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Steady-State and Transient Membrane Potentials in Human Red Cells Determined by Protonophore-Mediated pH Changes

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Summary. Protonophores have been used frequently to determine changes in membrane potential in suspensions of red cells, since such changes are reflected by changes in extracellular pH, due to proton and consequently protonophore reequilibration.

In a previous paper (Bennekou, P. 1988. *J. Membrane Biol.* **102**:225–234) a kinetic model for the translocation of a protonophore, CCCP, across the human red cell membrane was established. This model accounts for the protonophore reequilibration following abrupt changes in membrane potential.

In this paper the limitations of the method with regard to the estimation of transient membrane potentials are examined, using the transport model to simulate changes in extracellular pH in response to noninstantaneous changes in membrane potential. The temperature and time resolution calculated from the model are reported.

Furthermore, it is shown that the transport model established for CCCP is valid for another protonophore, TCS, thus indicating the general validity of the transport scheme for the entire class of protonophores.

Key Words protonophore · CCCP · human red cell · membrane potential

Introduction

In a number of reports, protonophores have been used as "probes" for the determination of human red cell membrane potential [1, 10]. The method, which originally was proposed by Macey et al. [4], is based upon introduction of an apparent proton conductance across the red cell membrane by addition of protonophores. Due to this apparent proton conductance, changes in membrane potential are reflected by relative fast changes in cellular and extracellular pH since proton redistribution according to the electrochemical gradient have been facilitated.

If the extracellular medium is unbuffered, the intracellular pH changes only very little when protons are redistributed in that the cytosol is heavily buffered, primarily due to hemoglobin. Consequently the change in extracellular pH reflecting the change in membrane potential, is maximal under these conditions, with a change of 1 pH unit equivalent to a potential change of −61.5 mV [4].

As far as the membrane potential changes from one steady-state potential to another (or to a quasi steady state), the kinetic characteristics of the proton carrier is of no concern, since the passively transported protons ultimately will reach equilibrium. When, however, transient responses are observed [9, 11] or the time dependence of the potential change is of interest, it is necessary to know the transport characteristics of the proton carrier to be able to judge if the observed pH transient truly represents the changing membrane potential, or is distorted as a consequence of a rate-limiting carrier-mediated proton redistribution.

In the present paper it is demonstrated that the frequency limit for potential determinations by this method is about one cycle every 120 seconds at 37°C (depending on the time dependence of the potential change) provided the extracellular medium is buffer free. The effective time resolution decreases progressively with lower temperatures.

Materials and Methods

Chemicals

All inorganic salts were purchased from Merck. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma, tetrachlorosalicylic anilide (TCS) from Kodak and valinomycin from Calbiochem. CCCP, TCS and valinomycin were administered as concentrated alcoholic solutions.

Cells

Freshly drawn, heparinized human blood from healthy donors was centrifuged, and the plasma and buffy coat removed by aspi-

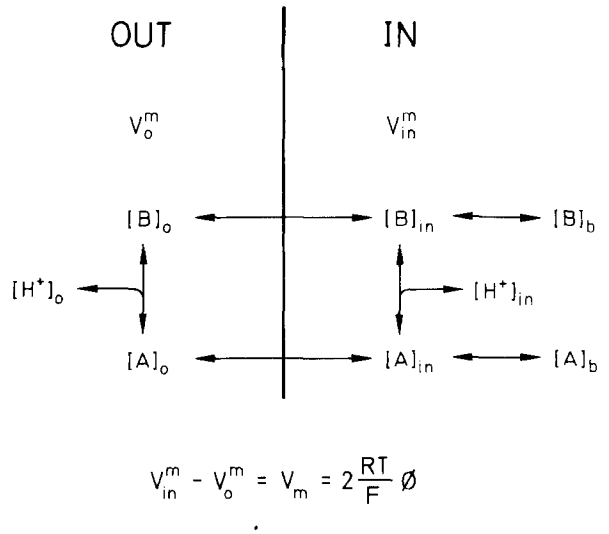

Fig. 1. The figure illustrates the transport scheme for protonophore translocation. *A* represents the undissociated protonophore (the acid), while *B* represents the anion (base). Binding is indicated by subscript *b*, and *OUT* and *IN* denotes the extracellular and intracellular phases, respectively

ration. The cells were washed three times in isotonic sodium chloride solution (156 mM), and left resuspended at a hematocrit of 20%, 37°C, at least 1 hr before use.

