Differential Effect of HOE642 on Two Separate Monovalent Cation Transporters in the Human Red Cell Membrane

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Key Words

Red blood cells • Erythrocytes • Cation fluxes • HOE642 • Low ionic strength • Cation channel • Cation antiporter • $K^+(Na^+)/H^+$ exchanger

Abstract

Residual K^+ fluxes in red blood cells can be stimulated in conditions of low ionic strength. Previous studies have identified both the non-selective, voltage-dependent cation (NSVDC) channel and the $K^+(Na^+)/H^+$ exchanger as candidate pathways mediating this effect, although it is possible that these pathways represent different modes of operation of a single system. In the present study the effects of HOE642, recently characterised as an inhibitor of the $K^+(Na^+)/H^+$ exchanger, on NSVDC has been determined to clarify this question. Radioisotope flux measurements and conductance determinations showed that HOE642 exerted differential effects on the NSVDC channel and the $K^+(Na^+)/H^+$ exchanger, confirming that the salt loss observed in low ionic strength solutions represents contributions from at least two independent ion transport pathways. The findings are discussed in the context of red blood cell apoptosis (eryptosis) and disease.

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Introduction

It has been long known that residual unidirectional Na^+ and K^+ fluxes (influxes and effluxes) as well as net salt loss of human red blood cells (RBCs) are significantly enhanced in low ionic strength (LIS) media, where NaCl (or KCl) is replaced by an isotonic quantity of sucrose [1-3]. At present, 10 different cation transport pathways and 5 Na^+ -dependent amino acid transporters have been characterised that can translocate Na^+ and/or K^+ across the membrane [4, 5]. Since fewer RBC transport pathways have been described for K^+ than for Na^+ , it is much easier to isolate residual K^+ fluxes. For K^+ , the residual flux is defined as the (ouabain + bumetanide + EGTA)-insensitive transport, where the Na^+/K^+ -pump, the $Na^+, K^+, 2Cl^-$ cotransporter (NKCC), and the Ca^{2+} -activated K^+ channel (Gardos channel) are respectively inhibited (or not activated). In addition, the K^+, Cl^- cotransporter (KCC) is silent under physiological conditions. Two further pathways for K^+ have been described in recent years, the non-selective, voltage-dependent cation (NSVDC) channel [6, 7] which is also permeable to Na^+ , and the $K^+(Na^+)/H^+$ exchanger [8, 9]. In different studies, both pathways have been identified as the

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principal pathway responsible for enhanced cation fluxes in LIS media. It is possible that, as a result of the different experimental approaches that have been used to characterise these two pathways, the observations could represent different manifestations of the same system. There is, however, evidence to suggest that two separate pathways underlie the two transport modes that have been described. In the present study, the effects of HOE642, a recently described inhibitor of $K^+(Na^+)/H^+$ exchange [10], on the NSVDC channel have been determined in order to clarify this question.

Materials and Methods

Freshly drawn blood from healthy human donors was used for the experiments. The RBCs were isolated by centrifugation (2000 x g, 5 min) at room temperature and the plasma and buffy coat removed by aspiration.

Influx measurements

The cells were washed three times with a physiological NaCl (HIS) solution containing 145 mM NaCl, 10 mM glucose, 10 mM morpholinoethane sulfonic acid/Tris-(hydroxymethyl)aminomethane (MES/Tris), pH 7.4 at room temperature. The RBCs were then washed once in the appropriate medium used for the flux experiment (HIS or LIS medium with or without HOE642). The LIS medium contained 250 mM sucrose, 10 mM glucose, 10 mM morpholinoethane sulfonic acid/Tris-(hydroxymethyl)aminomethane (MES/Tris), pH 7.4 at room temperature. In all experiments ouabain (100 μ M), bumetanide (100 μ M), and EGTA (100 μ M) were present in the solution during the flux measurement. HOE642 was dissolved in DMSO and employed at a range of final concentrations (0 - 1 mM). The amount of DMSO did not exceed 0.5% of the total flux volume.

KCC was activated by a 10% decrease of cell volume and of solution pH as previously described [11]. The modified HIS solution contained 125 mM NaCl, 10 mM glucose, 10 mM 4-morpholine propane sulfonic acid (MOPS), and the pH was adjusted with NaOH to 7.0. KCC activity was determined as the difference of the flux in Cl^- -containing modified HIS solution and the appropriate NO_3^- -containing (Cl^- free) modified HIS solution. In experiments in which NO_3^- replaced Cl^- , cells were additionally washed 6 times in the Cl^- free HIS solution.

