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Flux Ratio of Valinomycin-Mediated K^+ Fluxes across the Human Red Cell Membrane in the Presence of the Protonophore CCCP

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Summary. The ratio of valinomycin-mediated unidirectional K^+ fluxes across the human red cell membrane, has been determined in the presence of the protonophore carbonylcyanide *m*-chlorophenylhydrazone, CCCP, using the K^+ net efflux and ^{42}K influx. The driving force for the net efflux ($V_m - E_{K^+}$) has been calculated from the membrane potential, estimated by the CCCP-mediated proton distribution and the Nernst potential for potassium ions across the membrane. An apparent driving potential for the K^+ net efflux has been calculated from the K^+ flux ratio, determined in experiments where the valinomycin and CCCP concentrations were varied systematically. This apparent driving force, in conjunction with the actual driving force calculated on basis of the CCCP estimated membrane potential, is used to calculate a flux ratio exponent, which represents an estimate of the deviation of valinomycin-mediated K^+ transport from unrestricted electrodiffusion, when protonophore is present.

In the present work, the flux ratio exponent is found to be 0.90 when the CCCP concentration is 5.0 μM and above, while the exponent decreases to about 0.50 when no CCCP is present. The influence of CCCP upon the rate constants in the valinomycin transport cycle is discussed. The significance of this result is that red cell membrane potentials are overestimated, when calculated from valinomycin-mediated potassium isotope fluxes, using a constant field equation.

Key Words flux ratio · valinomycin · CCCP · red cells · membrane potential

Introduction

Determination of membrane potential in cells, where only one side of the membrane is accessible, is possible by indirect means only.

The methods used for this purpose are either equilibrium methods, based upon determination of distribution ratios, or dynamic methods, based upon determination of unidirectional fluxes of a charged compound, measured as tracer fluxes, with the provision that the compound in question conforms to unrestricted electrodiffusion. If this condition is fulfilled the ratio of the unidirectional fluxes is described by Ussing's flux ratio equation [13].

The former method has been used in a number of papers [2, 12, 14], mainly dealing with determination of the conductance through the Ca^{2+}-sensitive K^+ channel in human red cells. In these experiments the protonophore CCCP, carbonylcyanide *m*-chlorophenylhydrazone, as proposed by Macey et al. [11], has been used to mediate a change in extracellular pH, reflecting a change in membrane potential.

The latter method forms the basis for a series of papers [6–8] dealing with determination of human red cell anion permeability, where the estimation of membrane potential is based upon a constant field approach, as proposed by Hunter [5], after separate determination of K^+ tracer permeability in red cells suspended in K^+ equilibrium Ringer's in the presence of the K^+ ionophore valinomycin. In both cases, a set of requirements have to be fulfilled in order to obtain reliable results.

In the protonophore case it is assumed that the protonophore anion translocates through an electrodiffusional pathway only.

The applicability of flux ratio values for determination of membrane potentials is based upon independent ion fluxes. For the valinomycin-mediated potassium fluxes this independence can be questioned, since carrier-mediated fluxes conform to the Ussing flux ratio [9] only when the substrate concentration is well below saturating levels and if the translocation of the charged complex is slower than that of the unloaded carrier.

In the present paper, the driving force for valinomycin-mediated K^+ net efflux from human red cells has been calculated, using both the protonophore equilibrium method and the flux ratio approach, in order to evaluate the applicability of the respective methods.

Materials and Methods

Chemicals

All inorganic salts (p.a.) were purchased from Merck, Trizma and carbonylcyanide *m*-chlorophenylhydrazone was obtained from Sigma, valinomycin from Calbiochem, and di-*n*-butylphthalate from BDH. CCCP and valinomycin were administered as concentrated alcoholic solutions.

Cells

Freshly drawn, heparinized human blood from healthy donors was centrifuged, and plasma and the buffy coat were removed by aspiration. The cells were washed twice in 5 volumes of high potassium Ringer's solution (90 mM KCl, 66 mM NaCl, 0.15 mM $MgCl_2$ and 0.05 mM EGTA, pH 7.4), suspended in the washing medium at a hematocrit of about 15%, and stored on ice.

