$ exchanger in the human red blood cell membranes is provided by the effect of the three classical anion transport inhibitors (DIDS, DNDS, niflumic acid) on the K^+ efflux and H^+ influx. From Figs. 7 and 8 it is apparent that DIDS and DNDS are effective inhibitors of the acidification phase as well as the band 3-mediated alkalinization. Interestingly, niflumic acid had little effect on the pH recovery. Comparable inhibition of the K^+ efflux by these substances was shown (*see also* Jones & Knauf (1985) for the DIDS effect).

It has been previously reported that bovine and porcine erythrocytes do not exhibit an enhanced K^+ efflux in low ionic strength media (Erdmann et al., 1990). In fact, in our LIS solution (with a reduced ionic strength as compared to Erdmann et al. (1990)) there was only a small increase of the K^+ efflux from the erythrocytes of these species which correlated with the 10-fold reduction recorded for the H^+ influx.

Alternatives to a $K^+(Na^+)/H^+$ exchanger for explaining the described effects include: (i) a separate channel for K^+ and net $H^+(OH^-)$ transport via band 3; (ii) K^+/OH^- symport. The first possibility is supported by the model of Lew and Bookchin (1986) assuming that the membrane permeability for K^+ is increased under LIS conditions. Such a raised permeability will inevitably be inferred from flux measurements if only electrodiffusive processes are considered. However, should electrodiffusion underlie the LIS response for K^+, efflux of this ion would be predicted to be dependent on transmembrane potential. The results in the present study and from previous reports (e.g., Bernhardt et al., 1991) suggest that LIS-induced fluxes do not show such sensitivity, and support the proposal that an electroneutral $K^+(Na^+)/H^+$ exchanger is more likely. For such a mechanism, it would be unnecessary to invoke a change in membrane permeability to explain the LIS effect. Second, although a K^+/OH^- symport cannot be ruled out, a K^+/H^+ exchange is again more likely. This is based on the apparent dependence of the K^+, Na^+, and H^+ (or OH^-) fluxes on surface potential which our calculations predict (Richter et al., 1997, this paper). The surface potential of the erythrocyte membrane is more negative under LIS conditions than under normal conditions (e.g., Bernhardt, 1994). Therefore, in LIS media an increase of the cation concentration and a decrease of the anion concentration near the cell surface will arise. This would further lower the transport rate of a cation/anion symport under LIS conditions (consistent with the decrease of $K^+/Na^+/2Cl^-$ cotransport in LIS solution, which we have observed; *unpublished results*).

Jones & Knauf (1985) have suggested that the low ionic strength induced cation transport is mediated by band 3, acting as a low-conductivity channel for cations under these conditions. Although the possibility of a K^+ transport via band 3 cannot be entirely excluded, our results favor a $K^+(Na^+)/H^+$ exchanger separate from the band 3 protein. One reason to exclude a band 3 mediated process has been already indicated, namely the influence of surface potential. Second, the anion transport inhibitor niflumic acid, although able to retard LIS-induced alkalinization, has no effect upon the K^+ efflux and H^+ influx (Figs. 6 and 8). However, comparison of the effects of the various anion transport inhibitors, suggests that DIDS and DNDS directly inhibit the $K^+(Na^+)/H^+$ exchanger. Such an effect is not surprising since it is known that DIDS affects the K^+/H^+ exchanger proposed to operate in *Amphiuma* and trout red cells (Adorante & Cala, 1987; Fievet et al., 1993). Third, quinacrine, a known inhibitor of the $K^+(Na^+)/H^+$ exchanger in the mitochondrial membrane, inhibits the K^+ efflux and the H^+ influx in LIS solution but has no effect on the anion transport. Fourth, bovine and porcine red cells have normal band 3 activity, but no measurable fluxes equivalent to the proposed $K^+(Na^+)/H^+$ exchange in human red cells.

In conclusion, the present paper uses fluorimetric techniques to support the idea of the operation of a low ionic strength induced activation of a $K^+(Na^+)/H^+$ exchanger present in the human erythrocyte membrane. This $K^+(Na^+)/H^+$ exchanger operates after a fast initial change of pH_i in low ionic strength solutions mediated by the anion transporter (band 3).

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