Characterization of the $K^+(Na^+)/H^+$ Monovalent Cation Exchanger in the Human Red Blood Cell Membrane: Effects of Transport Inhibitors

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Abstract. The (ouabain + bumetanide + EGTA)-insensitive K^+ influx (defined as residual K^+ influx) in the human erythrocyte was investigated with respect to the characterization of the recently identified $K^+(Na^+)/H^+$ exchanger (Richter et al. 1997). In particular, the effects of selected ion transport inhibitors on this flux in physiological ionic strength (high ionic strength, HIS) as well as low ionic strength (LIS) solutions were qstudied. The stimulation of the K^+ influx observed in LIS medium was further enhanced when DIDS, phloretin, eosin-5-maleimide, furosemide, DIOA, NPPB, or DCDPC was present at a concentration of 0.1 mmol/l. This paradoxical, inhibitor-induced increase of the K^+ influx was more pronounced in LIS media where chloride (7.5 mmol/l) was replaced by nitrate. For DNDS, niflumic acid, and MK-196 (0.1 mmol/l) an enhanced K^+ transport could only be observed in nitrate-containing LIS solution.

Bumetanide and purine riboside, at a concentration of 0.1 mmol/l, did not cause significant changes of the K^+ influx in either chloride- or nitrate-containing LIS media. Dipyridamole and ruthenium red (0.1 mmol/l), which are positively charged, significantly reduced the K^+ influx in both chloride- and nitrate-containing LIS media. In nitrate-containing HIS solution only dipyridamole inhibited the K^+ influx.

The residual K^+ influx in LIS solution was significantly increased by removing internal $[Mg^{2+}]$, and decreased by quinacrine (1 mmol/l). In HIS solution, no effect of altering intracellular Mg^{2+} occurred but a stimulation of the flux by quinacrine

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was observed The results are discussed in terms of a more general surface charge effect of the used inhibitors on the $K^+(Na^+)/H^+$ exchanger

Key words: $K^+(Na^+)/H^+$ exchange — Residual transport — Red blood cells — Low ionic strength — Surface charge

Introduction

Recently, it was demonstrated that a major fraction of the "ground state permeability" of the red blood cell membrane to monovalent cations, which remains when all known transport pathways (Na^+/K^+ pump, various carriers, Ca^{2+}-activated K^+-channel (see e g Bernhardt et al 1988)) have been inhibited with appropriate specific inhibitors can be explained by a $K^+(Na^+)/H^+$ exchanger, rather than an electrodiffusive "leak" pathway (Richter et al 1997) Under low ionic strength (LIS) conditions (when NaCl is replaced by sucrose to maintain tonicity) residual K^+ transport (i e both K^+ influx and K^+ efflux) is markedly stimulated in human erythrocytes (e g Donlon and Rothstein 1969, Bernhardt et al 1991) It was possible to explain this effect on the basis of fluxes mediated by the $K^+(Na^+)/H^+$ exchanger, taking into account the influence of the ionic strength on the outer surface potential according to Gouy-Chapman-theory (i e the ion concentration near the membrane surface) (Richter et al 1997) The demonstration of this novel $K^+(Na^+)/H^+$ exchanger in the human red blood cell membrane, raises the important question of its pharmacological profile

Although many inhibitors, particularly of Cl^--dependent membrane transporters are known to be promiscuous (Cabantchik and Greger 1992), it is important to examine the effects of the classical inhibitors (e g 4,4′-diisothiocyanato-2,2′-stilbene disulfonic acid (DIDS), furosemide, 5-nitro-2-(phenylpropylamino)benzoic acid (NPPB)) on this transport process Furthermore, other inhibitors which have been shown to be effective on the mitochondrial $K^+(Na^+)/H^+$ exchanger (Kakar et al 1989, Jezek et al 1990) should be tested on the red blood cell system

The present aim, therefore, was to investigate the effects of a variety of transport inhibitors on $K^+(Na^+)/H^+$ exchange The results can be analysed in two separate ways Firstly, it is important to establish any specific pharmacology of this transporter, particularly in the context of the effect of quinacrine (Garlid et al 1986) on the mitochondrial system Secondly, there are possibilities of epiphenomena based on the actions of some of these molecules on other transporters, on the surface potential, membrane area etc Our final aim is to clarify the response of K^+ transport to these drugs in terms of actions on the $K^+(Na^+)/H^+$ exchanger, and to add support to its major role in contributing to the "leak" transport pathway Preliminary accounts of this work have been already published in abstract form (Bernhardt et al 1995, 1997)

Materials and Methods

Blood

Stored bank blood from healthy donors was used for the experiments Red blood cells were separated by centrifugation for 8 min at 1500 × *g* at room temperature Plasma and buffy coat were aspirated and the cells were washed 3 times with physiological (high ionic strength, HIS) solution containing (mmol/l) NaCl ($NaNO_3$, Na gluconate), 145, or $NaCH_3SO_4$, 165, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8, pH 7 4 at room temperature When the flux measurements were carried out in LIS solution, the cells in the final wash were suspended in a solution of the following composition (mmol/l) sucrose, 250, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8, pH 7 4 at room temperature All solutions had the same osmolarity (300 mosmol/l, measured with a vapour pressure osmometer) For the experiments with changed intracellular Mg^{2+}concentrations cells were washed 3 times with a solution containing (mmol/l) NaCl, 145, glucose, 10, 3-(N-morpholino)propanesulphonic acid (MOPS), 10, adjusted to pH 7 4 with tris(hydroxymethyl)amino-methane (Tris) (MOPS containing HIS solution) Then, the red blood cells were incubated for 15 min at 37°C (5% haematocrit) in this solution modified to include 30 mmol/l sucrose and 10 μmol/l A 23187 To alter the internal Mg^{2+} concentration, the incubation solution contained additionally 0 1 mmol/l ethylenediaminetetraacetic acid (EDTA) or 1 mmol/l Mg^{2+} After the incubation, the cells were washed 3 times at room temperature in the corresponding incubation solution which did not contain A 23187 followed by 3 washes in the MOPS-containing HIS solution When the flux measurements were carried out in LIS solution, the cells in the final wash were suspended in a solution of the following composition (mmol/l) sucrose, 250, glucose, 10, MOPS, 10, adjusted to pH 7 4 with Tris (MOPS-containing LIS solution)