Analysis

Initial values of cell water fraction and cellular contents of K^+, Na^+ and hemoglobin were determined as previously described [1].

In order to determine the variation of the cellular contents of ^{42}K, K^+, Na^+ and extracellular concentrations of K^+ during an experiment, the extracellular and cellular phases of samples were separated by the phthalate method [12]. 100-μl samples of cell suspension, hematocrit 3.1%, were transferred to ice-cold microcentrifuge tubes containing 875 μl of a solution (155 mM Trizma acetate, 2.0 mM EGTA, 3.3 mM $LiNO_3$, pH 7.7) layered on top of 400 μl di-*n*-butylphthalate. After 5 sec the tubes were centrifuged for 30 sec at 18,000 × *g*. The cells were now isolated as a pellet under the phthalate layer. Extracellular concentrations of K^+ were determined by flame photometry directly on the top phases of the phthalate tubes. The rest of the top phases and the phthalate layer were removed and the cell pellets processed for scintillation counting of the ^{42}K and determination of cellular K^+ and Na^+ content by flame photometry.

The extracellular potassium concentrations were used in a calculation according to a previously published model [1], dealing with changes in cell volume under net efflux conditions to obtain the potassium concentration per liter cell water. The K^+ Nernst potential, which varies with time under net efflux conditions, can then be calculated.

Experimental Procedure

1000 μl stock suspension of cells were centrifuged, washed once in the incubation medium, whereafter 100 μl packed cells were transferred to 3000 μl of this solution (154 mM NaCl + 2 mM KCl) containing ^{42}K, thermostatted at 37°C. The experiments were started by addition of 6 μl ethanolic solution of valinomycin and CCCP in various concentrations, and a sample of the suspension was taken for analysis every 10 to 15 secs. At the end of each experiment 100 μl of a 1% solution of Triton X-100 was added to the suspension, causing total hemolysis.

Electrodes and Amplifiers

pH in the suspensions were measured with a Radiometer G2222C glass electrode with a K701 calomel electrode as reference, connected to a Radiometer TTT 2 pH-meter. The K701 is a calomel electrode with an outer (Cs_2SO_4) salt bridge to prevent KCL efflux from the electrode to the medium.

Calculations

Since the cell suspension can be considered a closed system with regard to potassium and potassium isotope, mass conservation yields:

$$J_{net} = J_{ef} - J_{in} \tag{1}$$

where J_{net} represents the net flux and J_{ef} and J_{in}, unidirectional ef- and influxes.

Furthermore,

$$S_{in} + S_o = S_T \tag{2}$$

and

$$P_{in} + P_o = P_T \tag{3}$$

where S is the chemical compartment, P the tracer compartment, and subscripts *in*, *o* and *T* denote intracellular, extracellular and total compartments. Note that both the extracellular and cellular chemical compartments, as well as the isotope compartments, vary with time. The frame of reference, the compartments, is the amount of substance in question per liter cells.

The change with time of the extracellular tracer compartment can be written as

$$\frac{dP_o(t)}{dt} = -\frac{dP_{in}(t)}{dt} = J_{ef}\frac{P_{in}(t)}{S_{in}(t)} - J_{in}\frac{P_o(t)}{S_o(t)}. \tag{4}$$

Inserting Eqs. (1), (2) and (3) in Eq. (4), and solving for J_{in}, one arrives at

$$J_{in} = \frac{\left[\frac{dP_{in}(t)}{dt}\right] + J_{net}\left[\frac{P_{in}(t)}{S_{in}(t)}\right]}{\left[\frac{P_T - P_{in}(t)}{S_T - S_{in}(t)}\right] - \left[\frac{P_{in}(t)}{S_{in}(T)}\right]}. \tag{5}$$

The ratio between the unidirectional fluxes is:

$$Q = \frac{J_{ef}}{J_{in}} = \frac{J_{net} + J_{in}}{J_{in}}. \tag{6}$$

Finally, after insertion of Eq. (5) in Eq. (6) the flux ratio expression becomes

$$Q = \frac{\left[\frac{P_T - P_{in}(t)}{S_T - S_{in}(t)}\right] + \left[\frac{1}{J_{net}}\right] \cdot \left[\frac{dP_{in}(t)}{dt}\right]}{\left[\frac{P_{in}(t)}{S_{in}(t)}\right] + \left[\frac{1}{J_{net}}\right] \cdot \left[\frac{dP_{in}(t)}{dt}\right]}. \tag{7}$$

Equation (7), in which the individual elements can be determined experimentally, was used for calculation of the flux ratio under net flux conditions, where the chemical compartments, as well as the cellular and extracellular volumes, are subject to changes.