Protonophore Efflux Experiments

100 μl packed cells were transferred to a plastic vessel containing 3000 μl isotonic NaCl (154 mM) and KCl (2 mM) solution. 3 μl ethanolic protonophore solution, with varied concentrations, was added. When equilibrium was achieved, 3 μl ethanolic valinomycin solution was added to induce a voltage jump. The concentration of ethanol was thus 0.2% in all experiments. Intracellular pH was determined at the end of the experiment by addition of 100 μl detergent, either 1% of saponin or Triton X-100 in water, causing total hemolysis.

Electrodes and Amplifiers

A Radiometer G2222C glass electrode was used for measurement of extracellular pH in the suspension, with a Radiometer K 4112 calomel electrode as reference. The electrode amplifier output was fed into an A/D converter and the digitized pH-value *vs.* time stored for later analysis. The response time of the total system (95%) was below 1 sec.

Calculations

In a previous paper [2] dealing with the protonophore kinetics in a red cell suspension, it has been shown, that the protonophore translocation across the membrane can be represented schematically by Fig. 1, and described by

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

where

$$\beta = \frac{[A]}{[B]_{in}} = 10^{-(\mathrm{pH_{in}} - \mathrm{pK})}. \quad (1a)$$

Ar is the area of the membrane, P the permeability, Q a potential factor depending upon the electrodiffusion model [2], ϕ the reduced membrane potential divided by two, $[B]$ the concentration of protonophore anion and V the water volume. Subscripts o and in denote extracellular or intracellular parameters, α and α' are the ratios between the concentrations of bound and free protonophore, anion and protonated form respectively. $[A]$ is the concentration of undissociated protonophore, which is assumed to be in equilibrium across the membrane at any time; pH and pK have their usual meanings.

If the change in membrane potential is instantaneous, that is a square pulse starting at $t = 0$, Eq. (1) can be integrated and manipulated to give

$$\mathrm{pH}_o(t) = \mathrm{pH}_o(t = 0) + \log\left|\left\{\frac{P_\infty}{P_0} - 1\right\} \cdot \frac{1 - e^{-kt}}{1 + W_2 \cdot e^{-kt}} + 1\right| \quad (2)$$

where $\mathrm{pH}_o(t)$ is the extracellular pH at time t, P_0 is $\exp_{10}(\mathrm{pH}_o(0) - \mathrm{pK})$ and $P_\infty \exp_{10}(\mathrm{pH}_o(\infty) - \mathrm{pK})$. W_2 is a constant, characteristic for the individual experiment. The time constant, k, is a composite expression, which includes the protonophore permeability and the membrane potential factor, Q.

For a given driving force, the electrodiffusional flux depends on the potential energy profile along the translocation path, with $Q = -\phi/\sinh(\phi)$ under constant field conditions, and $Q = -1$ for a triangular (single barrier) profile. (For details, *see* ref. [2].)

$$k = -\mathrm{Ar} \cdot P \cdot Q \frac{V_o e^{-\phi} + \{V_{in}(1 + \alpha + \beta(1 + \alpha')) + V_o\beta\} \cdot e^{\phi}}{V_o V_{in}\left\{1 + \alpha + \beta\left(1 + \alpha' + \frac{V_o}{V_{in}}\right)\right\}}. \quad (2a)$$

Equation (2) gives the time dependency of the change in extracellular pH in a suspension of red cells, in response to an instantaneous change in membrane potential, when the suspending medium is buffer free. If, however, the change in membrane potential is slow, in the range from a couple of seconds and upwards, the exponential term in Eq. (1), as well as the membrane potential factor, Q, are functions of the time, i.e., $\phi = \phi(t)$, and consequently it is not possible to integrate Eq. (1) to arrive at Eq. (2). In the following section, Eq. (1) is manipulated to a form that can be solved numerically for the extracellular pH.