Flux measurements

Aliquots of washed erythrocytes were added to the flux media to give a final haematocrit of about 5 % All flux media contained ouabain (0 1 mmol/l) and ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′ tetraacetic acid (EGTA, 0 1 mmol/l) In addition, transport inhibitors were used at concentration of 0 1 mmol/l except where stated otherwise Stock solutions of the inhibitors were prepared in distilled water with Tris, or in dimethylsulphoxide (DMSO) When the cells were treated with substances dissolved in DMSO, equivalent amounts of DMSO were added to the control tubes 0 95 ml of the suspensions were preincubated in 1 5 ml Eppendorf tubes for 5 min at 37°C Then, 50 μl of the ^{86}Rb stock solution (in 150 mmol/l KCl, KNO_3, KCH_3SO_4, or K-gluconate) was added to each tube to give a final K^+ concentration of 7 5 mmol/l and a radioactivity of about 50 kBq/ml ^{86}Rb was used as a tracer for K^+ (cf Bernhardt et al 1991) After 30 min incubation at 37°C the flux was stopped by centrifugation at 12,000 × *g* (10 s), and the supernatants

were removed by aspiration The cells were then washed free of extracellular radioactivity by 4 successive centrifugations (12,000 × *g*, 10 s) and resuspensions in ice-cold washing solution containing (mmol/l) $MgCl_2$, 106, MOPS, 10 (pH 7 4) The cell pellet was lysed with 0 5 ml of 0 1% (v/v) Triton X-100 and the protein precipitated with 0 5 ml of 5% (w/v) trichloroacetic acid After the suspension was centrifuged for 5 min at 12,000 × *g*, the radioactivity of ^{86}Rb in the supernatant of the lysate was measured by Cerenkov counting in a liquid scintillation analyser (TRI-CARB 1600 TR)

The packed red cell equivalent of the suspension was calculated from the cyanmethaemoglobin with Drabkin's reagent on a 1 50 dilution of the cell suspension using OD_{540} packed cells = 246 (Hall and Ellory 1985)

In the sets of experiments involving pretreatment with inhibitors the cell suspensions were preincubated (haematocrit ~ 5%) for 15 min in 40 ml of HIS solution at 37°C containing 0 1 mmol/l ouabain, 0 1 mmol/l EGTA, and 0 01 mmol/l DIDS or 0 01 mmol/l furosemide The cells were washed 3 times with HIS solution to remove the extracellular inhibitors Where the flux was measured in LIS solution the final wash was in this solution Then, the red blood cells were resuspended in 5 ml of the flux media with or without inhibitors The following procedures were the same as described for flux measurement

Reagents

Inorganic salts, sucrose and glucose were of analytical grade MOPS was obtained from Serva Feinbiochemica GmbH & Co KG & (Heidelberg, Germany) Na- and K-methylsulphate were purchased from Hopkins & Williams Inc (Chadwell Heath, U K), and Na- and K-gluconate from BDH Chemicals Ltd (Poole, U K) Ouabain, furosemide, bumetanide, DIDS, phloretin, niflumic acid, purine riboside, quinacrine, EDTA, EGTA, and Tris were obtained from Sigma Chemical Company (St Louis, USA) [(Dihydroindenyl)oxyl]alkanoic acid (DIOA), NPPB, dipyridamole, and ruthenium red were purchased from Research Biochemicals Inc (Natick, USA) Eosin-5-maleimide (EM) was purchased from Molecular Probes Inc , Eugene (USA), and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) from Pfalz and Bauer Inc (Waterbury, USA) Dichlorodiphenyl-2-carboxylate (DCDPC) was obtained from Hoechst Pharmaceuticals (Frankfurt, Germany) MK-196 (indacrinone) was a kind gift of Dr W L Henkler, Merck Research Laboratories (Rahway, USA) ^{86}Rb (in RbCl) was a product by Amersham International (Amersham, U K)

Statistical treatment

The result of a single experiment was calculated from the average of triplicate samples Each value presented as the final result represents data from at least 3 separate experiments carried out on blood of different donors ± S E M 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

Effect of classical cotransport inhibitors on the residual K^+ influx

To characterize further the participation of a specific ion transport pathway, i e the $K^+(Na^+)/H^+$ exchanger, in the residual K^+ flux, the influence of a variety of transport inhibitors on the unidirectional K^+ influx in the presence of ouabain, bumetanide[1] and EGTA was studied in HIS and LIS media This includes classical transport inhibitors of ion cotransport pathways, anion transport (band 3 mediated transport) as well as ion channels Some of the experiments were carried out in chloride- as well as in nitrate-containing media

As expected, replacing Cl^- with NO_3^- in HIS solutions reduced ouabain-insensitive K^+ influx by about 90% in normal human red blood cells This component was completely sensitive to 0 1 mmol/l bumetanide or furosemide, (well-known ion cotransport inhibitors (Ellory and Stewart 1982, Forbush 3rd and Palfrey 1983, Kracke and Dunham 1987)) representing classical $Na^+/K^+/Cl^-$ cotransport (K^+/Cl^- cotransport is essentially silent in mature human erythrocytes under physiological conditions (Hall and Ellory 1986)) (Fig 1) From Fig 1 it can also be seen that in chloride containing HIS solutions MK-196 blocks this transport pathway nearly completely, whereas DIOA (a more selective K^+/Cl^- cotransport inhibitor (Garay et al 1988)) only poorly inhibits the $Na^+/K^+/Cl^-$ cotransport

