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Protonophore Anion Permeability of the Human Red Cell Membrane Determined in the Presence of Valinomycin

Poul Bennekou
Zoophysiological Laboratory B, August Krogh Institute, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

Summary. A transport model for translocation of the protonophore CCCP across the red cell membrane has been established and cellular CCCP binding parameters have been determined. The time course of the CCCP redistribution across the red cell membrane, following a jump in membrane potential induced by valinomycin addition, has been characterized by fitting values of preequilibrium extracellular pH *vs.* time to the transport model. It is demonstrated, that even in the presence of valinomycin, the CCCP-anion is "well behaved," in that the translocation can be described by simple electrodiffusion. The translocation kinetics conform to an Eyring transport model, with a single activation energy barrier, contrary to translocation across lipid bilayers, that is reported to follow a transport model with a plateau in the activation energy barrier. The CCCP anion permeability across the red cell membrane has been calculated to be close to 2.0×10^{-4} cm/sec at 37°C with small variations between donors. Thus the permeability of CCCP in the human red cell membrane deviates from that found in black lipid membranes, in which the permeability is found to be a factor of 10 higher.

Key Words erythrocytes · valinomycin · protonophore · CCCP · permeability · kinetics

Introduction

Ions, which are transported passively through cell membranes by electrodiffusion, distribute according to electrochemical equilibrium. If the steady-state membrane potential changes, the ion will redistribute and ultimately reach a new equilibrium.

A group of compounds, protonophores or uncouplers, induces an apparent proton permeability across the membrane, when added to a cell suspension. In a number of papers [1, 11, 16] a proton carrier from this group, carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) has been used as membrane potential probe of red cells suspended in unbuffered Ringer's solutions. The transport characteristics of CCCP, which translocates across the lipid moiety of the cell membrane by diffusion/electrodiffusion, have been extensively studied in lipid bilayer systems [8, 10, 13] and suspensions of liposomes [17, 18], but far less is known about the behavior in cell membranes. The rate of CCCP redistribution, following changes in membrane potential, is determined by the CCCP anion permeability. This is of no consequence in relation to the final equilibrium, but determines how rapid potential changes can be reflected by the pH shift in the extracellular medium.

In experiments with lipid bilayers LeBlanc [10] has determined the permeabilities of CCCP and found values of 11 cm/sec for the acid, and 2.0×10^{-3} cm/sec for the anion, while O'Shaughnessy and Hladky [13] have found the anion permeability to be 4.4×10^{-4} cm/sec. In experiments with red cells, Macey and Orme [12] have estimated the CCCP anion permeability to be one or two orders of magnitude lower than the corresponding figure for translocation across artificial lipid bilayers. In the present study a steady-state electrodiffusion transport model for CCCP translocation is established. The model allows calculation of CCCP anion permeability in red cells, and additionally offers opportunity to distinguish between different electrodiffusion scenarios. As a prerequisite cellular binding parameters for CCCP are determined.

Materials and Methods

Chemicals

All inorganic salts (analytic grade) were purchased from Merck. Carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) was obtained from Sigma and valinomycin from Calbiochem. CCCP and valinomycin were administrated as concentrated alcoholic solutions.

Cells

Freshly drawn, heparinized human blood from healthy donors was centrifuged, and plasma and the buffy coat removed by aspiration. The cells were washed three times in isotonic NaCl (156 mM), and kept resuspended at a hematocrit of 20% at 37°C at least 1 hr before use.

Changes in membrane potential were estimated from changes in extracellular pH as:

$$\Delta V_m = -2.303 \frac{RT}{F} \Delta \mathrm{pH}_o \tag{1}$$

which is valid for cells suspended in a buffer-free medium [11]. $\Delta \mathrm{pH}_o$ is the change in extracellular pH. R, T and F have their usual thermodynamic meaning.

An absolute calibration can be obtained by measurement of the pH after hemolysis of the cells by addition of a 1 o/o solution of saponin, in that the pH in the hemolyzate corresponds to the pH_o in the suspension if the membrane potential were 0 mV.

CCCP Binding

100 μl packed red cells were transferred to a plastic vessel containing 3000 μl 156 mM NaCl solution, under vigorous stirring. 3 μl ethanolic CCCP solution were added. When pH equilibrium was attained a sample was withdrawn, centrifuged, and the CCCP concentration in the supernate determined spectrophotometrically at 363 nm, where the absorption is independent of pH.

At the end of the experiment the cellular pH was determined by adding 50 μl saponin solution to the suspension, causing total hemolysis of the cells. 50 μl hemolysate was used for determination of the cellular hemoglobin content.

In experiments where the intracellular pH was varied, red cells, suspended in 156 mM NaCl solution at a hematocrit of 50%, were titrated to the desired pH by addition of 50 mM NaOH or HCl in a Ringer's solution.

CCCP Flux Experiments

100 μl of packed cells were transferred to a plastic vessel containing 3000 μl isotonic NaCl (154 mM) and KCl (2 mM) solution. 3 μl ethanolic CCCP solution (concentration varied) were added. When equilibrium was achieved, 3 μl ethanolic valinomycin solution (concentration varied) was added to increase the potassium conductance, thus inducing a voltage jump (hyperpolarization). The amount of ethanol was 2 0/00 in all experiments.