Equation (1a) inserted in the buffer equation for the protonophore in the extracellular phase gives

$$[B]_o(t) = \beta \cdot [B]_{in}(t) \cdot 10^{\mathrm{pH}_o(t) - \mathrm{pK}}. \quad (3)$$

Equation (3) is inserted in Eq. (1) to give

$$\mathrm{Ar} \cdot P \cdot Q \cdot [B]_{in}(t) \cdot \{e^{-\phi(t)} - \beta \cdot e^{\phi(t)} \cdot 10^{\mathrm{pH}_o(t) - \mathrm{pK}}\} = -V_{in} \cdot \left\{1 + \alpha + \beta\left(1 + \alpha' + \frac{V_o}{V_{in}}\right)\right\}\frac{d[B]_{in}}{dt}. \quad (4)$$

The intracellular free concentration of protonophore anion at time = t can be expressed in terms of the total amount of protonophore M and the parameters introduced previously in this section, *cf.* Eq. (A18) in [2], as follows

$$[B]_{in}(t) = \frac{M}{V_o\{10^{pH_o(t)-pK} + 1\} + V_{in}\{1 + \alpha + \beta(1 + \alpha')\}}. \quad (5)$$

Equation (5) is then substituted into Eq. (4), which after differentiation and rearranging yields

$$S_1 \cdot \{10^{pH_o(t)-pK} + S_2\} \cdot \{e^{-\phi(t)} - \beta \cdot e^{\phi(t)} 10^{pH_o(t)-pK}\} = \frac{d}{dt}\{10^{pH_o(t)-pK}\} \quad (6)$$

where

$$S_1 = \frac{Ar \cdot P \cdot Q}{1 + \frac{V_{in}}{V_o} \cdot \{1 + \alpha + \beta(1 + \alpha')\}} \quad (6a)$$

$$S_2 = -V_{in}\left\{1 + \alpha + \beta(1 + \alpha' + \frac{V_o}{V_{in}})\right\}. \quad (6b)$$

It is noted that generally $Q = Q(\phi(t))$, but degenerates to a constant, $Q = -1$ in the single-barrier case, which has been shown to describe CCCP translocation across the red cell membrane [2].

Finally, introducing $u(t) = \exp_{10}(pH_o(t) - pK)$ and substituting into Eq. (6)

$$S_1 \cdot \{u(t) + S_2\} \cdot \{e^{-\phi(t)} - \beta \cdot e^{\phi(t)} \cdot u(t)\} = \frac{du(t)}{dt}. \quad (7)$$

Equation (7) can be solved numerically to give $pH_o(t)$. The simulated curves presented in this paper are obtained from solutions to Eq. (7), calculated with a fourth order Runge-Kutta algorithm [7].

Results

If the K^+ conductance across the cell membrane is increased in human red cells suspended in a low potassium Ringer's solution containing protonophore, the extracellular pH increases since protons and protonophore redistribute as a consequence of the hyperpolarization of the membranes.

Like other protonophores, TCS increases the maximal valinomycin-mediated K^+ conductance attained, and as a consequence, the induced hyperpolarization increases, in the present experiments from about −80 mV at a nominal concentration of 10^{-6} M to about −110 at 10^{-5} M TCS, where the effect is maximal.

The redistribution time for a given protonophore is dependent, among other parameters, upon the protonophore anion permeability, acid strength and the binding parameters; *cf.* section on calculations and ref. [2]. For TCS, the binding parameter α was determined to be 108, at pH 7.0 and 37°C, and the pK-value was found to be 6.75 at 37°C (determined spectrometrically).