Under LIS conditions (low external Na^+ and anion concentration) there will be no classical $Na^+/K^+/Cl^-$ cotransport, the K^+ influx representing the increased K^+ transport via the $K^+(Na^+)/H^+$ exchanger described previously (Bernhardt et al 1991, Richter et al 1997) In LIS solutions, the presence of furosemide or DIOA causes a significant increase of the residual K^+ influx This stimulation is more pronounced in nitrate as compared with chloride-containing LIS solutions For MK-196 this stimulatory effect could be observed only in nitrate-containing LIS media (Fig 1) The dose-response curve for furosemide stimulation of the LIS-induced K^+influx (Fig 2, fitted as a Michaelis-Menten function and a linear component) shows that this substance increased the flux at concentrations much lower than 0 1 mmol/l (app $K_{0\,5} \sim 2$–$4\ \mu$mol/l) In addition, at higher concentrations (measured

[1] Originally we defined residual K^+ influx as the (ouabain, bumetanide or furosemide, and EGTA)-insensitive $K^+(^{86}Rb)$ influx Since the present paper includes investigations of the effects of bumetanide and furosemide on K^+ transport it is obvious that residual K^+ influx must be defined differently Therefore, in experiments with ion transport inhibitors, fluxes measured in LIS solutions were in low Na^+, low Cl^- media which effectively eliminate transport via the $Na^+/K^+/Cl^-$ cotransport pathway and is equivalent to having bumetadine present

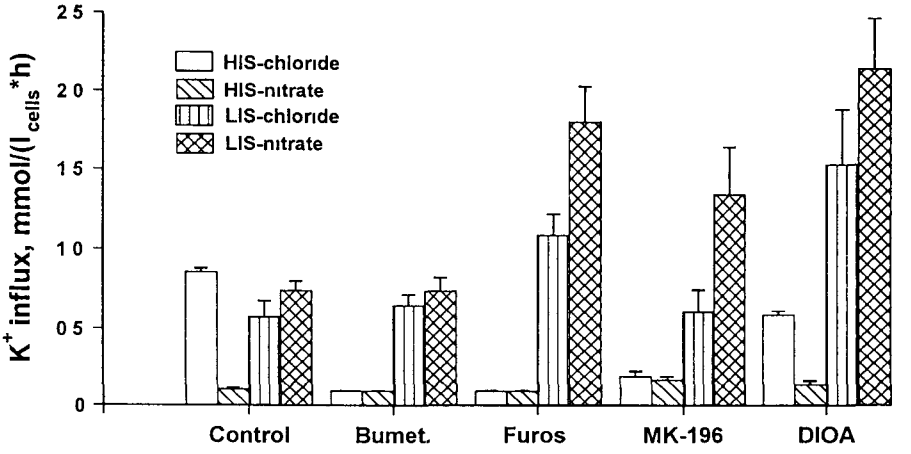

Figure 1. Effect of 0 1 mmol/l bumetanide, furosemide MK-196, or DIOA on K^+ influx in human red blood cells in physiological (HIS) and low ionic strength (LIS) solutions containing either chloride or nitrate Flux solutions contained (mmol/l) NaCl ($NaNO_3$), 145, or sucrose, 250, KCl (KNO_3), 7 5, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8 (pH 7 4) ouabain, 0 1, EGTA, 0 1 Results (mean ± S E M) are pooled data from at least 4 independent experiments

up to 1 mmol/l) a further linear increase of the residual K^+ influx could be observed In another series of experiments (data not shown) bumetanide was tested at concentrations up to 1 mmol/l Bumetanide was shown to be ineffective up to a concentration of 0 2 mmol/l At higher concentrations there was a slow progressive increase in K^+ influx (60% stimulation at 1 mmol/l bumetanide) as seen for high concentrations of furosemide (Fig 2)

Effect of anion transport inhibitors on the residual K^+ influx

Investigating the effect of classical anion transport inhibitors (see e g Deuticke et al 1990, Cabantchik and Greger 1992) at concentrations of 0 1 mmol/l on the residual K^+ influx, it can be seen from Fig 3 that the presence of DIDS, phloretin, and eosin-5-maleimide in the LIS solutions also caused a further stimulation of the LIS-induced K^+ influx, which was more pronounced in nitrate-containing as compared with chloride-containing LIS solutions For DNDS and niflumic acid this stimulatory effect could be observed only in nitrate-containing LIS media The presence of dipyridamole, on the contrary, led to a significant inhibition of the flux Dose-response curves for DIDS stimulation and dipyridamole inhibition of the residual K^+ influx in LIS solutions were constructed for in both chloride and nitrate media From Figs 4 and 5 it can be seen that both DIDS stimulation and dipyridamole inhibition were maximal at 0 1 mmol/l in chloride and nitrate

