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The effect of ATP, intracellular calcium and the anion exchange inhibitor DIDS on conductive anion fluxes across the human red cell membrane

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The influence of ATP depletion, the intracellular ionized Ca-concentration, anion substitution and DIDS on the conductive anion fluxes across the human red cell membrane has been examined. Under physiological or near physiological conditions it is not possible to observe conductive anion fluxes across the erythrocyte membrane in that anions totally dominate the membrane conductance. Consequently anions are at electrochemical equilibrium and the netflux is zero. However, conductive anion fluxes can be induced by raising the potassium conductance, either by addition of valinomycin, or by triggering the native calcium activated potassium channel by addition of the Ca^{2+} ionophore A23187 to cells suspended in a calcium containing medium. The interpretation of data from experiments with valinomycin induced netfluxes has normally been done according to a constant field model, and the results have consequently been given as permeabilities. Since it has been demonstrated recently, that these cation pathways do not conform to a constant field scheme (Bennekou, P. and Christophersen, P. (1986) J. Membr. Biol. 93, 221–227 and Vestergaard-Bogind, B., Stampe, P. and Christophersen, P. (1985) J. Membr. Biol. 88, 67–75), it has been chosen, instead of permeabilities, to calculate the ion conductances from net efflux data, using an independent estimate of the membrane potential. The main result reported, is that only one component is found for the conductive anion fluxes in the presence of DIDS using the latter theoretical framework, whereas a sizeable DIDS-insensitive component is found when the constant field analysis is used. Furthermore it is found that ATP and intracellular calcium do not influence the anion conductances.

Introduction

Two major transport modes are found for the translocation of anions across the human red cell membrane. Exchange transport, where an anion is exchanged against another anion, either as self exchange or as hetero exchange. For Cl^- self exchange an apparent permeability at pH 7.2 and 38°C of about $5 \cdot 10^{-4}$ is found [3].

In the net flux mode anions are translocated with a counter ion along an electrochemical gradient. Since the human red cell membrane normally is very impermeable to cations, it is necessary either to introduce or to activate a cation pathway to induce sizeable net fluxes.

At least two different methods are at hand for inducing an increased cation conductance in human red cells. Either addition of a cation ionophore, among which valinomycin is preferable, since it is highly K^+ selective, or by adding a calcium ionophore to increase the cellular Ca^{2+} level, thus activating the Ca^{2+}-activated K^+ channels.

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The standard method used to characterize the conductive anion flux across the membrane of human red cells has been to add valinomycin to a cell suspension to initiate a KCl efflux into Ringers with varied potassium concentrations. An apparent constant field permeability for the anion can then be calculated by a fit of the efflux data to the constant field equation, as proposed by Hunter [4]. In this treatment the membrane potential appears as an implicit variable.

A different approach has been to calculate the membrane potential from the protonophore-mediated electrochemical equilibrium distribution of protons across the membrane [5]. Using this method, an anion conductance can be calculated, which in turn can be expressed as an equivalent constant field permeability [6].

Although the results from these two methods differ considerably, the anion net flux permeability, however, is found to be four orders of magnitude below the exchange permeabilities, whichever of the methods is used.

The functional relationship between the exchange and net flux mechanisms has still not been finally determined. Among the various attempts which have been made to elucidate the relationship between anion exchange and net fluxes across the red cell membrane, a common technique has been to examine the effect of exchange inhibitors, especially DIDS, on both exchange and net fluxes.

DIDS binds highly selective to the anion exchange protein [7,8], and inhibits both exchange and net anion fluxes, which is taken to indicate a common step for these two translocation modes.

When using the constant field approach, the maximal extent of DIDS inhibition of the net fluxes have been reported to be 70–80%, thus leaving a DIDS insensitive component of 20–30% [9,10] under conditions where the exchange fluxes are inhibited almost 100% [11].