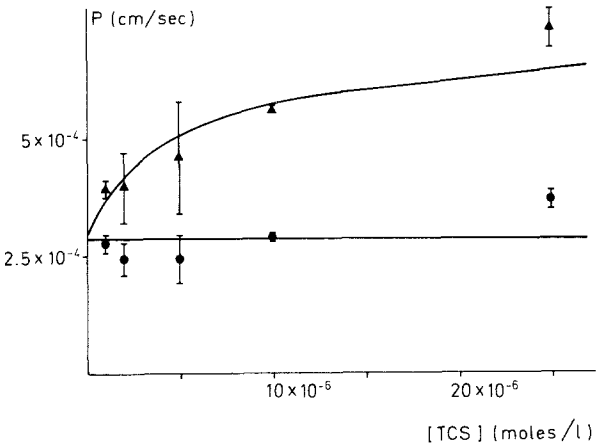

Fig. 2. TCS anion permeability calculated from a fit to Eq. (2) as a function of the nominal TCS concentration. The upper curve represents determinations under constant-field assumptions; the lower curve under single-barrier assumptions. Actual determinations (the mean of three experiments) are indicated by symbols. Error bars indicate ± 1 SD. Solid lines were drawn by eye. The abscissa axis is the TCS concentration calculated from the added amount; the ordinate axis the permeability in cm/sec

The TCS anion permeability has been calculated by fitting experimental determinations of $pH_o(t)$ to Eq. (2), with the time at addition of valinomycin taken as zero.

Figure 2 shows the TCS anion permeability calculated according to a constant field and a single-barrier transport model, respectively. It is seen, that whereas the constant field permeability increases with TCS concentration, the single-barrier permeability is far more constant, with a mean value of $(2.78 \pm 0.55) \times 10^{-4}$ cm/sec (SD).

In a preceding paper [2] the CCCP anion permeability was found to be about 2.0×10^{-4} cm/sec and the binding parameter α for CCCP 45 at pH = 7.0. Although the TCS anion permeability is about 35% higher than the permeability for CCCP, TCS equilibrates more slowly than CCCP (*not shown*), because the effect of the increase in permeability is more than counteracted by the effect on the time constant k of the increased binding, *cf.* Eq. (2a).

Influence of temperature: Figure 3 shows the temperature dependence of the CCCP anion permeability. In the temperature interval 15 to 37°C, the anion permeability of CCCP was found to increase from 5.5×10^{-5} cm/sec to 2.1×10^{-4} cm/sec. As can be seen from Fig. 3, a plot of the logarithm to the permeability *vs.* the reciprocal of the absolute temperature is curvilinear. The apparent activation

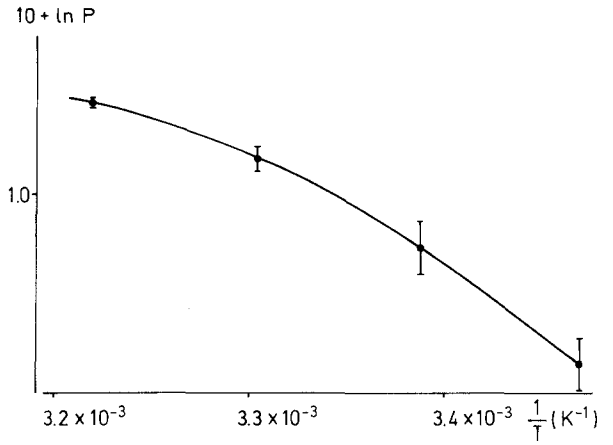

Fig. 3. Arrhenius plot of the calculated CCCP anion single-barrier permeability. Ordinate shows the logarithm to the permeability. The 10 is added to shift the curve to the first quadrant of the coordinate system. The abscissa axis is the reciprocal of the absolute temperature