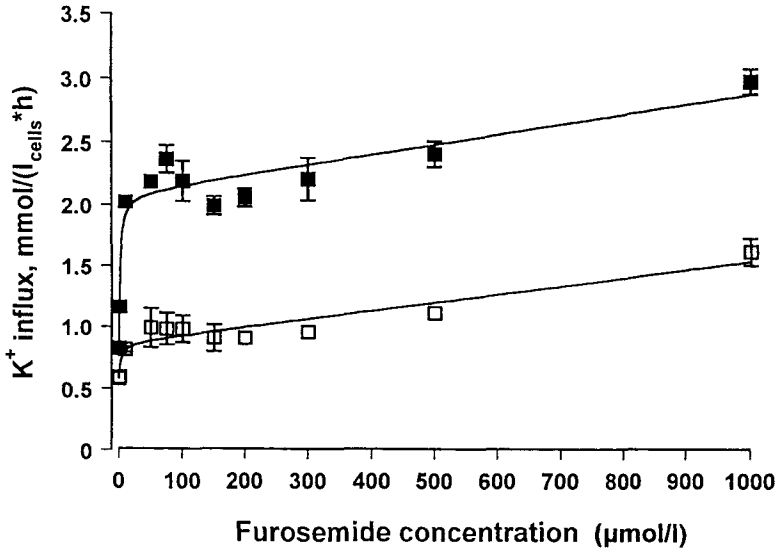

Figure 2. Dose-response curve for the furosemide effect on K^+ influx in human red blood cells in low ionic strength (LIS) media □ – chloride-containing solutions, ■ – nitrate-containing solutions The flux solution contained (mmol/l) sucrose, 250, KCl (KNO_3), 7 5, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8 (pH 7 4), ouabain, 0 1, EGTA, 0 1 Results (mean ± S E M) are pooled data from 3 independent experiments Where not shown, the error bars are smaller than the symbol

media (app. $K_{0\,5} \sim 20$–40 μmol/l for DIDS stimulation and ~ 5–10 μmol/l for dipyridamole inhibition). In a further series of experiments the anion dependence of the LIS-induced DIDS stimulation of the residual K^+ influx was investigated From the results presented in Table 1 it can be seen that under LIS conditions the replacement of 7.5 mmol/l KCl by KNO_3 resulted in a much more pronounced stimulation by DIDS as compared with the replacement of KCl by KCH_3SO_4 or K-gluconate.

In chloride-containing HIS solutions only the presence of phloretin caused a significant reduction of the K^+ influx (35%) probably due to partial inhibition of the $Na^+/K^+/Cl^-$cotransport The other classical anion transport inhibitors were without effect. The presence of DIDS, phloretin, or niflumic acid in nitrate-containing HIS solutions did not result in significant changes of the residual K^+ influx. However, in the presence of eosin-5-maleimide, a significant increase, and in the presence of dipyridamole a significant decrease, of the flux could be observed ($K_{0\,5} \sim 14$ μmol/l and 6 μmol/l, respectively; data not shown). Since dipyridamole is known to be a potent inhibitor of nucleoside transport in human red blood cells (Plage-

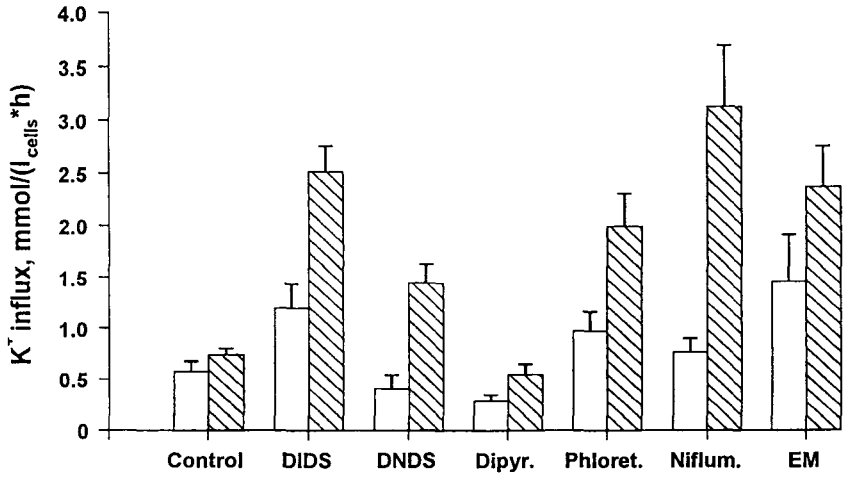

Figure 3. Effect of 0 1 mmol/l DIDS, DNDS, dipyridamole, phloretin, niflumic acid, or eosin-5-maleimide on K^+ influx in human red blood cells in low ionic strength (LIS) solutions For flux solutions see legend to Fig 2 The open columns represent values for chloride-containing solutions and the hatched columns for a medium where chloride was replaced by nitrate Results (mean ± S E M) are pooled data from at least 4 independent experiments

Table 1. Effect of anion composition of the medium on the DIDS-induced stimulation of the K^+ influx in human red blood cells in low ionic strength (LIS) solution

Anion in LIS solution	Residual K^+ influx (mmol/(l_{cells}*h))	
	Control	+DIDS
Chloride	0 69 ± 0 10	1 39 ± 0 37
Nitrate	1 04 ± 0 23	2 95 ± 0 20
Methylsulfate	0 79 ± 0 09	1 00 ± 0 07
Gluconate	1 02 ± 0 06	1 55 ± 0 29

Flux media contained (mmol/l) KCl (KNO_3, KCH_3SO_4, or K-gluconate), 7 5, sucrose, 250, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8, pH 7 4, ouabain, 0 1, EGTA, 0 1 DIDS concentration was 0 1 mmol/l Results (mean ± S E M) are pooled data from 3 independent experiments

mann and Woffendin 1987), purine riboside, another inhibitor of this transport pathway (Plagemann et al 1988) was tested There was no significant effect on the LIS-induced residual K^+ influx in either chloride- or nitrate-containing media, or in HIS solutions (data not shown). To exclude the possibility of an indirect effect