The electrode amplifier output was fed into an A/D converter and the digitized paired values of time and extracellular pH stored for later analysis.

Electrodes

Radiometer electrodes were used for measurement of pH in the suspension. The glass electrode was a G2222 C with a rise time (95% response) below 0.5 sec. A K4112 calomel electrode was used as reference.

Calculations

The electrodiffusional flux for an anion across the cell membrane is described by:

$$J_i = -P_i Q \{c_i^{\mathrm{in}} \cdot e^{-\phi} - c_i^o \cdot e^{\phi}\} \tag{2}$$

where P_i is the permeability, c_i the concentrations on the two sides of the membrane of the ith anion, ϕ the reduced membrane potential divided by 2, and Q a potential factor which is dependent on the electrodiffusion model chosen.

It can be shown (*see* Appendix) that the extracellular pH in a suspension of red cells in buffer-free Ringer's solution with CCCP added, following an instantaneous voltage change, can be described by:

$$\mathrm{pH}_o(t) = \mathrm{pH}(0) + \log \left| \left\{ \frac{P_\infty}{P_0} - 1 \right\} \frac{1 - e^{-kt}}{1 + W_2 e^{-kt}} + 1 \right| \tag{3}$$

where $\mathrm{pH}_o(t)$ is the extracellular pH at the time t, P_0 is $\exp_{10}(\mathrm{pH}_o(0) - \mathrm{pK})$ and P_∞ $\exp_{10}(\mathrm{pH}_o(\infty) - \mathrm{pK})$. W_2 is a constant depending on the binding parameters and pH. The time constant k is a composite expression, containing the protonophore anion permeability, binding parameters, cell area, cellular and extracellular volumes, and the membrane potential factor Q, the value of which depends on the electrodiffusion model (*see* Appendix).

The protonophore anion permeability was determined from a nonlinear fit of experimental values of t, $\mathrm{pH}(t)$ to Eq. (3) using a simplex procedure with a least-squares estimator, for the determination of the three independent parameters P_0, P_∞ and the permeability P.

CCCP Binding

The amount of CCCP bound to intracellular components is calculated as:

$$M^B = M - [C]_T^o \left| V_o + V_{\mathrm{in}} \frac{1 + 10^{\mathrm{pH_{in}} - \mathrm{pK}}}{1 + 10^{\mathrm{pH}_o - \mathrm{pK}}} \right| \tag{4}$$

where M^B is the amount of bound CCCP, M the total amount of CCCP added to the suspension and $[C]_T^o$ the total concentration of CCCP (acid and corresponding base) in the extracellular medium. V_o and V_{in} are the extracellular and cellular water volumes. M^B is used in the calculation of the binding parameters, α and α', which are the amount of CCCP bound per liter cell water, divided by the cellular concentration (moles/liter cell water) of free base or acid, respectively.

Unstirred Layers

The calculated permeabilities have not been corrected for the effect of unstirred layers. With a cell volume fraction of about 0.03, the mean distance between cells in the suspension is 20 μm, which gives a maximum thickness of the unstirred layer of 10 μm in a homogenous suspension. If the diffusion coefficient of the CCCP anion in water is taken to be 5×10^{-6} cm^2/sec [10], the error in the determination of the membrane permeability is about 4%, and since the system is well stirred the actual error must be negligible.

Results

CCCP Binding

When CCCP had been added to a suspension of red cells, it was found that the total amount of CCCP, calculated from the extracellular concentrations under equilibrium conditions, was less than the amount added, the difference representing the amount bound. When different amounts of CCCP were added to a red cell suspension, the bound amount varied linearly with the total amount, up to a nominal concentration of 3×10^{-5} moles/liter total water (Fig. 1*A*).

Furthermore, a variation in binding of CCCP is seen with variation in intracellular pH, the binding increasing with decreasing pH (Fig. 1*A*). Since CCCP is a weak monovalent acid, with a pK value around 6 (*see below*) it is present both as free acid and free anion. In Fig. 1(*B*) is shown the ratios between the concentration of found CCCP and free anion α, and bound CCCP and free acid α', with varying cellular pH.

In contrast the binding parameters α and α' do not vary with variations in extracellular pH, when the intracellular pH is constant (*not shown*).

As the protonated and anionic forms of CCCP have different spectra in the ultraviolet, the pK of CCCP can be calculated after spectrophotometric determination of the ratio between acid and base in buffers with varying pH. The pK value was found to be 6.10 ± 0.03 at 24°C and 5.93 ± 0.03 at 38°C, in media with ionic strength 150 mM, which gives a $\Delta pH/\Delta T = -0.012 \pm 0.003$ in this temperature interval.

Immediately after addition of valinomycin to a suspension of red cells, with CCCP added as membrane potential probe, the extracellular pH begins to change. After about 30 sec a new steady-state value is reached, reflecting the hyperpolarization induced by valinomycin (Fig. 2).