Red blood cells were suspended at haematocrit of approximately 5% in a total volume of 1 ml of flux medium (HIS, modified HIS, LIS, or Cl^- free solution) contained in an Eppendorf 1.5 ml microcentrifuge tube. The cell suspension was equilibrated at the flux temperature of 37°C for 5 min, after which tracer solution ($[KCl + ^{86}RbCl]$) or ($[KNO_3 + ^{86}RbCl]$), 7.5 mM final concentration, was added. The duration of exposure of cells to isotope (i.e. the flux time) was 30 min. Isotope uptake was terminated by centrifugation at 15,000 x g (10 s), and the supernatant removed by aspiration. The cells were then washed free of extracellular radioactivity by four successive

resuspensions and centrifugations (15,000 x g, 10 s) in ice-cold medium consisting of $MgCl_2$ (106 mM), N-morpholinopropane sulfonic acid (MOPS; 10 mM), with pH adjusted to 7.4. The cell pellet was lysed with 0.5 ml of 1% (v/v) Triton X-100 and the protein precipitated by addition of 0.5 ml of 5% (w/v) trichloroacetic acid followed by centrifugation at 15,000 x g for 5 min. The activity of ^{86}Rb in the supernatant was determined by Cerenkov counting in a liquid scintillation analyser (Packard Tri-Carb 2900). Haemoglobin content of RBC suspensions was determined as cyanmethaemoglobin using Drabkin's reagent.

Statistical Treatment of Results

Each experiment was repeated at least 3 times on blood from different donors. Data are presented as the mean $\pm$ SD of independent experiments.

Conductance determinations:

Packed RBCs were injected into either an unbuffered salt solution (NR, 154 mM NaCl, 2 mM KCl) or a solution where NaCl was substituted by sucrose, (SR, 264 mM sucrose, 2 mM KCl) to a haematocrit of 3.1%. In all experiments, solutions were supplemented with 20 μ M CCCP and 10 μ M NS1652 (final concentrations, from stock solutions prepared in DMSO). In some experiments, solutions were supplemented with HOE642 (final concentration 200 μ M) from stock solutions prepared in DMSO.

The membrane potential of the cells was estimated by continuous monitoring of extracellular pH (pH_{out}) in the presence of the protonophore CCCP [12] using a conventional pH electrode and calculated using the equation:

$$V_m = 61.5 \text{ mV} * (pH_{in} - pH_{out}). \quad \text{Eq. 01}$$

pH_{in} was determined as the pH in the solution after the cells were lysed by addition of 100 μ l Triton X-100 (1% v/v) in 3 M NaCl solution.

The cation conductance was calculated as:

$$g_+ = g_{Cl} \frac{E_{Cl} - V_m}{V_m - E_{K^+}}, \quad \text{Eq. 02}$$

while the chloride conductance was determined after a valinomycin induced hyperpolarization, as previously described [13] using the equation:

$$g_{Cl} = g_{K, val} \frac{E_{K^+} - V_m}{V_m - E_{Cl^-}}. \quad \text{Eq. 03}$$

Reagents

HOE642 ((4-Isopropyl-3-methylsulfonyl-benzoyl)-guanidine methanesulfonate) was a gift from Sanofi/Aventis (Germany), NS1652 (2-(N'-trifluoromethylphenyl)ureido)benzoic acid was a gift from Neurosearch A/S (Denmark). DMSO, CCCP (Carbonylcyanide-m-chloro-phenyl-hydrazine), nitrendipine, valinomycin, bumetanide, EGTA, MOPS, inorganic salts, sucrose, glucose and Triton X-100 were supplied by Sigma Chemical Co. Ouabain and MES was supplied by SERVA GmbH (Germany) and Tris by Carl Roth GmbH & Co. (Germany).

Fig. 1. Dose-response curves for HOE642 on the (ouabain + bumetanide + EGTA)-insensitive K^+ influx in HIS (■) and LIS (□) solution. Flux values were corrected for haemolysis (< 1%). Note that the actual values of the K^+ influx in LIS solution are 7.6 - 10.5 times higher than in HIS solution. Data are means $\pm$ SD of the K^+ influx from RBCs of 4 different healthy donors and regression curves of the type $y = y_0 + a \cdot \exp(-b \cdot x)$. The half saturation values of the inhibitable component are 156 μ M and 277 μ M for HIS and LIS solution, respectively.

Fig. 2. Representative trace of the potential response (calculated membrane potential) following injection of packed red blood cells into a SR solution in the absence or presence of 200 μ M HOE642. The broken horizontal line shows the initial chloride Nernst potential (104 mV). The arrow indicates addition of 1 mM $CaCl_2$ (final concentration). It should be noted, that the Cl^- Nernst potential is shifted approximately 18 mV towards negative values after Ca^{2+} addition, the result of an increase in the extracellular Cl^- concentration.

^{86}Rb (in $RbCl$) was produced by Amersham Biosciences (U.K.).