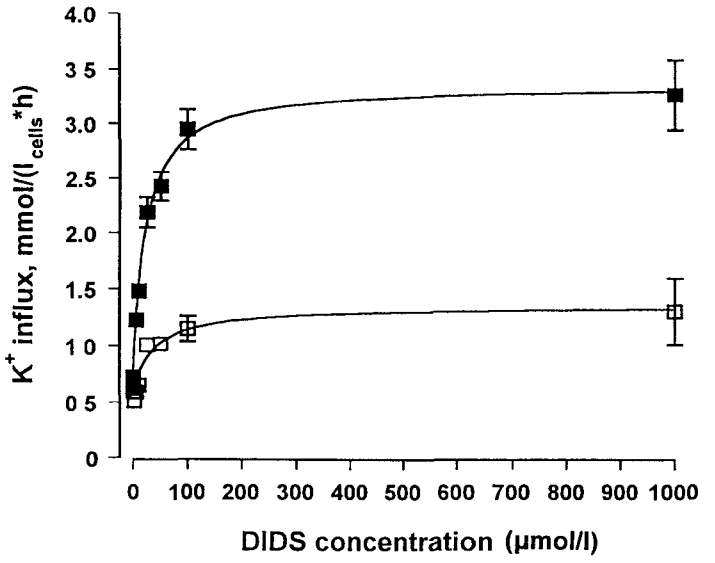

Figure 4. Dose-response curve for the DIDS effect on K^+ influx in human red blood cells in low ionic strength (LIS) media □ chloride-containing solutions, ■ – nitrate-containing solutions For flux solutions see legend to Fig 2 Results (mean ± S E M) are pooled data from 3 independent experiments Where not shown, the error bars are smaller than the symbol

of the selected transport inhibitors acting by inhibiting chloride / phosphate and chloride / bicarbonate exchange and thereby altering intracellular anion content and pH, experiments were carried out where the phosphate buffer was replaced by a less permeant buffer (10 mmol/l MOPS-Tris) Under these conditions stimulation by DIDS and furosemide of the residual K^+ influx was again observed (data not shown)

Effect of selected ion channel inhibitors on the residual K^+ influx

The investigation of the LIS-induced residual K^+ influx was extended to selected ion transport inhibitors known to affect ion channels (Gogelein and Greger 1986, Gogelein and Pfannmuller 1989, Cabantchik and Greger 1992, Ma 1993) Fig 6 shows that NPPB, DCDPC, and ruthenium red are only poor inhibitors of $Na^+/K^+/Cl^-$ cotransport in chloride-containing HIS media However, the presence of NPPB or DCDPC in LIS solutions also leads to a significant enhancement of the K^+ influx, again more pronounced in nitrate-containing solutions In contrast, ruthenium red inhibits the flux both in chloride- and nitrate-containing LIS solution

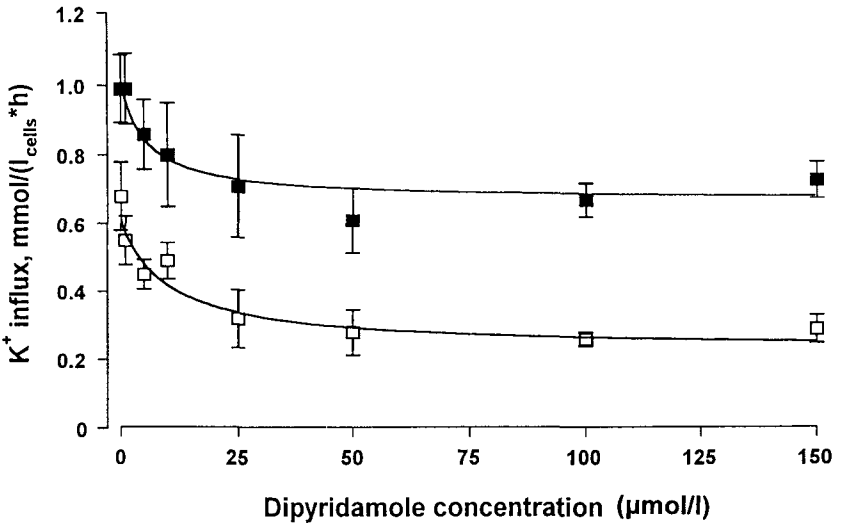

Figure 5. Dose response curve for dipyridamole effect on K^+ influx in human red blood cells in low ionic strength (LIS) media □ – chloride-containing solutions, ■ – nitrate-containing solutions For flux solutions see legend to Fig 2 Results (mean ± S E M) are pooled data from 3 independent experiments

Effect of DIDS or furosemide on residual K^+ influx in LIS media after preincubation of the cells with these substances under HIS conditions

The question arises as to whether the stimulating effect of selected transport inhibitors under LIS conditions is due to their presence in the flux solution or whether the effect can be also observed after preincubating and washing the cells in HIS solution. We investigated this problem for DIDS and furosemide. DIDS was chosen since it has been shown under physiological conditions that its inhibitory effect on the anion transport system is due to the existence of two types of DIDS interaction with the band 3 protein: covalent and noncovalent (Passow et al. 1982; Passow 1986; Gasbjerg et al. 1993). Furosemide was studied since it interacts with at least two different ion transport systems in the human red blood cell membrane. It competes with chloride for the substrate binding site on band 3 (Brazy and Gunn 1976; Ellory and Stewart 1982) and is also able to affect a modifier site (Cabantchik et al. 1978). In addition, furosemide also binds directly to other transport proteins, e.g. $Na^+/K^+/Cl^-$ cotransporter (Brugnara et al. 1986; Kracke and Dunham 1987). As can be seen from Fig. 7 the stimulatory effect of DIDS on the residual K^+ influx in LIS media occurred only when it was present during the flux measurement. Preincubation of the cells with 10 µmol/l DIDS under HIS conditions (see Methods) did