For ions moving independently through the membrane,

$$Q = \frac{J_{ef}}{J_{in}} = \frac{C_{in}}{C_o} e^{\frac{zF}{RT}V_m} = e^{\frac{zF}{RT}(V_m - E_{K^+})} \tag{8a}$$

which is Ussing's flux ratio equation for unrestricted electrodiffusion [13]. C_{in} and C_o denotes cellular and extracellular K^+ concentrations, V_m the membrane potential, and E_{K^+} potassium Nernst potential. z, R, T and F have their usual meanings.

For transport mechanisms, which do not fulfill the independence requirements [9], Q can be expressed as

$$Q = e^{n\frac{zF}{RT}(V_m - E_{K^+})}. \tag{8b}$$

An apparent driving force for the valinomycin mediated K^+ flux can be estimated from

$$n(V_m - E_{K^+}) = \frac{RT}{F} \ln_e Q. \tag{9}$$

Membrane Potential

Changes in membrane potential were estimated from the CCCP-mediated shift in extracellular pH, according to

$$V_m = (pH_{cell} - pH_o) \cdot 61.5 \text{ mV} \tag{10}$$

where V_m is the membrane potential corresponding to a measured change in the pH in the extracellular medium. At 37°C Eq. (10) is valid, provided the cells are suspended in a medium with low buffer capacity less than 1% of the buffer capacity in the cells [10]. The intracellular pH is obtained by measurement of the pH in the hemolyzate after addition of detergent.

Conductances were calculated according to Hodgkin and Huxley [4], from

$$i_{K^+} = g_{K^+}(V_m - E_{K^+}) \tag{11}$$

where i_{K^+} is the current density (FxJ_{net}) due to the flux of potassium ions, and g_{K^+} the conductance, or from an equivalent expression for chloride.

Cell area was taken as 4.2×10^7 cm²/kg dry matter.

Results

When valinomycin and CCCP were added to a suspension of red cells, with ^{42}K in the extracellular medium a net efflux of KCl started immediately, concurrent with a ^{42}K tracer influx (*see* Fig. 1). In the initial period the extracellular pH increased, reflecting the hyperpolarization caused by the valinomycin-induced increase in K^+ conductance. When the extracellular pH had reached a plateau, after about 30 sec (*see* Fig. 1), the membrane potential could be calculated from Eq. (10) and at the same point the K^+ flux ratio could be calculated from Eq. (7). Since the K^+ efflux caused by valinomycin is practically instantaneous, the time from valinomycin addition to the plateau in extracellular pH is reached is the time in which CCCP redistributes.

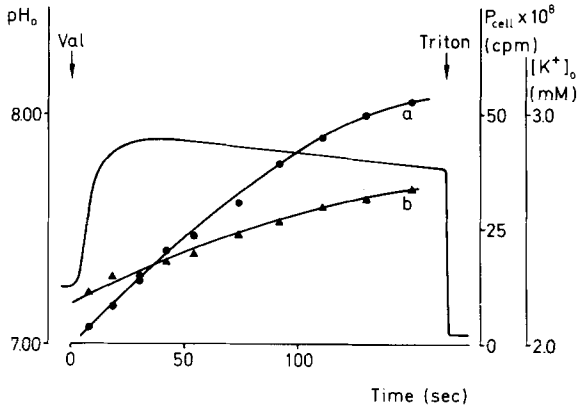

Fig. 1. Time course of the extracellular pH (solid line), extracellular potassium concentration (curve *b*) and intracellular ^{42}K activity (curve *a*) after addition of valinomycin to the cell suspension at time 0 (arrow). At the end of the experiment, Triton X-100 is added to the suspension (arrow), causing hemolysis of the cells. The cellular tracer activity, P_{cell}, is given in counts/min · liter cells

Three types of experiments have been performed: valinomycin dose-response and CCCP dose-response experiments under net efflux conditions, with the extracellular K^+ concentration initially at 2 mM, and CCCP dose-response experiments under equilibrium conditions, where the extracellular potassium concentration was 100 mM.