However, these estimates have been obtained using the constant field method [4], and since it has been shown [1] that some of the assumptions used in this approach are invalid, we have used the protonophore-mediated steady-state pH, attained following an increase in potassium conductance, for estimation of the membrane potential to characterize the effect of DIDS on the anion net fluxes.

The results presented here show that the maximum inhibition of the conductive anion fluxes is at least 95%, a finding which questions the existence of a DIDS-insensitive component of the normal anion net flux pathway.

Materials and Methods

Chemicals. All inorganic salts (pro analysis) and di-*n*-butylphthalate were purchased from Merck, Darmstadt. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonate) and the ionophores CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), valinomycin and A23187 were from Sigma, St. Louis. Sucrose (Aristar) were from BDH, Poole. The test combination for determination of hemoglobin was from Boehringer, Mannheim.

Cells. Freshly drawn blood from healthy human donors was heparinized and centrifuged and the plasma and buffy coat were removed by aspiration. The cells used for the valinomycin experiments were washed three times in isotonic Ringers solution. In experiments where the cation conductance was increased by opening of the Ca^{2+}-activated K^+ channel, the cells were first washed twice in 5 vol. K^+-equilibrium Ringer (90 mM KCl/66 mM NaCl/150 μM $MgCl_2$ (pH $\approx$ 7.4)) containing 50 μM EGTA. The cells were thereafter depleted of ATP and 2,3-diphosphoglycerate as previously described [12], and washed three times in K^+-equilibrium Ringer.

In the experiments where chloride was replaced by other anions, the cells were washed three times in an equivalent salt solution in which the chloride was substituted by the appropriate anion. When Cl^- was substituted by NO_3^- or SO_4^{2-} the cellular phase was titrated for chloride to ensure that the replacement was complete.

The cells were then loaded with Ca marked with ^{45}Ca using 0.5 to 1 μmol of ionophore A23187 per litre cells, see Ref. 13 for details.

Phthalate method. The cellular contents of K^+, Na^+, and ^{45}Ca and the extracellular concentration of K^+ were determined using the phthalate method as previously described [2].

During an experiment 100-μl samples of the cell suspension (hematocrit $\approx$ 3.1%) were transferred to the phthalate tubes, and 5 s later the

tubes were centrifuged for 30 s at 18000 × g. Approx. 3.1 μl of cells were now isolated as a pellet under the phthalate layer. The extracellular concentration of K^+ was determined by flame photometry on the top phases of the phthalate tubes. The rest of the top phases and the phthalate were removed and the cell pellets were processed for flame photometric determination of the K^+- and Na^+-content and, in experiments with Ca^{2+}-loaded cells, for scintillation counting of the ^{45}Ca content.

Membrane potential. Changes in membrane potential (V_m) were determined according to the method of Macey et al. [5]. The experiments were carried out with cells suspended in a buffer-free salt solution at a hematocrit of 3.1% in the presence of 20 μM of the protonophore CCCP, which mediates a fast electrochemical equilibration of protons across the cell membranes. Since the intracellular phase is heavily buffered, a change in V_m results in a shift in the extracellular pH to a new equilibrium value, determined by the constant intracellular proton activity and the membrane potential. At the end of an experiment Triton X-100 was added, resulting in immediate hemolysis of all cells. Since all buffering capacity was confined to the cellular phase, the pH of the hemolysate reflected the original cellular pH which remained constant during the experiment. Absolute V_m values were then calculated from the differences between the peak values of extracellular pH and the corresponding cellular pH values.

Experimental procedure. In all experiments one volume of packed cells were transferred into 30 vol. of buffer-free isotonic salt solution, containing 3 mM KX and 153 (123.5 when X, the anion, was sulphate) mM NaX with 20 μM CCCP and varied concentrations of DIDS, thermostatted at 37 °C and vigorously stirred.

In the experiments with Ca^{2+}-loaded cells the efflux started instantaneously since the cells were transferred with open K^+ channels into the salt solutions. When non ATP depleted cells were used, the efflux was initiated by addition of valinomycin.