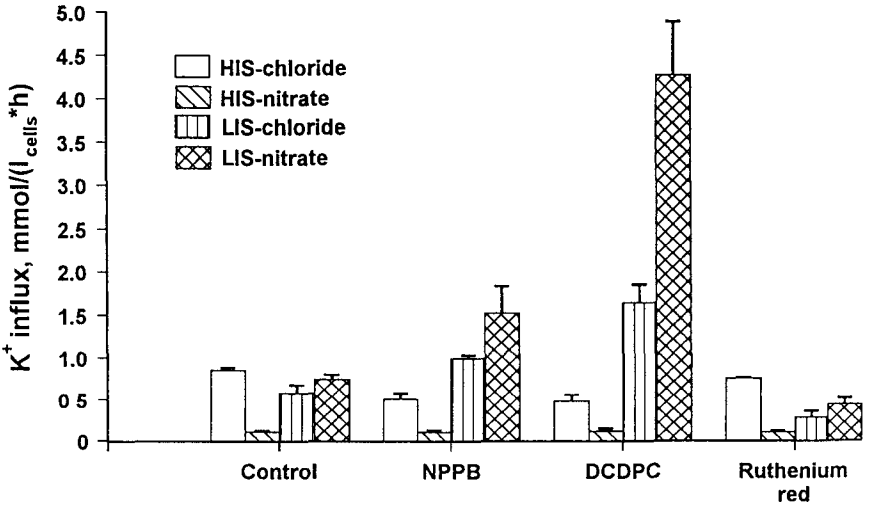

Figure 6. Effect of NPPB, DCDPC, or ruthenium red on K^+ influx in human red blood cells in physiological (HIS) or low ionic strength (LIS) solutions containing either chloride or nitrate Key shows the solution used The flux solutions contained (mmol/l) NaCl ($NaNO_3$), 145, or sucrose, 250, KCl (KNO_3), 7 5, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8 (pH 7 4), ouabain, 0 1, EGTA, 0 1 Results (mean ± S E M) are pooled data from at least 4 independent experiments

not affect the subsequent cation flux, indicating complete reversibility of the effect Thus, the covalent binding site(s) for DIDS on band 3 protein is (are) not involved The same observation as for DIDS was made for furosemide, showing a significant stimulation of the residual K^+ influx only when present in the LIS flux solution (data not shown)

Comparison of properties of the $K^+(Na^+)/H^+$ exchanger in the red blood cell membrane with that known to exist in the mitochondrial membrane

It is known that a $K^+(Na^+)/H^+$ exchanger is present in the mitochondrial membrane (e g Kakar et al 1989, Jezek et al 1990) This transporter is inhibited by quinacrine and by an increase of internal Mg^{2+} (Garlid et al 1986, Garlid 1994) To compare the properties of the $K^+(Na^+)/H^+$ exchanger of the human red blood cell membrane and of the mitochondrial membrane we investigated the influence of altering intracellular Mg^{2+} concentration as well as that of quinacrine on the residual K^+ influx Lowering the internal Mg^{2+} concentration with ionophore and EDTA did not alter the residual K^+ influx in HIS solutions (0 31 ± 0 02 vs 0 30 ± 0 15 mmol/(l_{cells} h) for 0 and 1 mmol/l Mg^{2+}, respectively) In LIS media, however, there was a significant increase of the flux upon the reduction of the internal

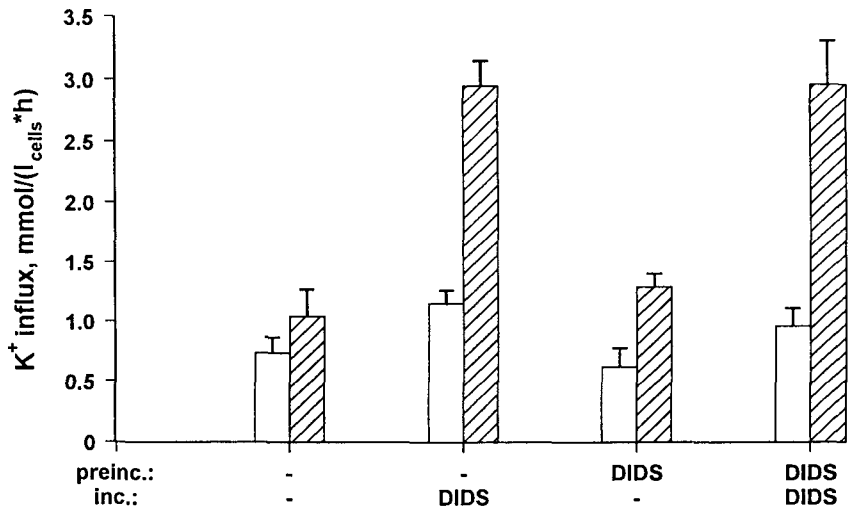

Figure 7. Effect of 0 1 mmol/l DIDS on K^+ influx in human red blood cells in low ionic strength (LIS) media after preincubation of the cells for 15 min with 10 μmol/l DIDS in physiological solutions (see Methods) Composition of the media for preincubation (mmol/l) NaCl ($NaNO_3$), 145, glucose 10, NaH_2PO_4/Na_2HPO_4, 5 8, pH 7 4 and for flux measurements (mmol/l) sucrose, 250, KCl (KNO_3), 7 5, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8, pH 7 4, ouabain, 0 1, EGTA, 0 1 Open columns represent flux values for chloride containing media, hatched columns for nitrate-containing solutions Results (mean ± S E M) are pooled data from 4 independent experiments

Table 2. Effect of 1 mmol/l quinacrine on the K^+ influx in human red blood cells in physiological (HIS) or low ionic strength (LIS) solution