Valinomycin Dose-Response Experiments

With CCCP kept constant at 2.0×10^{-5} moles/liter H_2O, and the valinomycin concentration varied, in the interval 2.5×10^{-8} to 1.0×10^{-6} moles/liter H_2O, the membrane potential ranges from −30 to −75 mV. Since the K^+ Nernst potentials at the time where the extracellular pH reaches a plateau, about 30 sec after addition of valinomycin (*see* Fig. 1), are nearly identical (−106 to −108 mV) in these experiments, the driving force for the K^+ net efflux ranges from 30 to 75 mV.

Figure 2 shows the appararent driving potential (flux ratio based, Eq. (9)) plotted against $V_m - E_{K^+}$, where the membrane potential is estimated from the CCCP-mediated changes in extracellular pH. The flux ratio exponent n, is found as the slope to the curve shown in Fig. 2. Estimated by linear regression, n is found to be 0.90.

CCCP Dose Response

When the protonophore was added to a suspension of red cells, an increase in KCl net efflux as well as

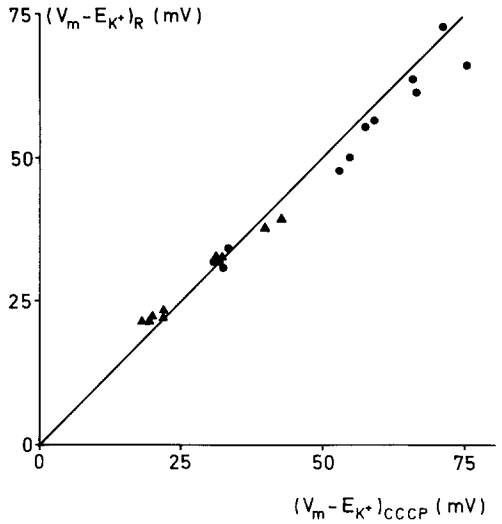

Fig. 2. Driving potentials for K^+ net efflux, determined from the flux ratio equation, ordinate axis, plotted against the driving potential determined as the difference between the membrane potential estimated from the changes in pH and the potassium Nernst potentials (abscissa). Data from CCCP (▲) and valinomycin dose-response experiments (●). The solid line is the theoretical value for a flux ratio exponent equal to one. Units are in millivolts

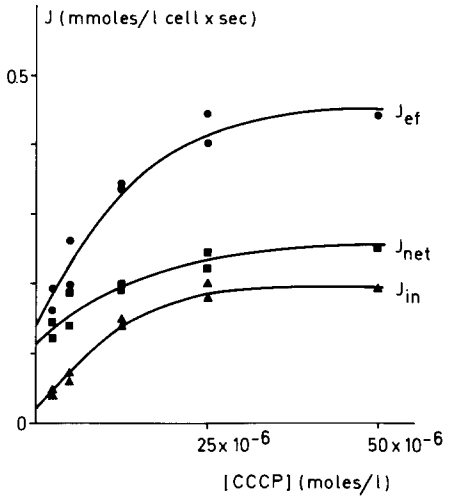

Fig. 3. CCCP dependence of potassium net flux (■) and unidirectional influxes (▲) and effluxes (●) in mmol/liter cells · sec (ordinate). Abscissa axis is CCCP concentration in moles/liter H_2O. The valinomycin concentration was $0.5 \cdot 10^{-6}$ moles/liter H_2O in all experiments. Lines were drawn by eye

in the unidirectional fluxes were observed with increasing protonophore concentration (*see* Fig. 3). Furthermore, the protonophore increases the valinomycin-induced hyperpolarization (*see* Fig. 4). In these experiments, the CCCP concentration ranged from 1.0 to 50 μM, with valinomycin kept constant at 0.5 μM.