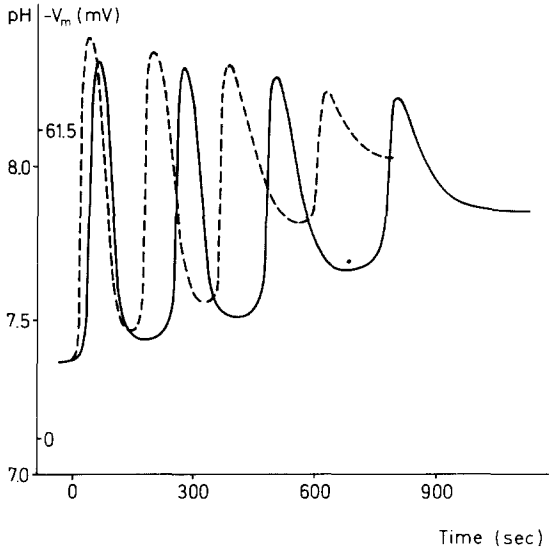

Fig. 4. CCCP-mediated changes in extracellular pH, caused by membrane potential oscillations in red cells. The oscillations were induced by addition of the Ca^{2+} ionophore A23187 at zero time, to a calculated concentration in the cell suspension of 2.3×10^{-6} (——) and 3.8×10^{-6} (-----) moles/liter H_2O. The abscissa axis is time in seconds, the ordinate axis the actual changes in the pH in the extracellular phase (left) and the membrane potential calculated from the pH changes (right)

energy for the redistribution process in the temperature interval 30–37°C is found to be 7.4 kcal/moles from an Arrhenius plot, and 14.9 kcal/mole over the interval from 15–22°C.

Discussion

When the TCS flux is described by a single-barrier transport model, where the potential factor Q equals -1 (*see* Calculations and ref. 2), the calculated anion permeability is found to be constant with varying membrane potential. This single-barrier translocation scheme is in accordance with what have been found for the TPA, tetraphenylarsonium cation [5] and the CCCP anion [2], and could be expected, since TCS, like TPA and CCCP translocates across the lipid moiety of the red cell membrane, and therefore, in the case of the anions, traverses the same potential energy barrier. The result thus confirms the conclusion from the earlier reports, that the potential energy profile for translocation of small lipophilic (an)ions in the human red cell membrane is triangular rather than rectangular.

The ability of the transport model, developed for CCCP, to describe the TCS redistribution across the red cell membrane, indicates that all protonophores that translocate as monomers [6, 8] can be described by this model.

The influence of TCS upon valinomycin-induced hyperpolarization, which increases with increasing concentrations of TCS, parallels the effect of CCCP, but the concentration at half-maximal hyperpolarization is about 1×10^{-6} moles/liter, one order of magnitude less than found for CCCP under otherwise similar experimental conditions. This excess hyperpolarization is caused by an augmented valinomycin-induced K^+ conductance, and reflects a direct influence of the protonophore upon the rate constants in the valinomycin transport cycle [3].

Since the temperature dependence for the translocation of protonophores is a function of at least two temperature-dependent variables, namely the distribution coefficient for the protonophore between the water and lipid phases and the rate constant for translocation, the apparent activation energy does not yield information about the potential energy barrier, but only about the actual reequilibration times.

When valinomycin is added to a suspension of red cells, the membrane potential attains a new (quasi) steady-state value, which changes slowly (depolarizes), because the potassium and chloride Nernst potentials decrease under the induced net efflux condition.

If protonophore has been added to the cell suspension in order to estimate the membrane potential, protons and protonophore will redistribute as a consequence of the hyperpolarization, and ultimately the extracellular pH reaches a new (quasi) steady-state value, corresponding to the hyperpolarization. Under these conditions, the time taken