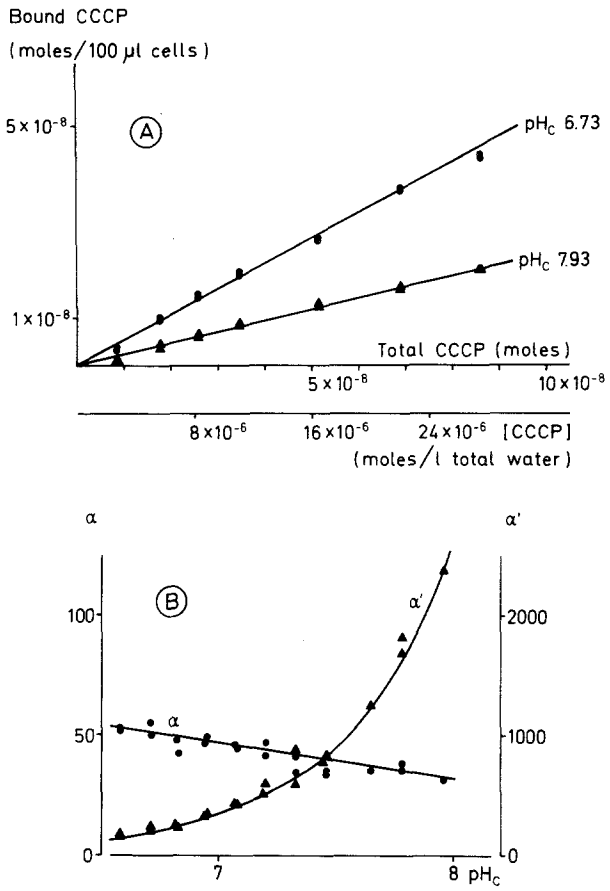

Fig. 1. (*A*) Bound CCCP plotted against total amount CCCP added at two different values of intracellular pH. The upper abscissa axis is total amount of CCCP added to the cell suspension, while the lower abscissa axis is the corresponding nominal CCCP concentration. The ordinate axis is bound CCCP, calculated according to Eq. (4). Symbols (filled circles and triangles) represent experimental determinations, the solid lines are linear regression curves for the experimental points. (*B*) Ratio between concentration of bound CCCP and intracellular free CCCP anion, α (left ordinate axis, filled circles), and ratio between concentration of bound CCCP and intracellular free protonated CCCP, α' (right ordinate axis, triangles), plotted against intracellular pH. The straight line is from linear regression

The potassium net efflux on the other hand, attains the highest level immediately following addition of valinomycin, and declines with time since the potassium and chloride Nernst potentials change with the progress in KCl efflux. Within the experimental uncertainty, however, the efflux is linear for the first 45 to 60 sec [2]. The time elapsing after valinomycin addition and before the K^+ efflux has reached the maximum value is between 0.5 and 1.0 sec, estimated from measurements of the change in extracellular potassium concentration, using a K^+-sensitive electrode (*not shown*). This interval is considered to represent a maximum value

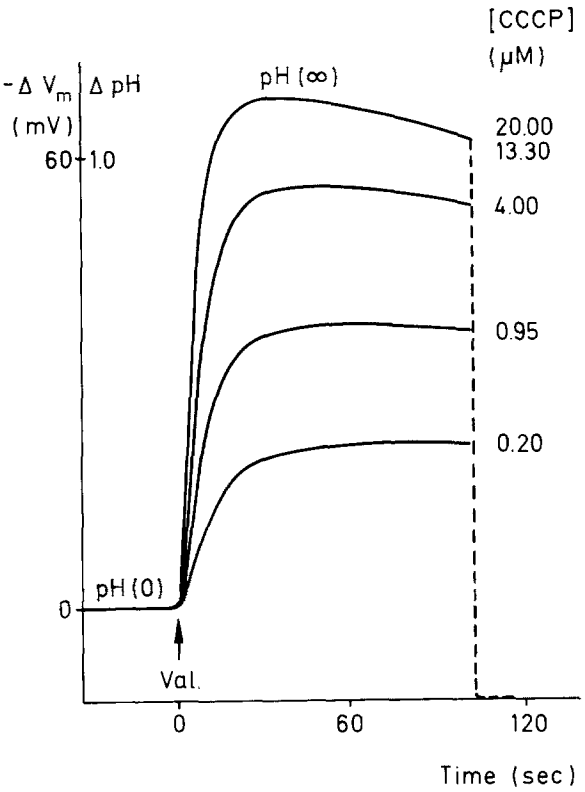

Fig. 2. pH traces from experiments with various amounts of CCCP added to the cell suspension; the concentration in μmol/liter H_2O is indicated to the right. At the end of the experiments saponin was added, resulting in total hemolysis of the cells (broken line). The ordinate axis shows extracellular pH and calculated equivalent change in membrane potential; the abscissa axis time in seconds. Valinomycin addition at time zero, indicated by arrow. The decline in pH and consequently membrane potential after the new steady-state pH has been reached, pH(∞), is due to the KCl efflux which leads to a decreasing K^+ Nernst potential

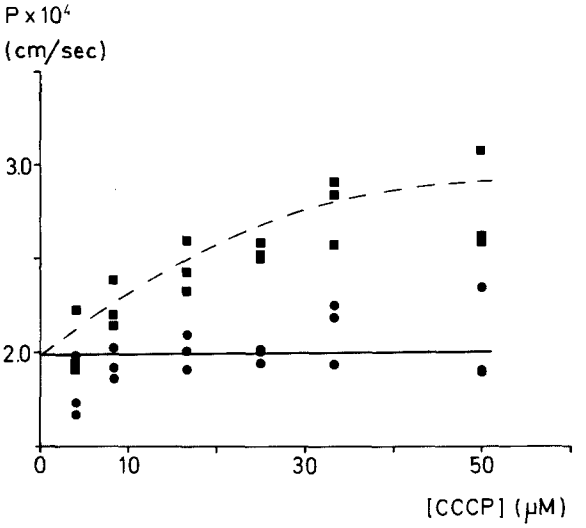

Fig. 3. Calculated CCCP anion permeabilities as function of CCCP concentration. The valinomycin concentration was 0.1 μM in all experiments. ■ represent constant field permeabilities, ● single-barrier permeabilities. Dashed line drawn by eye. Ordinate axis is permeability in cm/sec; abscissa axis nominal CCCP concentration in μmol/liter total water

for the time in which mixing and uptake in the cells is incomplete.