During the first approx. 90 seconds of each experiment 100-μl samples of suspension were transferred to cold-stored phthalate tubes (see above) at intervals of about 7 s, to determine the net efflux of K^+ from the cells, while the extracellular pH was recorded. At the end of an experiment samples were taken for determination of the total compartments of K^+ and hemoglobin (compartments, whether intracellular, extracellular or total, are here taken as an amount per litre of cells). The total hemoglobin compartment was used as a control for the amount of cells, and the hemoglobin concentration in the extracellular phase was measured and used for calculation of hemolysis during the experiment. Only experiments in which the hemolysis was below 1% were used. In each experimental series the initial cellular contents of water and K^+ were determined on samples from the packed cells stored on ice.

Calculations

Since the transport mediated by both valinomycin and the native K^+ channel is highly K^+ selective, the salt lost from the cells is KX (X representing the anion). In that electroneutrality is maintained, the net efflux of anion in mmol/litre cells per hour can be calculated from the initial, linear decrease in cellular K^+ content. The amount of cells in each experiment was determined from the concentration of total hemoglobin in the suspension. Due to the rather low net fluxes in the present experiments, from 1200 mmol/litre cells per hour to 50, the initial loss of cellular anion will increase the Nernst potential, E_X, with about 1 mV within the first 30 s. Since this change is within the experimental uncertainty, it has not been considered necessary to calculate the individual corrections as previously described [6], and the initial Nernst potentials were used in the calculations instead of the values at the peak membrane potential. The initial value, E_X, was calculated from the difference in cellular and extracellular pH before the cells were transferred to the reaction vessel.

The membrane potential (V_m) was calculated from CCCP-mediated new electrochemical equilibrium of protons across the cell membranes (see section on methods). A pH-difference ($pH_{ex} - pH_c$) of one unit is equivalent to a membrane potential of 61.5 mV (inside negative).

The anion conductance, g_X was calculated from the equation of Hodgkin and Huxley [14]

$$I_X = z_X \cdot J_X \cdot F = (V_m - E_X) \cdot g_X \quad (1)$$

where I_X is the current of anions across the membrane, z_X is the charge, J_X is the anion netflux and F is the Faraday constant.

Apparent constant field permeabilities were calculated by multiplying the constant field flux equation with Faradays number and the charge number, and equating with the Hodgkin-Huxley current equation (Eqn. 1) see Ref. 15 for details.

The red cell membrane area was taken to be $1.75 \cdot 10^7$ cm^2 per litre of packed cells, when the cells were suspended in 156 mM NaCl and $pH_{cell} = 7.2$, 37 C°.

Results

In order to induce sizeable net fluxes from human red cells into low potassium Ringers the very low 'resting' conductance for K^+ across the human red cell membrane was increased, either by using ATP-depleted Ca^{2+}-loaded cells, a treatment which opens the Ca^{2+}-activated K^+ channels, or by adding the K^+-ionophore valinomycin to the cell suspension. Using either of these two methods a highly selective KCl efflux is induced, and for all practical purposes the K^+ net efflux is identical to the Cl^- net flux, since electroneutrality is maintained.

Since both Ca^{2+}-loaded (ATP-depleted) cells and cells with normal physiological Ca^{2+} content were used in the experiments, the effect of the intracellular Ca^{2+} concentration and the presence of ATP on the chloride concentration was examined.

It was found, that neither the intracellular Ca^{2+} level in the interval up to 240 μM ionized calcium (see Fig. 1), nor the ATP depletion (not shown) had any effect on Cl^- conductance.

In three series of experiments with varying intracellular Ca^{2+} concentrations, see Fig. 1, the chloride conductance was found to be independent of the Ca_{cell}. Mean values and standard deviations for the chloride conductance and equivalent constant field permeability are shown in Table I.