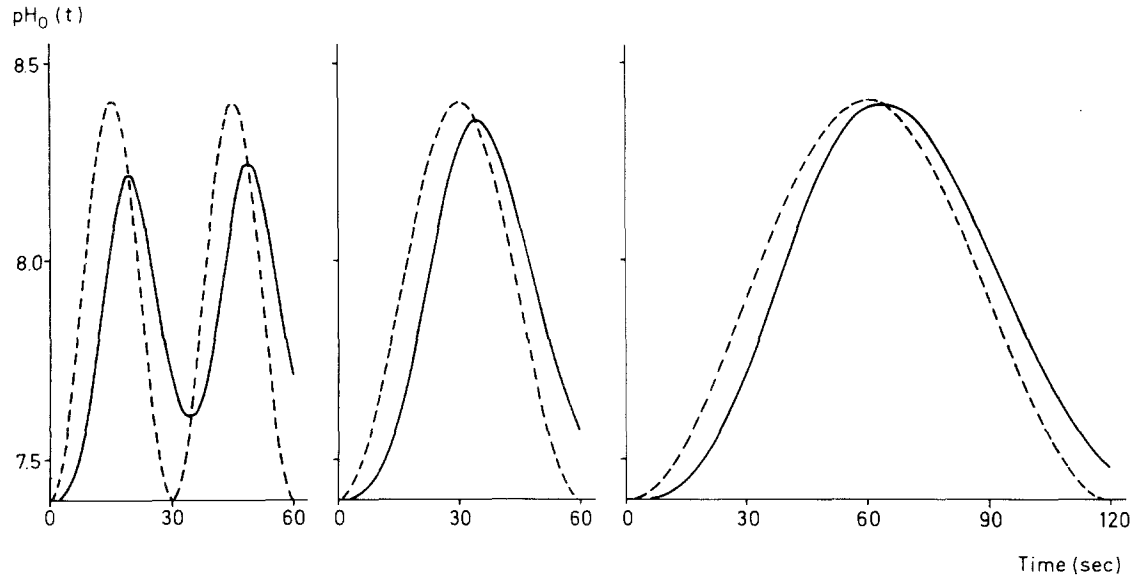

Fig. 5. Simulated CCCP-mediated changes in extracellular pH (——), caused by sinusoidal membrane-potential changes (-----) with frequencies of one cycle every 30, 60 and 120 sec. Initial pH_{in} is assumed to be 7.2 and pH_{ii} 7.4. Abscissa axis time is in seconds, ordinate axis in pH

for the ionophore redistribution is of minor consequence only.

If protonophores are used for estimations of membrane-potential transients, however, the time lag between potential change and protonophore-mediated pH equilibration becomes critical.

It has been shown [11], that relatively fast oscillations in red cell K^+ conductance can be induced under certain experimental conditions, using the Ca^{2+} ionophore A23187 to bring about an opening of the Ca^{2+}-sensitive K^+ channel. The oscillating K^+ conductance results in an oscillating membrane potential, *see* Fig. 4, with an upper frequency of about one cycle every two minutes. The question then arises how faithfully the changes in extracellular pH reflect the changes in membrane potential.

Figure 5 shows a simulation of CCCP-mediated changes in extracellular pH, calculated according to Eq. (5). A sinusoid variation in membrane potential, with an amplitude of −61.5 mV has been assumed, which, when steady state has been attained, results in an extracellular alkalinization of one pH unit. (It is not intended to imply any specific physiological significance to the choice of a sine function, only mathematical convenience.) The CCCP permeability is 2.00×10^{-4} cm/sec, and the binding parameter, α, 45.

At a frequency of one cycle every 120 sec, the phase shift is only small, about 5 sec, and the attenuation at the peak value is insignificant, *see* Fig. 5, right panel, but with increasing frequency the phase shift and attenuation increases too, meaning that the extracellular pH change becomes a more and more distorted representation of the changing membrane potential (Fig. 5, middle and left panels).

Consequently, the pH oscillations with the shortest period, shown in Fig. 4, must be expected to show a time lag of about 5 sec with respect to the generating potential change.

Lowering the temperature decreases the limiting frequency, i.e., the frequency where the distortion is insignificant (*see* Fig. 6) in that the CCCP permeability decreases with decreasing temperature. At 15°C, the peak value in extracellular pH is shifted about 20 sec to the right, with an attenuation of the peak value of 20%. At 15°C, the limiting frequency thus decreases to a cycle every 8 to 10 min.

It should be noted, however, that these values are minimum values attained with a buffer-free medium, under which conditions the transport model is valid. If buffer is present in the extracellular medium, the minimum cycle times increase. Furthermore, it is important to realize that phase shift and attenuation is dependent upon the waveform of the potential change that induces the change in the extracellular pH. If the change in membrane potential is instantaneous, which can be considered to be the case when valinomycin is added to a suspension of red cells, the full change in extracellular pH is evident only after about 30 sec [2].