Solution	Residual K^+ influx (mmol/(l_{cells}*h))	
	Control	+ Quinacrine
HIS	0 08 ± 0 01	0 34 ± 0 13
LIS	0 69 ± 0 12	0 14 ± 0 06

Flux media contained (mmol/l) NaCl, 145, or sucrose, 250, KCl, 7 5, glucose, 10, NaH_2PO_4/Na_2HPO_4, 5 8 (pH 7 4), ouabain, 0 1, bumetanide, 0 1, EGTA, 0 1 Results (mean ± S E M) are pooled data from 4 independent experiments

Mg^{2+} concentration (the equivalent flux values being 1 22 ± 0 33 and 0 50 ± 0 21 mmol/(l_{cells} h), respectively) Also, the addition of 1 mmol/l quinacrine led to a reduction of the residual K^+ influx in LIS solution (Table 2) Surprisingly, in the presence of quinacrine under HIS conditions there was a stimulation of the flux

to a value greater than the value measured in LIS solution. So, the presence of quinacrine resulted in an "inverse low ionic strength effect".

Discussion

It is now well-established that when human erythrocytes are suspended in isotonic LIS media containing sucrose, both unidirectional residual K^+ fluxes (as well as Na^+ fluxes) and net K^+ efflux are increased significantly (e.g. Donlon and Rothstein 1969; Bernhardt et al. 1991). This increased transport cannot be explained on the basis of electrodiffusion and its characteristics suggest the involvement of the $K^+(Na^+)/H^+$ exchanger, a novel specific transport pathway in the human red blood cell membrane described by us recently (Bernhardt et al. 1991; Denner et al. 1993; Richter et al. 1997).

It was shown that the increase of K^+ and Na^+ transport via the $K^+(Na^+)/H^+$ exchanger in LIS solutions was due solely to reduction of the ionic strength of the extracellular solution (Richter et al. 1997). The decrease of the ionic strength led to an increase of the absolute value of the negative surface potential resulting in an enhancement of the cation concentration near the membrane surface, and hence near the transport binding site. This ion concentration near the transport binding site is of importance for ion transport via the carrier mechanism (Richter et al. 1997). However, it is still possible that another transport pathway was activated in LIS media, as suggested by other authors (see below).

In previous experiments, as in the present study, we used ouabain to eliminate Na^+/K^+ pump involvement, and EGTA to rule out the Ca^{2+}-activated K^+ (Gardos) channel (also supported by earlier experiments in different species (Erdmann et al. 1990)), and anion-dependence (i.e. chloride- *vs.* methylsulfate-containing LIS solution) and/or bumetanide to inhibit $Na^+/K^+/Cl^-$and K^+/Cl^- cotransport (see also Bernhardt et al. 1991).

One system that could be activated under conditions resembling LIS treatment of human erythrocytes was described by Halperin et al. (1989). This voltage-sensitive channel was shown to open irreversibly when the transmembrane potential increased to +20 mV and higher. It was shown to exist in human and HK, but not in LK sheep red blood cells (Halperin et al. 1990), and to be permeable to Na^+, K^+, and Ca^{2+}. In contrast, the LIS-induced effect on the residual K^+ flux was found for LK, but not for HK sheep red blood cells (Erdmann et al. 1991), and was shown to be fully reversible (Bernhardt et al. 1984). Furthermore, Ca^{2+} influx is not enhanced under LIS conditions (Ellory et al. 1988). For these reasons it is unlikely that the flux pathway in the present work is the same as that studied by Halperin et al. (1989; 1990).

The present work has shown that the LIS-stimulated residual K^+ influx can be activated further by a variety of transport inhibitors (including inhibitors of

ion cotransport and channel pathways) (Figs 1 4, 6) many of which affect anion transport via band 3 protein (see e g Deuticke et al 1990, Cabantchik and Greger 1992) One possible explanation for these results (and therefore supporting the concept of Halperin et al (1989)) is that inhibition of net anion movement via band 3 protein by these inhibitors alters the transmembrane potential, modifying the driving force for unidirectional cation tracer fluxes across the red blood cell membrane Evidence against such an indirect effect of transmembrane potential causing the present phenomena includes the fact that altering transmembrane potential progressively from −8 mV to about +45 mV by decreasing NaCl (or NMDG-Cl) / increasing sucrose gave a monotonic increase in *both* influx and efflux of K^+ as well as Na^+ (Bernhardt et al 1991, Denner et al 1993, Richter et al 1997) Further, isotonic (i e HIS) gluconate, or tartrate media alter the transmembrane potential to the same extent as does LIS solution, but fail to elicit the response (Richter et al 1997) In addition, the residual K^+ influx is more (or only) stimulated by the transport inhibitors in nitrate- as compared to chloride-containing LIS solution On the basis of the transmembrane potential effect one would expect the opposite result In fact, Halperin et al (1989) were able to show that the transmembrane potential of human erythrocytes suspended in chloride-containing LIS media decreased from positive values in the absence of DIDS to negative values in the presence of DIDS In nitrate-containing LIS solutions the decrease of the potential in the presence of DIDS was much less pronounced reaching values of about 0 mV only These data were obtained from estimates of the proton distribution ratio between the inside and the outside of the cells suspended in an unbuffered medium in the presence of the proton ionophore carbonyl cyanide *m* chlorophenylhydrazone (CCCP) Furthermore, the concept of an indirect transmembrane potential effect is hard to defend since dipyridamole, also known to be an inhibitor of band 3 mediated anion transport (Deuticke 1970, Renner et al 1988, Legrum and Passow 1989) decreases the residual K^+ influx in LIS solutions (Figs 3, 5) in contrast to the other classical anion transport inhibitors investigated