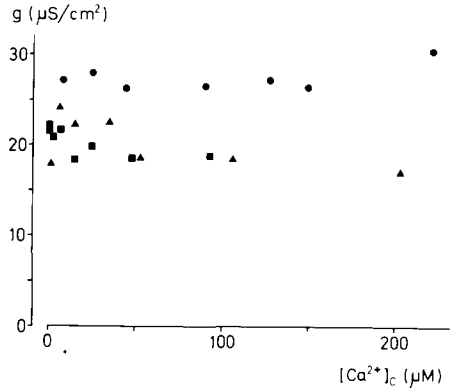

Fig. 1. The figure shows the effect of cellular Ca^{2+} on the chloride conductance. The different symbols represent dose-response experiments on three batches of cells. The ordinate is the anion conductance in μS/cm^2, the abscissa is the cellular concentration of ionized Ca in μmol per litre cell water.

When chloride was replaced by SO_4^{2-}, Br^-, NO_3^- and I^- the ratio between the conductances was found to be 0.48 : 1.0 : 1.48 : 2.12 : 2.49 for the sequence sulphate, chloride, bromide, nitrate and iodide. See Table II for the absolute values of conductances and equivalent constant field permeabilities.

When DIDS is added to a suspension of cells with induced high potassium conductance, an increasing saturating hyperpolarization is seen with increasing DIDS concentration (see Fig. 2), whereas the KCl efflux attains a minimum at about 6 μM DIDS, and thereafter it seems to

TABLE I

CONDUCTANCE AND PERMEABILITY

The three columns showing the permeabilities correspond to the columns showing the conductances. The variation in conductance between the three series is in the accordance with the interdonor variation reported previously [6]. The high value of the permeability in the middle column is due to a lowered extracellular chloride concentration (140 mM) compared to the normal 156 mM. The apparent constant field permeabilities are very sensitive to changes in the chloride ratio, compared to the conductances [25].

	g_L (μS/cm^2)			P_{Cl} (10^{-8} cm/s)		
Expt.	1	2	3	1	2	3
Mean	20.3	27.5	19.3	4.20	8.70	4.67
S.D.	1.52	1.49	3.55	0.27	0.46	0.93
n	8	7	8	8	7	8

TABLE II

CHORD CONDUCTANCE AND PERMEABILITY

Chord conductances, g_X, and the corresponding equivalent constant field permeabilities, P_X.

	g_X ($\mu S/cm^2$)	P_X (10^{-8} cm/s)
SO_4^{2-}	11.0	1.87
Cl^-	27.6	7.63
Br^-	41.0	11.3
NO_3^-	61.6	16.8
I^-	68.6	18.9

re-increase slightly with increasing DIDS concentration (see Fig. 2). Accordingly the calculated apparent conductance passses through a minimum too at about 6 μM DIDS, where the maximum inhibition is found (see Fig. 3).

Although the conductances for Cl^-, NO_3^- and SO_4^{2-} are different under comparable conditions, the DIDS inhibition of the net fluxes of these ions show identical patterns, and the normalized conductances as function of the DIDS concentration can be described by a single curve (see Fig. 4).

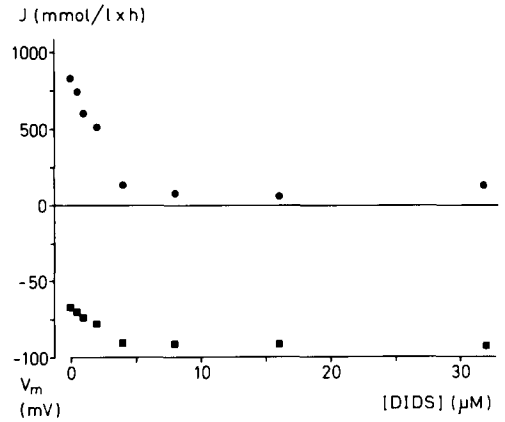

Fig. 2. The figure shows (upper half) the chloride net flux and membrane potential (lower half) as function of the DIDS concentration. The upper ordinate axis is the net flux in mmol/litre cells per hour, the lower ordinate axis is the membrane potential in mV. The abscissa axis is the DIDS concentration in μmol per litre extracellular water. The DIDS concentrations were calculated from the total amounts added to the suspension. With the hematocrit used (3.1%), the number of DIDS molecules at a 1 μM concentration is about two times the number of band 3 binding sites, and the free DIDS-concentration was thus below the nominal concentration, especially at the lower end of the concentration range.