When the valinomycin-induced potassium conductance is calculated according to Eq. (11), with the membrane potential calculated from Eq. (10), and the K^+ Nernst potential calculated from the flame photometric determination of extracellular and intracellular K^+ concentrations, the conductance is found to be an increasing, saturating function of the CCCP concentration (*see* Fig. 4).

In Fig. 2, the apparent driving potentials for the efflux, calculated from the observed flux ratios according to Eq. (9), are plotted against the driving potentials calculated from the CCCP estimated membrane potentials and the potassium Nernst potentials.

In this case too, the flux ratio exponent is found to be about 0.9, but with indications of a decrease at the lowest CCCP concentrations.

Separate dose response experiments were therefore performed with the CCCP concentration ranging from 0 to 5.0 μM. At these low CCCP concentrations, the flux ratio exponent decreases from a value near unity to an extrapolated value of about 0.5 (*see* Fig. 5).

Under equilibrium conditions, that is $E_{K^+} = E_{Cl^-} = V_m$, CCCP has only very little, if any, influence upon the tracer fluxes of potassium, in contrast to the effect upon the net fluxes. When the CCCP concentration is raised from 0 to 5 μM, the rate of isotope equilibration is increased at most 1.5 times (*not shown*).

All experiments were performed with cells and Ringer's in equilibrium with atmospheric carbon dioxide. To test if bicarbonate/chloride exchange mediated by the anion exchange mechanism influenced proton equilibration by the protonophore, control experiments were done with carbon dioxide depleted cells.

A vigorously stirred suspension of red cells were depleted of carbon dioxide in a stream of argon. The depletion was followed by monitoring the accompanying alcalinization of the suspension. When a new steady-state pH value was reached, the cells were titrated to the original pH with HCl/NaCl solution. Degassing was then continued for 30 min, in which time no further changes in pH was observed.

In CCCP dose-response experiments, where the valinomycin concentration was kept constant at 0.25 μM, only insignificant differences in pH were found between carbon dioxide-depleted cells suspended in carbon dioxide-free Ringer and cells and Ringer in equilibrium with atmospheric carbon dioxide. Identical changes in estimated membrane po-

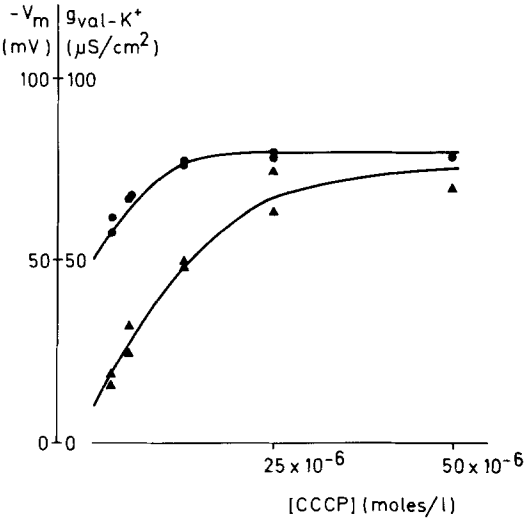

Fig. 4. Membrane potential (●) estimated by the extracellular pH changes, and the calculated K^+ conductance (▲) as function of the CCCP concentration. Figs. 3 and 4 are based upon the same experiments. Note that the left ordinates show the negative of the membrane potential. Lines were drawn by eye

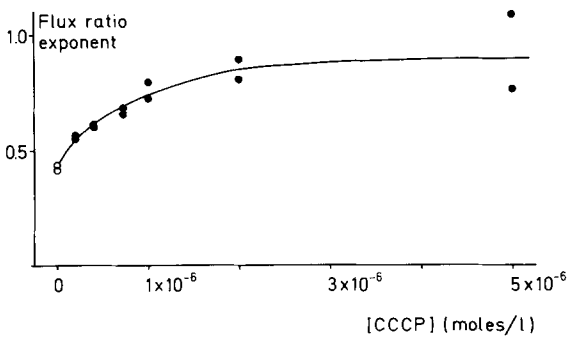

Fig. 5. Flux ratio exponent (ordinate) as function of the CCCP concentration (abscissa). The flux ratio exponents at zero CCCP concentration (open circles) are calculated using an extrapolated value of the CCCP-estimated membrane potential. Line was drawn by eye