Results

In a recent paper, members of a family of compounds designed to inhibit Na^+/H^+ exchange (HOE694 and HOE642) were reported to also inhibit residual K^+ influx [10]. These substances showed a remarkable inhibitory effect in both HIS and LIS solution. The highest efficacy was found for 1 mM HOE642 with respectively 40% and 65% inhibition of the (ouabain + bumetanide + EGTA)-insensitive K^+ influx in HIS and LIS solutions. In the present study, the concentration dependence of this compound on residual K^+ influx was therefore determined (Figure 1). HOE642 exhibited a dose-dependent inhibition of influx in both solutions, with estimated half saturation values of the inhibitable component of 156 μ M in HIS solution and 277 μ M in LIS solution. This is the first

compound that has been found to significantly reduce residual K^+ influx levels in both HIS and LIS solutions.

It was consequently decided to test the effect of HOE642 on RBCs suspended in a sucrose substituted Ringer solution (SR) in the presence of the chloride conductance blocker NS1652, conditions in which the NSVDC channel has been shown to be highly activated [14]. Contrary to the inhibitory effect of HOE642 on residual K^+ influx, this compound was found to stimulate the K^+ conductance mediated by the NSVDC channel. In the presence of 200 μ M HOE642, the re-polarization following NSVDC channel voltage activation was more pronounced compared to the control experiment, and the hyperpolarization due to the Gardos channel activation caused by Ca^{2+} entry through the open NSVDC channel appeared to be more pronounced. Furthermore, the deactivation, which is a consequence of Ca^{2+} pump-mediated extrusion of Ca^{2+} was slower and of longer duration (Figure 2).

HOE642 (μM)	g_{NSVDC} (g_{Cl^-} units)
0	0.3619
12.5	0.3956
25	0.3750
50	0.4329
100	0.6487
200	0.9284

Table 1. Calculated RBC conductance (g_{NSVDC}) in g_{Cl^-} units (Eq. 02) as a function of HOE642 concentration. Under the experimental conditions employed, the chloride conductance is about $2 \mu\text{S}/\text{cm}^2$.

Addition of 1 mM CaCl_2 to cells injected into NR solution containing 200 μM HOE642 did not perturb membrane potential from the normal resting value of -12.5 mV (data not shown), demonstrating that HOE642 itself does not exert Ca^{2+} ionophore properties.

The calculated RBC cation conductance (Eq. 02) as a function of HOE642 concentration is shown in Table 1. In all experiments, 10 μM nitrendipine was present to eliminate any contribution from the Gardos channel to the cation conductance. Note that the calculated conductances are expressed in g_{Cl^-} units which, in the presence of 10 μM NS1652, has been determined to be approximately $2 \mu\text{S}/\text{cm}^2$ [13]. HOE642 concentrations no higher than 200 μM were employed in these dose-response experiments, since the buffer capacity conferred to the system at higher concentrations renders impossible the application of the CCCP method.

In order to ensure that the effect of HOE642 observed for cells suspended in SR originated from interaction with the NSVDC channel, and not indirectly from an increased inhibition of chloride conductance, the effect of this compound on cells suspended in a high ionic strength Ringer (NR) was determined. In the presence of valinomycin, HOE642 enhanced the induced hyperpolarization (data not shown), indicating either a weak chloride conductance block, or an increase of the valinomycin induced K^+ conductance. In the presence of NS1652, HOE642 however showed no effect, indicating that a small inhibition of chloride conductance underlies the HOE642 action which, in the presence of 10 μM NS1652, is not observed. An alternative system that could be implicated in the residual K^+ influx in HIS and/or LIS solution is KCC. Although it has been shown previously that there is no Cl^- -dependent component to the residual K^+ transport in either physiological (that is, HIS) or LIS solutions, we tested the influence of 0.5 mM HOE642 on

Fig. 3. The (ouabain + bumetanide + EGTA)-insensitive K^+ influx in modified HIS solution and the appropriate NO_3^- -containing (Cl^- free) modified HIS solution (see Materials and methods) in the absence and presence of HOE642 (as % of control value). Note that KCC activity is calculated as the difference in the flux in Cl^- -containing and the appropriate NO_3^- -containing (Cl^- free) solution. Data are means $\pm$ SD, $n = 4$.

KCC activity. Under conditions where KCC is activated (see Materials and methods), no effect of HOE642 on this transporter was observed (Figure 3). That failure to detect a decrease in the flux in the modified HIS solution supplemented with HOE642 is the result of the large magnitude of KCC-mediated transport fraction in comparison with the residual component that persists.