The almost instantaneous rise in potassium net efflux, and consequently in induced K^+ conductance, indicates that valinomycin induces a jump in membrane potential, and the initial ascending phase in extracellular pH thus represents CCCP equilibration, which is too slow to reflect the instantaneous voltage change.

Membrane Potential

The change in extracellular pH used for estimation of the membrane potential (*see* Eq. (1)), was calculated from pH(∞) using P_∞ from the fit to the transport model in the case of the flux experiments, otherwise from the maximum pH_o deflection (*see* Fig. 2). The value of the membrane potential calculated from the fit was within ±1.2mV from the value calculated from the peak value, corresponding to a difference of 0.02 pH units.

The new steady-state pH, $pH_o(\infty)$, in the extracellular phase, and the corresponding membrane potential calculated from the pH difference across the membrane show a considerable dependence both upon valinomycin concentration, and CCCP concentration (*see* Fig. 2). The variations in membrane potential with either valinomycin or CCCP concentration are saturating functions, as previously reported [1].

CCCP Anion Permeability

CCCP anion permeability was determined as best fit to Eq. (3), from paired observations of time (time for valinomycin addition taken as zero) and extracellular pH, in experiments where either the CCCP or valinomycin concentration was varied. The standard deviation of the experimental pH values compared to the model was typically 0.01 pH unit.

Figure 3 shows the $CCCP^-$ permeability, calculated from experiments where the valinomycin concentration was constant, 1.0×10^{-7} M, and CCCP varied, and Fig. 4 the permeability calculated from experiments, with a constant CCCP concentration of 3.33×10^{-5} M, and the valinomycin concentration varied.

For constant field diffusion, the potential factor Q has the value $-\phi/\sinh\phi$, where ϕ is the reduced

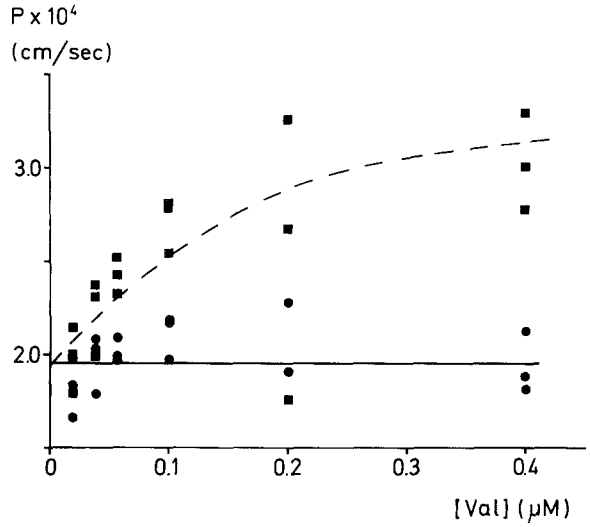

Fig. 4. Calculated CCCP anion permeabilities as function of valinomycin concentration. The CCCP concentration was 33.3 μM in all experiments. Symbols and ordinate axis as in Fig. 3. Abscissa axis, nominal valinomycin concentration in μmol/liter total water

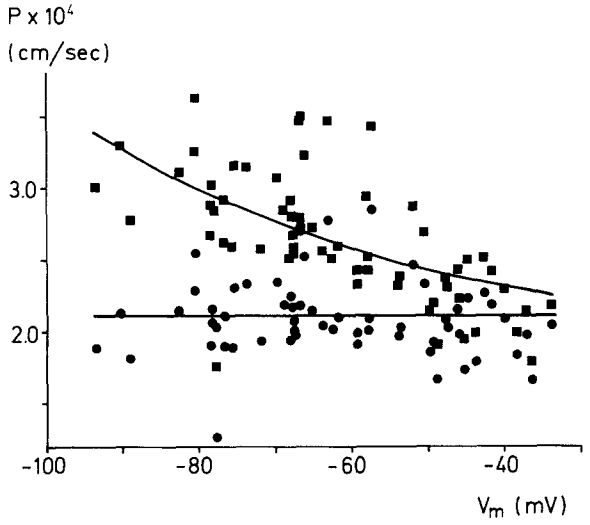

Fig. 5. CCCP anion permeabilities *vs.* calculated membrane potential. Symbols and ordinate axis as in Fig. 3. Lower line gives the mean of the single-barrier permeabilities; upper curve the calculated apparent constant field permeabilities. Abscissa axis represents the membrane potential in millivolts

membrane potential divided by 2. For a single-barrier electrodiffusion model $Q = -1$.