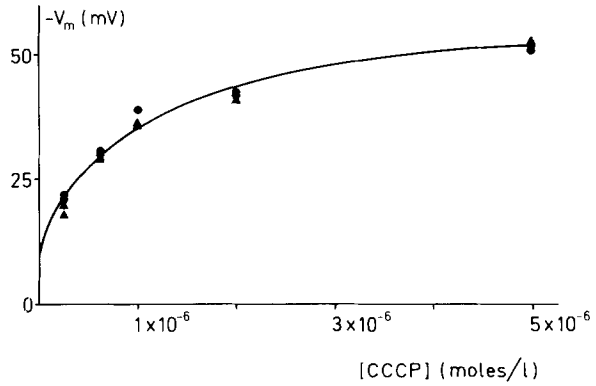

Fig. 6. Membrane potential estimated by the extracellular pH changes as function of the CCCP concentration. (▲) carbon dioxide-depleted cells and (●) cells in equilibrium with atmospheric carbon dioxide. Line was drawn by eye

tential with changes in CCCP concentration are thus found (*see* Fig. 6).

Discussion

Since valinomycin is a translatory carrier, the ratio between the unidirectional potassium fluxes across the red cell membrane in the presence of valinomycin cannot be expected to obey Ussing's flux ratio equation, Eq. (8), but must be described by an expression like Eq. (8b), where n represents the deviation from the flux ratio for electrodiffusion.

In limiting cases, however, with substrate concentrations well below $K_{1/2}$, where the carrier is far from saturation, and the rate-limiting process is the translocation of the charged substrate-carrier complex, n goes to 1.

In the present experiments, such a limiting case seems to exist when the CCCP concentration is above 5 μM.

It could be argued that the membrane potentials determined from the valinomycin-K^+ flux ratio represent the genuine membrane potentials, in which case the membrane potentials determined from the CCCP-mediated change in extracellular pH are underestimates due to an insufficient amount of CCCP (less than 5 μM) for development of the full change in extracellular pH.

Since the apparent membrane potential, judged from the flux ratio, is almost constant from 0 to 50 μM CCCP, while the net flux increases with a factor of about four (*see* Fig. 3), both the K^+ and Cl^- conductances should increase in parallel with the same factor.

Such an effect of CCCP is considered extremely unlikely in that the potassium and chloride ions have opposite charges and are translocated via different transport paths, for K^+, a carrier floating through the lipid bilayer and for Cl^-, a protein plug penetrating the membrane.

Furthermore, as the chloride conductance when using the CCCP-estimated membrane potential is found to be constant (*see* Fig. 7), it is required that the underestimate of the membrane potential by chance parallels a CCCP-dependent change in KCl net efflux. It is considered that such a coincidence is extremely unlikely, and the constant chloride conductance is taken as an indication of the

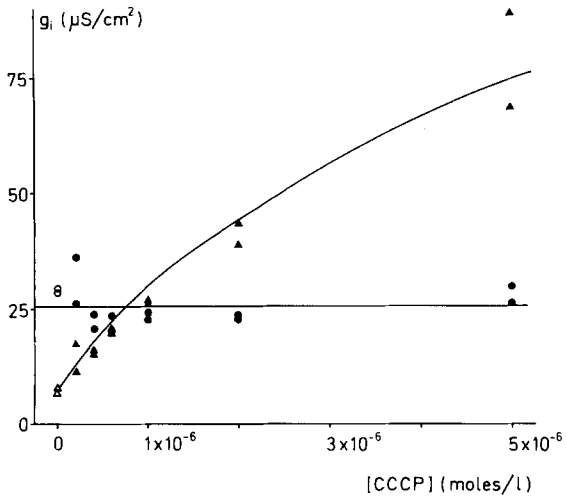

Fig. 7. K^+ (▲) and Cl^- (●) conductances calculated from the K^+ net efflux and the CCCP-estimated membrane potentials plotted as function of the CCCP concentration. The open symbols at zero concentration are based upon extrapolated values of the membrane potential. Lines were drawn by eye

validity of the membrane potentials estimated by the CCCP-mediated change in extracellular pH.

