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Membrane Potential, Anion and Cation Conductances in Ehrlich Ascites Tumor Cells

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Summary. The fluorescence intensity of the dye 1,1'-dipropyloxadicarbocyanine ($DiOC_3$-(5)) has been measured in suspensions of Ehrlich ascites tumor cells in an attempt to monitor their membrane potential (V_m) under different ionic conditions, after treatment with cation ionophores and after hypotonic cell swelling. Calibration is performed with gramicidin in Na^+-free K^+/choline$^+$ media, i.e., standard medium in which NaCl is replaced by KCl and cholineCl and where the sum of potassium and choline is kept constant at 155 mM. Calibration by the valinomycin "null point" procedure described by Laris *et al.* (Laris, P.C., Pershadsingh, A., Johnstone, R.M., 1976, *Biochim. Biophys. Acta* **436**:475–488) is shown to be valid only in the presence of the Cl^--channel blocker indacrinone (MK196). Distribution of the lipophilic anion SCN^- as an indirect estimation of the membrane potential is found not to be applicable for the fast changes in V_m reported in this paper. Incubation with $DiOC_3$-(5) for 5 min is demonstrated to reduce the Cl^- permeability by 26 ± 5% and the NO_3^- permeability by 15 ± 2%, while no significant effect of the probe could be demonstrated on the K^+ permeability. Values for V_m, corrected for the inhibitory effect of the dye on the anion conductance, are estimated at −61 ± 1 mV in isotonic standard NaCl medium, −78 ± 3 mV in isotonic Na^+-free choline medium and −46 ± 1 mV in isotonic $NaNO_3$ medium. The cell membrane is depolarized by addition of the K^+ channel inhibitor quinine and it is hyperpolarized when the cells are suspended in Na^+-free choline medium, indicating that V_m is generated partly by potassium and partly by sodium diffusion. Ehrlich cells have previously been shown to be more permeable to nitrate than to chloride. Substituting NO_3^- for all cellular and extracellular Cl^- leads to a depolarization of the membrane, demonstrating that V_m is also generated by the anions and that anions are above equilibrium. Taking the previously demonstrated single-file behavior of the K^+ channels into consideration, the membrane conductances in Ehrlich cells are estimated at 10.4 $\mu S/cm^2$ for K^+, 3.0 $\mu S/cm^2$ for Na^+, 0.6 $\mu S/cm^2$ for Cl^- and 8.7 $\mu S/cm^2$ for NO_3^-. Addition of the Ca^{2+}-ionophore A23187 results in net loss of KCl and a hyperpolarization of the membrane, indicating that the K^+ permeability exceeds the Cl^- permeability also after the addition of A23187. The K^+ and Cl^- conductances in A23187-treated Ehrlich cells are estimated at 134 and 30 $\mu S/cm^2$, respectively. The membrane potential is depolarized in hypotonically swollen cells, confirming that the increase in the Cl^- permeability following hypotonic exposure exceeds the concommitant increase in the K^+ permeability. In control experiments where the membrane potential $V_m = E_K = E_{Cl} = E_{Na}$, it is demonstrated that cell volume changes has no significant effect on the fluorescence signal, apparently because of a large intracellular buffering capacity. The increase in the Cl^- conductances is 68-fold when cells are transferred to a medium with half the osmolarity of the standard medium, as estimated from the net Cl^- efflux and the change in V_m. The concommitant increase in the K^+ conductance, as estimated from the net K^+ efflux, is only twofold.

Key Words membrane potential · Cl^- conductance · NO_3^- conductance · K^+ conductance · fluorescent probe · regulatory volume decrease · volume regulation · ionophore A23187 · quinine · indacrinone · Ehrlich ascites mouse tumor cells

Introduction

Knowledge of the electrical potential difference across the cell membrane and the chemical gradients for the ions transported across the membrane permits conclusions about the nature of the transport of these ions as well as the relative permeabilities of the membrane to these ions.

Various approaches have been used to estimate the resting membrane potential (V_m) of Ehrlich cells with variating results. Lassen et al. (1971), Hoffmann, Simonsen and Sjøholm (1979), and Dawson and Smith (1987) report membrane potentials in cells suspended in standard solutions in the range −20 to −28 mV, as measured directly by microelectrodes. Gstrein, Paulmichl and Lang (1987), on the other hand, find a much larger membrane potential at −57 mV, using the same technique. Indirect techniques for estimation of the cell membrane potential include the use of (*i*) lipid-soluble cations (dibenzyldimethylammonium), the distribution of which indicates a membrane potential of −17 to −23 mV (Cespedes & Christensen, 1974), (*ii*) lipophilic anion (SCN^-), the distribution of which indicates a membrane potential of −19.6 mV (Smith & Robin-

son, 1989) and (*iii*) fluorescent cyanine dyes. The membrane potential of Ehrlich cells has been assessed with two fluorescent probes 3,3'-dipropylthiodicarbocyanine ($DiSC_3$-(5)) and 1,1'-dipropyloxadicarbocyanine ($DiOC_3$-(5)), and the values obtained using these dyes were all in the range −35 to −60 mV (*see* Laris et al., 1976; Burckhardt, 1977; Philo & Eddy, 1978; Hoffmann & Lambert, 1983), except in cells incubated for a longer period (30 min to 2 hr) in which values in the range −18 to −42 mV were recorded (Laris et al., 1976). Where most of the microelectrode measurements may have given erroneously low values for the membrane potentials due to leakage caused by the microelectrode impalements, the results obtained with the dyes have, on the other hand, been questioned for the possible effects of the dye on the ionic conductances as well as errors introduced by the calibration of the dyes using the "valinomycin null-point" method (*see* Johnstone, Laris & Eddy, 1982; Smith, 1982).