Discussion

The first evidence for the involvement of two separate pathways in the enhancement of unidirectional Na^+ and K^+ fluxes across the membrane of human RBCs suspended in LIS solutions came from studies characterising the NSVDC channel and the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchanger in varying experimental conditions. Although it is now recognised that the first pathway is electrogenic [6, 7] whereas the latter is electroneutral [8, 9], a common pathway underlying these two transport modes cannot be entirely discounted [14]. However, as has been speculated elsewhere [15, 16], it was assumed that the increase of the cation fluxes for RBCs suspended in LIS solutions is mediated by the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchanger. Only after reducing the extracellular ionic strength to very low val-

Table 2. Divergent properties of the $K^+(Na^+)/H^+$ exchanger and the NSVDC channel [8, 16, 18-22], *Bennekou P, personnel communication.

	$K^+(Na^+)/H^+$ exchanger	NSVDC channel
rat erythrocytes	present [18]	absent *
NEM	activation in HIS, no effect in LIS [19]	inhibition [20]
decrease of extracellular pH	inhibition of K^+ influx [8]	no effect [21]
Cl-dependence	no [22]	yes [16]
NaCl replaced by NMDG-Cl	no effect [8]	inhibition [16]
NaCl replaced by Na-gluconate	no effect [8]	activation [16]

ues (which have not been employed in experiments in which the $K^+(Na^+)/H^+$ exchanger has previously been characterised), does the NSVDC channel start to play a significant role.

The results presented here clearly show that HOE642 exerted differential effects on the NSVDC channel and the $K^+(Na^+)/H^+$ exchanger. Furthermore, the addition of HOE642 to a modified HIS solution did not result in a change of the Cl⁻-dependent K^+ flux mediated by the KCC.

It is important to note that, after transferring cells from HIS to LIS solutions, a new steady-state is quickly established, associated with an increase of the internal pH from 7.2 to approximately 7.7 [9] which results in a depolarization from -10 mV to 18.5 mV. Although this calculation (which assumes a Nernst distribution of H^+) will be an underestimation given that in both extracellular and intracellular compartments there will be a significant buffer capacity (the extracellular solution was buffered in the investigations of the low ionic strength effect), the transmembrane potential calculated here for cells in LIS solution is below the level at which significant activation of the NSVDC channel will occur [4]. Depolarizing the RBC membrane from negative to positive values, the NSVDC channel starts to open at potential values more positive than 30 mV. It seems likely that such a value is not achieved in LIS solution.

Characterisation of the NSVDC channel conductivity showed that it was activated from positive potentials of about 100 mV and consequently achieves a high activity state [17], such that channel-mediated K^+ flux will dominate salt loss. Since the conductance is calculated from the electrogenic properties only, a contribution from the electrically-silent $K^+(Na^+)/H^+$ exchanger are not apparent. This means that the effects of HOE642 on the NSVDC channel that have been observed must be independent of any activity of the $K^+(Na^+)/H^+$ exchanger.

The notion that the $K^+(Na^+)/H^+$ exchanger and the NSVDC channel are separate pathways is supported by a comparison of divergent properties reported in the literature that are summarised in Table 2.

In a fundamental investigation of the low ionic strength effect by Donlon and Rothstein [23], where the authors decreased the extracellular ionic strength to a very low level (close to 0), the increase of the net K^+ loss was described as a triphasic response (a curve with two inflection points). The first inflection point (estimated at 45 mV) probably reflects the transition from a situation in which $K^+(Na^+)/H^+$ exchange dominates to one in which the NSVDC channel is most important. The second reflection point at very low ionic strength (at a calculated transmembrane potential of around 170 mV) may represent the beginning of an electrical break-down of the membrane.

Our studies of the effect of HOE642 on residual K^+ transport and on the conductance properties of a RBC suspension clearly show that the salt loss observed in LIS solutions represents contributions from at least two independent ion transport pathways, the electroneutral $K^+(Na^+)/H^+$ exchanger and the electrogenic NSVDC channel.

This finding might be of relevance in identifying the processes underlying red cell apoptosis (eryptosis) and disease. A variety of reports have identified an important role for the NSVDC channel in eryptosis induced by osmotic shock, oxidative stress, and energy depletion [24-28]. In addition, sickle cells, thalassemic cells, and glucose-6-phosphate dehydrogenase deficient erythrocytes have been shown to be more sensitive to these pathophysiological conditions [29]. In all cases, eryptosis is induced following an increase of cytosolic Ca^{2+} that subsequently activates the lipid scramblase, thereby translocating phosphatidylserine from the inner to the outer leaflet of the membrane. Furthermore, in a recent

publication it has been demonstrated that the Na⁺/H⁺ exchanger is involved in phosphatidylserine exposure in human platelets during activation [30]. Whether the

K⁺(Na⁺)/H⁺ exchanger plays a significant role in eryptosis and red blood cell disease remains to be elucidated.

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