As shown in Figs. 3 and 4, the constant field permeability, as calculated from the fit to Eq. (3), is an increasing function of both CCCP- and valinomycin concentration, whereas the single-barrier permeability seems to be constant regardless of both CCCP- and valinomycin concentration. When the valinomycin concentration is held constant at 0.1 μM the single-barrier permeability is found to be $(1.98 \pm 0.16) \times 10^{-4}$ cm/sec (SD, $n = 18$), while the permeability is found to be $(1.95 \pm 0.23) \times 10^{-4}$ cm/sec (SD, $n = 18$) when the CCCP concentration is held constant and the valinomycin concentration varied.

In that the membrane potential is dependent on the concentrations of CCCP and valinomycin, and the calculated CCCP anion permeability depends on the membrane potential, the $CCCP^-$ permeability can be related to the membrane potential (Fig. 5). Because no a priori information exists either regarding the distribution function for the calculated permeabilities, or a straight line relationship between the calculated CCCP permeability and the membrane potential, a nonparametric description of the correlation has been used.

The Spearman rank correlation coefficient [4] for membrane potential *vs.* $CCCP^-$ permeability for a pooled group of experiments has been found to be 0.686 in the constant field case, $n = 61$, which is significant at the 0.1% level, while the correlation coefficient for the same data is found to be 0.115 in the single-barrier case. This means that the $CCCP^-$ permeability calculated with the assumption $Q = -1$ is independent of the membrane potential. The mean value of the $CCCP^-$ permeability in the single-barrier case is $(2.11 \pm 0.28) \times 10^{-4}$ cm/sec (SD, $n = 61$). The single-barrier permeability for the CCCP anion is thus a constant with an overall mean value of 2.11×10^{-4} (Fig. 5).

Discussion

Binding of CCCP to red cells is a pH-dependent nonsaturable process at least up to a concentration of 3.3×10^{-5} moles/liter, as can be seen from Fig. 1. In that CCCP is a weak acid, with a pK of about 6.0 depending on the temperature, the acid base ratio too varies with pH. The binding parameters α and α', that is the ratio between bound CCCP and free anion, respectively free acid, is shown in Fig. 1(*B*). As can be seen from the Figure, α, which is the binding constant calculated on the basis of the intracellular concentration of free $CCCP^-$, is a slowly decreasing, linear function of pH, while α', the binding constant calculated on the basis of free acid is an increasing, nonlinear function of pH.

Apart from the fraction of CCCP present in the intra- and extracellular spaces, a third fraction must exist in the lipid phase of the membrane.

Since the total amount of CCCP calculated from the extracellular concentration and the binding parameters at equilibrium with varied extracellular pH

is found to be constant, and identical to the added amount, this fraction is numerically insignificant, and does not influence the analysis of the redistribution.

The shape of the binding curves (*see* Fig. 1*B*) suggests that the CCCP anion is bound to an intracellular titratable component, for which hemoglobin seems to be a likely candidate in view of the almost linear acid-base titration curve [15], and the number of binding sites required. At the highest CCCP concentrations used, up to one mmol of CCCP is bound per liter cells. If the bound species were the acid, the pH dependence of the calculated binding parameter α' would be difficult to explain, and this parameter is considered to be an artifact, resulting from the linear binding of the anion. The actual binding mode however, is of no consequence for the calculation of the transport parameters, which is dependent on the total amount bound only.

In the commonly used kinetic model for transport of protonophore anions across artificial lipid bilayers, it is assumed that the anion does not leave the membrane in the experimental period (which for lipid bilayer experiments is of the order of milliseconds), and that the species transported between the compartments is protons, which associate/dissociate with the carrier anion at the membrane interface [3, 10].

In the present paper, the quasi steady-state transport in red cells has been characterized under experimental conditions, which are quite different from the lipid bilayer case. Another ionophore, valinomycin, was present and the transport was followed for a period of up to 30 sec. In this period a considerable redistribution of CCCP between the inner and outer compartments occurred. The size of the redistribution depends on the size of the potential jump, and both uncharged CCCP and the anion are supposed to move across the interface boundaries as part of this process.

In the analysis, it is assumed that addition of valinomycin to a red cell suspension induces a change in membrane potential with a rectangular profile. This assumption is based on the rapid increase (less than 1 sec) of the KCl efflux to a maximal value, indicating a reasonably short rise time. This efflux is nearly constant for the first 30 to 45 sec, whereafter it decreases [2].

The degree of constancy of the membrane potential during an experiment (20 to 30 sec) can be estimated from the linear change in extracellular pH following the peak value (*see* Fig. 2). The deviation from a constant value is found to be 3 to 4 mV in the experiments with the highest induced fluxes (high concentrations of valinomycin and CCCP), and decreasing with decreasing levels of ionophores.

It has been attempted to fit the data to the model, but with $t + \Delta t$ substituted for t. The resulting parameters did not change significantly, however, and Δt was not found to be significantly different from 0.

In conclusion a square-wave profile seems to be a reasonable approximation for the change in membrane potential induced by valinomycin.

It should be noted, that a previous analysis has demonstrated, that under the present experimental conditions the anion exchange systems do not interfere with the CCCP-mediated pH equilibration, and consequently do not bias the membrane potential calculated from pH(∞), even at CCCP concentrations below 1 μM [2]. It has been observed [6, 14], that addition of CCCP to valinomycin-treated cells accelerates the valinomycin-mediated potassium efflux. This acceleration has been interpreted, either as a direct coupling between valinomycin-K^+ and the CCCP anion, thus forming an electroneutral complex [17, 18], whereby K^+ and $CCCP^-$ efflux bypasses the energy barrier for charge translocation, or as a direct CCCP influence upon valinomycin K^+ conductance [1], through a lowering of the potential barrier.