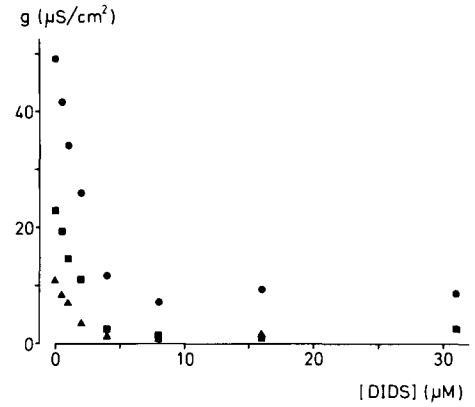

Fig. 3. Anion conductances as function of the nominal DIDS concentration. (■) Cl^-, (●) NO_3^- and (▲) SO_4^{2-}. The ordinate axis is $\mu S/cm^2$ and abscissa axis DIDS concentration in μmol per litre extracellular water.

Discussion

Since it was originally established, that cation fluxes across the human red cell membrane could be anion restricted [16,17], attempts have been made to characterize the anion net translocation pathway and its relation to the exchange pathway.

It was originally proposed by Hunter [4], that a fit of efflux data for valinomycin induced KCl fluxes to a constant field equation for K^+ and Cl^- could be used for a calculation of the chloride

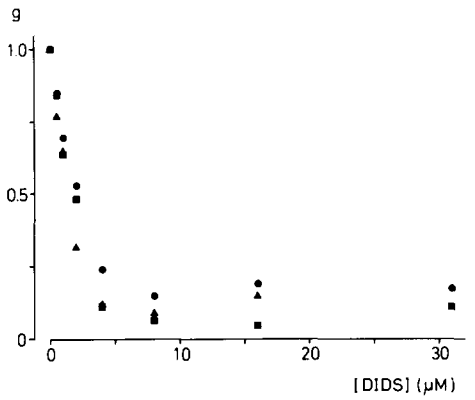

Fig. 4. The figure shows (ordinate axis) normalized anion conductances (that is the conductance at a given DIDS concentration divided by the uninhibited value) as function of the DIDS concentration. Symbols and abscissa axis as in Fig. 3.

permeability. The reservation inherent in this theoretical approach is of course that both chloride and potassium ions should follow constant field kinetics.

For chloride no information concerning the translocation mechanism exists. Furthermore it has been shown in experiments with lipid membranes, that the valinomycin-induced K^+ fluxes conform to a carrier kinetics scheme, which only under certain limiting conditions can be approximated to free electrodiffusion [18].

It has recently been shown, that the valinomycin-induced K^+ fluxes across the erythrocyte membrane do not conform to such limiting conditions [1], and it is not feasible to use a constant field treatment. Consequently, although this treatment gives an order of magnitude estimate of the chloride net permeability, as stated by Hunter [19] it is not sufficiently accurate to allow detailed (quantitative) comparisons between anion net and exchange fluxes.

A different approach has therefore been used for the characterization of anion net fluxes. Since it has been demonstrated, that red cell membrane potentials can be estimated using protonophore mediated pH changes [5] and KCl net fluxes can be estimated from concentration data, it is possible to calculate a chord conductance according to the treatment given by Hodgkin and Huxley [14]. To facilitate references to earlier works, apparent constant field permeabilities based on the conductances are given too, but in our opinion no physical significance can be attached to these permeabilities.