Conclusion

Although estimation of change in membrane potential, from one steady-state value to another, using

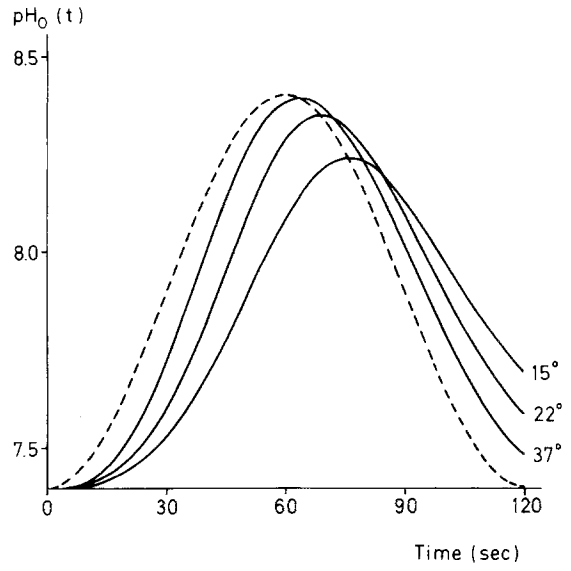

Fig. 6. Simulated CCCP-mediated changes in extracellular pH (——), at various temperatures caused by a sinusoidal membrane-potential change (-----) with a frequency of one cycle every 120 sec. Initial pH values and coordinate axes are as in Fig. 5

the concomitant protonophore-mediated shift in extracellular pH, presents no difficulties, extreme care is necessary if the change in membrane potential is transient.

Using CCCP at 37°C, the pH response following changes in membrane potential is significantly phase shifted and attenuated, when the transients become faster than one cycle every 2 min, and it becomes increasingly difficult or impossible to estimate the relationship in time between the calculated membrane potential and other cellular events.

This situation becomes progressively worse at temperatures lower than 37°C, because the time required for CCCP equilibration increases.

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References

1. Bennekou, P. 1984. K^+-valinomycin and chloride conductance of the human red cell membrane: Influence of the membrane protonophore carbonylcyanide *m*-chlorophenylhydrazone. *Biochim. Biophys. Acta* **776:**1–9
2. Bennekou, P. 1988. Protonophore anion permeability of the human red cell membrane determined in the presence of valinomycin. *J. Membrane Biol.* **102:**225–234
3. Bennekou, P., Christophersen, P. 1986. Flux ratio of valinomycin-mediated K^+ fluxes across the human red cell membrane in the presence of the protonophore CCCP. *J. Membrane Biol.* **93:**221–227
4. Macey, R.I., Adorante, J.S., Orme, F.W. 1978. Erythrocyte membrane potentials determined by hydrogen ion distribution. *Biochim. Biophys. Acta* **512:**284–295
5. Macey, R.I., Orme, F.W. 1980. Permeability of red cells to lipid soluble ions. *In:* Membrane Transport in Erythrocytes. Alfred Benzon Symposium 14. U.V. Lassen, H.H. Ussing and J.O. Wieth, editors. pp. 498–512. Munksgaard, Copenhagen
6. McLaughlin, S.G.A., Dilger, J.P. 1980. Transport of protons across membranes by weak acids. *Physiol. Rev.* **60:**825–864
7. Press, W.H., Flannery, B.P., Teukolsky, S.A., Vetterling, W.T. 1986. Numerical Recipes. Cambridge University Press, Cambridge
8. Neumcke, B., Bamberg, E. 1975. The action of uncouplers on lipid bilayer membranes. *In:* Membranes: A Series of Advances. Vol. 3, pp. 215–254. G. Eisenman, editor. Marcel Dekker, New York
9. Sadykov, Y.H., Holmuhamedov, E.L., Evtodienko, V. 1984. Effect of pH and proton buffer on oscillations of ion fluxes in rat erythrocytes. *Eur. J. Biochem.* **143:**369–371
10. Vestergaard-Bogind, B. 1983. Spontaneous inactivation of the Ca^{2+}-sensitive K^+ channels of human red cells at high intracellular Ca^{2+} activity. *Biochim. Biophys. Acta* **730:**285–294
11. Vestergaard-Bogind, B., Bennekou, P. 1982. Calcium-induced oscillations in K^+ conductance and membrane potential of human erythrocytes mediated by the ionophore A23187. *Biochim. Biophys. Acta* **688:**37–44

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