If a direct coupling between valinomycin-K^+ and $CCCP^-$ to an electroneutral complex were responsible for the potassium net efflux acceleration, there must, with a nearly constant extracellular pH, be an electrodiffusional reflux of $CCCP^-$ of the same size as the K^+ efflux acceleration.

When the calculated $CCCP^-$ permeability is extrapolated to zero concentration of either CCCP or valinomycin (*see* Figs. 3, 4), it gives a value unbiased by a possible coupling. If the net electrodiffusion influx of $CCCP^-$ is calculated, using this unbiased permeability the size of the influx is found to be one order of magnitude lower than necessary to account for the increase in K^+ net efflux. Consequently the coupling does not contribute significantly, if at all, to the $CCCP^-$ redistribution in the presence of valinomycin. $CCCP^-$ translocation across the red cell membrane can thus safely be treated in an electrodiffusional framework.

This conclusion is directly supported by a flux ratio analysis of the valinomycin-induced K^+ fluxes in the presence of CCCP [2], which shows that no acceleration of the K^+ exchange fluxes is found under equilibrium conditions. According to this analysis, the acceleration under net flux conditions is due to an effect of CCCP upon the rate constants in the valinomycin transport cycle.

The transport of an ion through the membrane can be treated either as an electrodiffusion process using the integrated Nernst-Planck flux equation, or alternatively, according to absolute rate theory, as a series of translocations across barriers in activation energy, from one local minimum in potential energy

to the next [19]. In both cases, the resulting flux equation can be represented by the left part of Eq. (1), where Q depends on the specified potential energy profile, or series of activation energy barriers, respectively.

In the Eyring formalism [19], the single-barrier scenario means that the anion experiences only a single activation energy barrier, when it is translocated across the membrane and $Q = -1$. In the Nernst-Planck formalism, the potential energy barrier for translocation can be represented by a trapezoid [5, 7, 9], characterized by the ratio between the minor and major bases r. If the trapezoid becomes rectangular, $r = 1$, the constant field equation with $Q = -\phi/\sinh\phi$ ensues, while when degeneration into a triangle, $r = 0$, the flux equation in the Nernst-Planck formalism has the same dependence of the membrane potential as the Eyring formalism with a single activation energy barrier, i.e. $Q = -1$.

When the CCCP anion permeability is calculated according to constant field conditions, a significant rise in permeability is seen in dose-response experiments (*see* Figs. 3, 4). If, on the other hand, CCCP anion permeability is calculated according to a single-barrier model, where the flux has a steeper dependence upon the membrane potential, the CCCP anion permeability is found to be a constant, regardless of CCCP or valinomycin concentration.

The single-barrier behavior of the CCCP efflux, found in the present work is in accordance with the membrane potential dependence reported by Macey and Orme [12] for translocation across the red cell membrane of the positively charged tetraphenyl-arsonium ion, and indicates that the plateau in the activation energy profile across the red cell membrane is very narrow. In this respect the red cell membrane seems to differ from artificial lipid bilayers, where O'Shaughnessy and Hladky [13] and Kasianowich, Benz and McLaughlin [8] report a value of 0.57 for r in a study of CCCP, and $r = 0.65$ in experiments with the protonophore FCCP (*p*-trifluoromethoxyphenylhydrazone) [3].

The value of the CCCP anion permeability, 2.11×10^{-4} cm/sec, as reported in the Results section, is one magnitude lower than the value reported by LeBlanc [10] for artificial lipid bilayers, but in agreement with the estimate given by Macey and Orme [12] for translocation across the red cell membrane.

Conclusion

CCCP translocation across the human red cell membrane, following a fast hyperpolarization induced by valinomycin, can be described by an electrodiffusion transport model. The transport kinetics conform to a single barrier, rather than to a constant field, scenario. This finding, which is in accordance with other red cell studies using lipid-soluble cations, questions the validity of constant field calculations, which traditionally have been used for human red cell transport studies.

Since the steady-state translocation of CCCP is a "well behaved" process, CCCP seems to be an appropriate choice for a lipid-soluble anion to probe the human red cell membrane, and supports the notion that genuine membrane potentials can be estimated using this compound.

That CCCP translocation across the human red cell membrane can be described by electrodiffusion furthermore indicates that $CCCP^-$ coupling to a valinomycin-K^+ complex is not a significant part of the translocation process across the erythrocyte membrane.

The deviation of the CCCP permeability and transport mode from what is found in experiments with synthetic lipid bilayer membranes do indicate that results from such experiments are only partly valid for translocation across the human red cell membrane.

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Appendix

CCCP Binding

The amount of bound CCCP can be expressed as:

$$M^B = M - M^F \tag{A1}$$

where M is the total amount of CCCP, M^F the amount of free, and M^B the amount of bound CCCP.

As CCCP is a weak monovalent acid, the ratio between the concentration of free anion and free acid is given by:

$$\mathrm{pH} - \mathrm{pK} = \log \frac{[\mathrm{CCCP}^-]}{[\mathrm{CCCP}]} = \log \frac{[B]}{[A]} \tag{A2}$$

which is valid on both sides of the membrane.