Ca^{2+} and ATP

It has been reported, that ATP depletion and Ca^{2+} loading of human red cells inhibit the monovalent anion exchange [20] and sulphate exchange [21], in the latter case with an apparent inhibition constant of about 6 μM. Within the experimental uncertainty, which is about 10%, no effect of depletion to a level below 1 μM ATP or Ca loading up to 240 μM ionized calcium is seen on the calculated chloride conductances. Thus the present experiments do not support that a raised intracellular Ca^{2+} level increases the chloride net permeability as proposed by Bürgin and Schatzmann [22]. A possible explanation for this discrepancy could be that the Ca^{2+} concentration used in the present experiments do not exceed 240 μM, which may be well below the concentrations used by Bürgin and Schatzmann [22] (who used 1 mM in the extracellular solution), but more probably that that they estimate the membrane potential from the Nernst potential for potassium ions. Since the highest obtainable K^+ conductance across the red cell membrane through the Ca^{2+}-activated K^+ channel is only 3 to 4 times the chloride conductance [13], the membrane potential will differ from the K^+ Nernst potential if the K^+ and Cl^- Nernst potentials differ.

Selectivity sequence

Although the absolute conductance found in the present paper deviate from other determinations (due to the different theoretical approach) the selectivity sequence is in agreement with the sequence found earlier [19], and conform to an Eisenmann sequence I, representing a weak binding site for anions [19,23]. The absolute value of the equivalent constant field permeability for chloride, calculated from the conductance (see Table II), are 2 to 4 times higher than the values previously reported [9,10,19,24]. In our opinion the reason for this is that earlier works, using the constant field approach have overestimated the membrane potential, and thereby the driving force for the efflux, and thus underestimated the permeability [1].

DIDS inhibition

The almost complete inhibition of the anion conductance in the presence of DIDS, which is found in the present experiments, indicates that in the normal human red cell, only one major transport pathway exists for the net anion flow. That the inhibition for the different ions can be described by a single curve, see Fig. 4, supports the notion, that this pathway is a common pathway for these inorganic anions.

A maximum inhibition of 90 to 95% is found at a nominal DIDS concentration of about 6 μM. When the DIDS concentration is increased further, the apparent conductance increases again due to an increase of the net flux, whereas the membrane potential still show maximal hyperpolarization (see Figs. 2 and 3). This indicates that

DIDS, at higher concentrations, induces a non-conductive pathway for chloride, and that the inhibition of the normal conductive anion pathway could approach the 99 + % level found at maximal inhibition of the exchange pathway. At present we have no information regarding this tentative non-conducting pathway.

This result is at variance with earlier works, which reports a sizeable, about 33%, DIDS-insensitive component [9,10]. A comparable inhibitor insensitive fraction has been reported for a related inhibitor, DNDS [24].

In our opinion this DIDS-insensitive component appears as a consequence of the constant field treatment, which introduces an error in the (implicit) estimate of the driving force for the flux as discussed above. This error becomes smaller the closer the membrane potential is to the K^+ Nernst potential, that is the more hyperpolarized the membrane is, and the final (inhibited) permeability is thus compared to an initial (uninhibited) value which is to low. This is illustrated by the high normal values reported in the present paper (see Table II).

Although more work is necessary before it is possible to discuss the significance of the present results for the various mechanisms proposed for the anion net flux pathway and the functional relation to the exchange protein, the parallel effects of DIDS on the exchange and net anion fluxes are indicative of a close relationship, but at present no explanation exists for the differences between exchange and net fluxes with regard to the effects of ATP and calcium.

Conclusion

The present work represents a first attempt to reevaluate the characteristics of the conductive pathway for anions in human red cells, a reevaluation which has become necessary in that it has been shown, that an earlier, commonly used theoretical model for the transport is insufficient.

It has been demonstrated, that the DIDS inhibition of the conductive fluxes of a selection of different inorganic anions follows the same pattern, thus supporting the notion that these ions use a common pathway.

The main result however, is that only one pathway exists for the conductive anion translocation across the human red cell membrane.

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