It has previously been shown that bicarbonate/chloride exchange through the anion exchange protein does not interfere with the CCCP-mediated pH equilibration in red cell suspensions at CCCP concentrations as high as 20 μM [1].

Since rather low CCCP concentrations are used in the present experiments, CCCP-estimated potential changes, caused by addition of valinomycin, in carbon dioxide depleted cells were compared to changes in cells in equilibrium with atmospheric carbon dioxide (*see* Fig. 6).

Identical increases in hyperpolarization with the CCCP concentration were observed in both types of experiments, and only insignificant differences in absolute values were seen.

Consequently, it is concluded that CCCP-estimated membrane potentials are not biased by bicarbonate cycling.

The kinetics of the valinomycin mediated transport of the cations K^+, Rb^+ and Cs^+ has been described by a transport model [10] and extensively characterized in experiments with lipid bilayer membranes [3].

A theoretical expression for the flux ratio of valinomycin K^+ fluxes has been derived by Läuger [9]. If the flux ratio exponent is estimated using this expression and the rate constants from experiments with transport of Rb^+ across monoolein bilayers [3] where the translocation of the uncomplexed valinomycin is rate limiting, the exponent is estimated to be in the range 0.70 to 0.80, going from 10 to 100 mM Rb^+ in the medium, thus decreasing with decreasing Rb^+ concentration.

As far as it is possible to compare lipid bilayer experiments with results obtained from red cells, the flux ratio exponent found when the CCCP concentration is zero, is in agreement with the bilayer data.

For lipid bilayers, the rate-limiting step in the transport cycle is the back transport of the uncomplexed valinomycin molecule. For red cells this could be the case, too, when no CCCP is present.

The present experimental determination of the flux ratio exponent indicates that the presence of CCCP in the red cell membrane increases the rate constant for the translocation of the uncomplexed valinomycin molecule relative to the rate constant for translocation of the charged complex, since only a very small effect of CCCP is found under equilibrium conditions, in contrast to the pronounced effect upon the net flux.

The lack of a CCCP effect upon ^{42}K equilibration under equilibrium conditions rules out the mechanism for the net flux acceleration from lipid vesicles proposed by Yamaguchi and Anraku [15], according to which the valinomycin-K^+ complex should bypass the activation energy barrier for translocation by coupling to the CCCP anion, resulting in an electroneutral complex, because such a mechanism would be expected to increase significantly the equilibrium exchange rate for potassium.

CCCP could, however, affect other rate constants in the valinomycin transport cycle, but under the present experimental conditions it is not possible to determine the individual rate constants or propose a mechanism for the CCCP influence. The effect of the changes in rate constants must, however, be an increase in conductance and flux ratio exponent, while the equilibrium exchange rate is relatively unaffected.

The found flux ratio exponent for valinomycin-K^+ of about 0.5 when no CCCP is present have implications for determination of red cell chloride permeability according to the Hunter approach [5], since the condition for using this method is that the valinomycin-mediated K^+-fluxes can be described in a constant-field context.

According to the present results, such an approach is not possible, and estimates of net chloride permeability will tend to be an underestimate, since the potassium permeability has been overestimated as a consequence of the exchange component in the valinomycin-mediated potassium fluxes.

Conclusion

The flux ratio for valinomycin-mediated K^+ fluxes has been calculated under varied experimental conditions, causing the driving potential for the K^+ efflux to range from 20 up to 75 mV.

The relationship between an apparent driving force, calculated from the flux ratio, and the independently calculated driving force, using the membrane potential estimated by the CCCP distribution and the Nernst potential for potassium, can be described by a flux ratio exponent of 0.90 when CCCP is present (concentrations above 5 μM).

This flux ratio exponent represents the degree of deviation shown by the actual carrier transport system from free electrodiffusion, for which the exponent is identical to 1.

When the CCCP concentration decreases, the flux ratio exponent decreases, too, to a value of about 0.5 when no CCCP is present, indicating that a considerable exchange component is part of the valinomycin-mediated fluxes across the human red cell membrane.

The significance of this is that determination of membrane potentials and permeabilities in red cells by a constant-field treatment of valinomycin-induced net fluxes, is not feasible.

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