M^F can then be expressed as:

$$M^F = V_o[A]\{1 + 10^{\mathrm{pH}_o - \mathrm{pK}}\} + V_{\mathrm{in}}[A]\{1 + 10^{\mathrm{pH}_{\mathrm{in}} - \mathrm{pK}}\} \tag{A3}$$

where V_o and V_{in} are extracellular and intracellular water volume.

Equation (A3) is valid, when the distribution of CCCP has come to equilibrium, since the concentration of free acid is then identical on the two sides of the membrane.

The concentration of free acid is then:

$$[A] = \frac{[C]_T^o}{1 + 10^{\mathrm{pH}_o - \mathrm{pK}}} \tag{A4}$$

where $[C]_T^o$ is the total extracellular concentration of CCCP (acid and base).

Inserting Eqs. (A3) and (A4) in (A1) yields:

$$M^B = M - [C]_T^o \left| V_o + V_{\mathrm{in}} \frac{1 + 10^{\mathrm{pH}_{\mathrm{in}} - \mathrm{pK}}}{1 + 10^{\mathrm{pH}_o - \mathrm{pK}}} \right|. \tag{A5}$$

The binding parameters α and α' are related to the amount bound by

$$\alpha = \frac{[B]_{\mathrm{in}}^B}{[B]_{\mathrm{in}}^F}; \qquad \alpha' = \frac{[A]_{\mathrm{in}}^B}{[A]_{\mathrm{in}}^F} \tag{A6a,b}$$

where the bound amount is taken on basis of 1.0 liter cell water.

CCCP Flux

The amount of CCCP anion crossing the cell membrane can be written as:

$$Ar \cdot J \approx -V_{\mathrm{in}}(1 + \alpha) \frac{d[B]_{\mathrm{in}}}{dt} - (V_o + V_{\mathrm{in}}(1 + \alpha')) \frac{d[A]}{dt} \tag{A7}$$

assuming that intracellular CCCP binding is linearly proportional to the concentration of the free anion, and that the rate of association/dissociation is fast compared to the rate of transport (*see* Fig. 6). Ar is the membrane area; remaining symbols are as previously defined. Under the present experimental conditions, where the only extracellular buffer capacity is represented by minute amounts of bicarbonate and extracellular water and CCCP, the error introduced by equating the left and right sides in Eq. (A7) is negligible.

The electrodiffusional ion flux across the membrane can be expressed as:

$$J_i = -P_i Q(c_i^{\mathrm{in}} e^{z\phi} - c_i^o e^{-z\phi}). \tag{A8}$$

Q is a potential factor that depends on the transport model [7, 9, 12]. For a single-barrier model $Q \approx -1$, for a constant field model $Q = -\phi/\sinh\phi$, with $\phi = FV_m/2RT$ (anion).

Since the undissociated acid is very permeable, compared to the anion [10], it is assumed that the undissociated acid is in

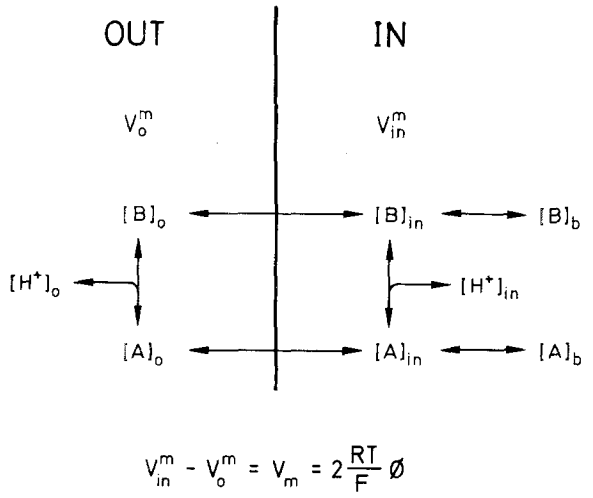

Fig. 6. This Figure illustrates the assumed transport scheme, on which the model is based. (*A*) represents the undissociated CCCP, while (*B*) represents the anionic (base) form

(quasi) equilibrium across the cell membrane. Consequently the right sides of Eqs. (A7) and (A8) can be equated.

Since

$$\frac{[B]_{in}}{[A]} = 10^{pH_{in}-pK} = \frac{1}{\beta} \tag{A9}$$

if $[A]$ is substituted into Eq. (A7), this leads to

$$Ar \cdot P \cdot Q([B]_{in}e^{-\phi} - [B]_o e^{\phi}) = -V_{in}\left\{1 + \alpha + \beta\left(1 + \alpha' + \frac{V_o}{V_{in}}\right)\right\}\frac{d[B]_{in}}{dt}. \tag{A10}$$

Since the total CCCP compartment is closed:

$$[A]\{V_o + V_{in}(1 + \alpha')\} + [B]_o V_o + [B]_{in}V_{in}(1 + \alpha) = M \tag{A11}$$

which is valid for all t.