Another interesting finding was published by Zade-Oppen et al (1988) These authors have demonstrated that in the case of a high (inside positive) transmembrane potential at an external pH of 6 4, the efflux of both Na^+ and K^+ from human red blood cells was significantly higher in the presence than in the absence of furosemide This result was discussed in the context of the stimulation of the $Na^+/K^+/Cl$ cotransporter However, it is difficult to explain the stimulation of the residual K^+ influx by different ion transport inhibitors (including furosemide) described in the present paper as a result of the activation of the $Na^+/K^+/Cl^-$ cotransport system, since this ion transport pathway shows a strict anion preference for chloride over nitrate or methylsulfate (Dunham et al 1980, Ellory and Stewart 1982, Brugnara et al 1986) In addition, phloretin, known to inhibit (80%

at 0.1 mmol/l) this transport pathway weakly (Chipperfield 1981) stimulates the LIS-induced residual K^+ influx (Fig. 1). Furthermore, the low concentrations of the substrate ions in LIS solutions (especially Na^+ and Cl^-) induced a decrease of the ion fluxes through the $Na^+/K^+/Cl^-$ cotransport system (Canessa et al. 1986). Finally, LK sheep red blood cells lack $Na^+/K^+/Cl^-$ cotransport (Dunham and Ellory 1981), but show the LIS effect (Erdmann et al. 1991).

Similarly, it is difficult to explain the current results as a specific effect of the activation of other known transport pathways for ions and organic osmolytes in the red blood cell, by the majority of the present inhibitors, with inhibition by certain others. The only two substances found to inhibit the residual K^+ influx under LIS conditions, dipyridamole and ruthenium red (Figs. 3,5,6) (quinacrine is discussed separately, see below), are known as effective inhibitors of nucleoside (Plagemann and Woffendin 1987) and Ca^{2+} transport pathways (pump and channels) (Hinds et al. 1981; Ma 1993), respectively. Both substances are positively charged, in contrast to all the other inhibitors used, which are negatively charged at pH 7.4. The inhibitory effect of dipyridamole is of particular interest since it was also observed in nitrate- containing HIS solutions (in the presence of ouabain and EGTA). This also demonstrates clearly that, as defined, the residual K^+ influx can be further decreased under HIS conditions.

A surprising feature of the present results which requires explanation is the *stimulation* of residual cation transport by many different anionic inhibitors of specific membrane transport systems. An explanation may be sought in terms of a more general surface potential effect of all these molecules. External surface potential of red blood cells only becomes relevant when ionic strength is lowered significantly (see e.g. Bernhardt 1994). The reduced ionic strength of the solution leads to an increase (more negative) of the surface potential. Therefore, the monovalent cation concentration near the cell surface will increase, whereas the surface concentration of anions is decreased. Anionic inhibitors will increase the surface concentration of the monovalent cations. Correspondingly, cationic drugs would cause an opposite effect. Such a surface potential effect (due to inhibitor charges and/or LIS) on the residual K^+ transport, i.e the transport via the $K^+(Na^+)/H^+$ exchanger is in agreement with experimental findings already discussed in a previous paper (Richter et al. 1997).

An interesting result of the present work is that the residual K^+ influx is higher in nitrate- in comparison with chloride-containing LIS solution. The effect becomes more pronounced in the presence of a variety of negatively charged transport inhibitors. For the residual K^+ efflux, a decrease was reported when chloride was replaced by nitrate in a LIS solution (Zade-Oppen et al. 1988). These results are consistent with the finding that chloride replacement by nitrate results in an acidification of the interior of the red blood cell by shifting the effective isoelectric point for the intracellular charges to a lower value (Payne et al. 1990) and the view

that the residual K^+ transport is realised by the $K^+(Na^+)/H^+$ exchanger (Richter et al. 1997).

One aim of the present work was to compare properties of the $K^+(Na^+)/H^+$ exchanger present in the mitochondrial membrane with properties of the $K^+(Na^+)/H^+$ exchanger described in the human red blood cell membrane. Like the mitochondrial system, the human red blood cell $K^+(Na^+)/H^+$ exchanger in LIS media is stimulated by removing internal Mg^{2+} and inhibited by quinacrine (Table 2). One should take into account that a significant proton flux via the human red blood cell $K^+(Na^+)/H^+$ exchanger occurs only in LIS solutions. In HIS solution there is a (K^+ + Na^+) influx component compensated for almost completely by the (K^+ + Na^+) efflux (Richter et al. 1997). Therefore, the stimulating effect of quinacrine in HIS solution is surprising and needs further elucidation. Interestingly, both inhibition and activation of the Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles by quinacrine was found depending on the experimental conditions (De La Pena and Reeves 1987).

In summary, the present work supports the idea that the ionic strength of the extracellular solution has significant effects on the residual transport of monovalent cations across the red blood cell membrane, i.e. for the transport via the $K^+(Na^+)/H^+$ exchange mechanism. Depending on the charge on the molecule at pH 7.4, the addition of classical transport inhibitors to a red blood cell suspension in LIS solution will cause an increase (negatively charged substance) or a decrease (positively charged substance) of the K^+ and Na^+ fluxes mediated by the $K^+(Na^+)/H^+$ exchanger. It seems, therefore, that most likely the transport inhibitors investigated do not act specifically on the $K^+(Na^+)/H^+$ exchanger. In addition, based on the investigations of the residual K^+ influx at different intracellular Mg^{2+} concentrations and with and without quinacrine it seems likely that the $K^+(Na^+)/H^+$ exchanger of the human erythrocyte membrane seems to show similarities with the $K^+(Na^+)/H^+$ exchanger present in the mitochondrial membrane.

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