Insertion of Eq. (A9) in Eq. (A11) gives:

$$[B]_{in}\{V_{in}(1 + \alpha + \beta(1 + \alpha')) + \beta V_o\} + [B]_o V_o = M \tag{A12}$$

and solving for $(B)_o(t)$:

$$[B]_o(t) = \frac{M - [B]_{in}(t)\{V_{in}(1 + \alpha + \beta(1 + \alpha')) + \beta V_o\}}{V_o}. \tag{A13}$$

Insertion of Eq. (A13) in Eq. (A10) leads to a first-order differential equation. After separation of the variables we have:

$$d\ln\{[B]_{in}C - Me^{\phi}\} = -kdt \tag{A14}$$

where

$$V_o e^{-\phi} + \{V_{in}(1 + \alpha + \beta(1 + \alpha')) + V_o\beta\}e^{\phi} = C \tag{A14a}$$

and

$$\frac{Ar \cdot P \cdot Q}{V_{in}V_o\left\{1 + \alpha + \beta\left(1 + \alpha' + \frac{V_o}{V_{in}}\right)\right\}} \cdot C = -k. \tag{A14b}$$

Equation (A14) is integrated and rearranged to give:

$$[B]_{in}(t) = \left\{[B]_{in}(0) - \frac{M}{C}e^{\phi}\right\}e^{-kt} + \frac{M}{C}e^{\phi}. \tag{A15}$$

As CCCP equilibrates, the pH in the extracellular medium, which is buffer free, changes. Expressing the concentration of protonated CCCP in the extracellular and intracellular water phases according to the buffer equation and assuming the acid to be in equilibrium across the membrane leads to:

$$[B]_o(t) = \beta[B]_{in}(t) \cdot 10^{pH_o(t)-pK} \tag{A16}$$

which is inserted in Eq. (A15) to give:

$$\beta[B]_{in}(t) \cdot 10^{pH_o(t)-pK} = \frac{M - [B]_{in}(t)\{V_{in}(1 + \alpha + \beta(1 + \alpha')) + \beta V_o\}}{V_o}. \tag{A17}$$

which is solved for $(B)_{in}(t)$ to give:

$$[B]_{in}(t) = \frac{M}{V_o\{10^{pH_o(t)-pK} + 1\} + V_{in}\{1 + \alpha + \beta(1 + \alpha')\}}. \tag{A18}$$

Inserting Eq. (A18) in Eq. (A15) and rearranging gives:

$$10^{pH_o(t)-pK} = \frac{M}{\left\{\left\{[B]_{in}(0) - \frac{M}{C}e^{\phi}\right\}e^{-kt} + \frac{M}{C}e^{\phi}\right\}\beta V_o - \frac{V_{in}(1 + \alpha + \beta(1 + \alpha'))}{V_o} - 1}. \tag{A19}$$

For convenience

$$\frac{C}{\beta V_o e^{\phi}} = W_1; \quad \frac{[B]_{in}(0) \cdot C}{Me^{\phi}} - 1 = W_2; \quad \frac{V_{in}(1 + \alpha + \beta(1 + \alpha'))}{V_o} + 1 = W_3 \tag{A19a,b,c}$$

are introduced.

These expressions, which are constants characteristic of the individual experiments are inserted in Eq. (A19) to give:

$$10^{pH_o(t)-pK} = \frac{W_1}{W_2e^{-kt} + 1} - W_3. \tag{A20}$$

For $t = 0$ and $t \to \infty$ Eq. (A20) reduces to:

$$10^{pH_o(\infty)-pK} = W_1 - W_3 = P_\infty \tag{A21a}$$

$$10^{pH_o(0)-pK} = \frac{W_1}{W_2 + 1} - W_3 = P_0 \tag{A21b}$$

which, when solved for W_1 and W_3 yield:

$$W_1 = \frac{W_2 + 1}{W_2}(P_\infty - P_0)$$

$$\text{and } W_3 = (P_\infty - P_0)\frac{1}{W_2} - P_0. \tag{A22a,b}$$

Equation (A20) can now be written as:

$$pH_o(t) = pH(0) + \log\left|\left(\frac{P_\infty}{P_0} - 1\right)\frac{1 - e^{-kt}}{1 + W_2 e^{-kt}} + 1\right|. \tag{A23}$$

Insertion of Eq. (A21b) in Eq. (A16) gives:

$$[B]_o(0) = P_0\beta[B]_{in}(0). \tag{A24}$$

Inserting Eqs. (A12), (A14a) and (A24) in Eq. (A19b) we get:

$$W_2 = \frac{e^{-2\phi} - \beta P_0}{\frac{V_{in}}{V_o}(1 + \alpha + \beta(1 + \alpha')) + \beta(1 + P_0)}. \tag{A25}$$

Since

$$P_\infty = 10^{pH_o(\infty)-pK}, \text{ and } \frac{FV_m}{RT} = 2\phi = -(pH_o(\infty) - pH_{in}) \cdot \ln 10$$

Eq. (A25) can finally be written as:

$$W_2 = \frac{\beta(P_\infty - P_0)}{\frac{V_{in}}{V_o}(1 + \alpha + \beta(1 + \alpha')) + \beta(1 + P_0)}. \tag{A26}$$

Equation (A23), to which the data are fitted, contains two constants, k and W_2, that are dependent on the binding parameters. It is apparent from inspection of these constants, Eqs. (A14b) and (A26), that their values are unchanged even if the interpretation of the binding mode (binding of the protonophore anion) do not hold, since in that case the binding parameter for binding of undissociated acid should be used, whereby numerically the same constants appear.