In the present report, the fluorescence of the dye $DiOC_3$-(5) is calibrated in Na^+-free media using gramicidin instead of valinomycin to increase the cation permeability of the cell membrane. The influence of the fluorescent dye on the chloride conductance is examined indirectly from measurements of changes in cell volume by a technique that has previously been described (*see* Hoffmann, Lambert & Simonsen, 1986) and the membrane potential is corrected accordingly. The effect of cell volume changes and variation in cell density on the fluorescence signal has been investigated.

Swelling in hypotonic media or addition of the Ca^{2+}-ionophore A23187 to Ehrlich ascites tumor cells in isotonic medium activate separate, conductive K^+ and anion transporting pathways, resulting in a substantial net loss of KCl and associated loss of cell water (*see* Hoffmann, Simonsen & Lamberts, 1984, Hoffmann et al., 1986). The regulatory volume decrease (RVD), following the hypotonic swelling, and the A23187-induced cell shrinkage are both (*i*) inhibited by quinine and by Ba^{2+}, which block the Ca^{2+}-activated K^+ channel (Lambert, 1984; Lambert, Simonsen & Hoffmann, 1984; Hoffmann et al., 1986) and (*ii*) inhibited by the chloride channel inhibitor indacrinone (Aabin & Hoffmann, 1986). In order to quantify the increase in the conductances for K^+ and for Cl^- during RVD and after increase in the free cellular Ca^{2+}, we have estimated the cell membrane potential in suspensions of Ehrlich cells after transference to hypotonic solutions or after addition of A23187. Furthermore, the effect of channel blockers on the membrane potential has been investigated.

Materials and Methods

Cell Suspensions and Incubation Media

Ehrlich ascites tumor cells were obtained from white Theiller mice seven days after intraperitoneal implantation. The mice were killed by dislocation of the cervical vertebras, and the tumor cells were immediately afterward harvested in standard incubation medium containing heparin (2.5 IU/ml). The cells were washed by centrifugation (700 × *g*, 45 sec), once with standard medium and twice in the appropriate experimental solutions. The composition of the standard medium (300 mOsm) was 150 mM Na^+; 5 mM K^+; 150 mM Cl^-; 1 mM Mg^{2+}; 1 mM Ca^{2+}; 1 mM sulfate; 1 mM inorganic phosphate; 3.3 mM MOPS (3-(N-morpholino) propane sulfonic acid); 3.3 mM TES (N-tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid; 2-(2-hydroxy-1,1-*bis*(hydroxymethyl)ethyl) aminoethane sulfonic acid) and 5 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid. Hypotonic media (225 and 150 mOsm) were prepared by diluting the isotonic media with distilled water containing the buffers alone. $NaNO_3$ medium was prepared by substituting the Na^+ and the K^+ salts of NO_3^- for NaCl and KCl. In choline$^+$ and K^+ media, choline chloride and KCl, respectively, were substituted for NaCl in equimolar amounts. N-methyl-D-glucamineNO_3 medium, where N-methyl-D-glucammonium$^+$ was substituted for Na^+ and NO_3^- was substituted for Cl^-, was prepared from a N-methyl-D-glucamine stock solution titrated with equimolar amounts of HNO_3. The pH was adjusted to 7.4 in all media. The temperature was kept at 37°C under all conditions.

Measurement of Ion Concentration and Water Content

Cell suspension samples (1 ml) were transferred to preweighed vials for determination of ion content and cell water. The vials were centrifuged (20,000 × *g*, 60 sec), thereafter 100 μl samples of the supernatant were diluted 10 times with perchloric acid (7% final concentration) and saved for determination of extracellular ion content. Excess supernatant was removed by suction and the wet wt of the cell pellet was determined by reweighing the samples. The packed cells were then lysed in 800 μl distilled water, deproteinized by addition of 100 μl perchloric acid (70%) and centrifuged (20,000 × *g*, 10 min). Supernatant samples (600 μl) were saved for determination of cellular ion content before removal of excess supernatant by suction. The dry wt of the perchloric acid precipitate, determined by drying the samples for 48 hr at 90°C followed by reweighing, was converted to cell dry wt by multiplication by the factor 0.77 (1 g dried perchloric acid precipitate were in 20 separate experiments found to correspond to 0.77 ± 0,02 g cell dry wt).

Na^+ and K^+ were determined by atomic absorption flame photometry (Perkin Elmer atomic absorption spectrophotometer, model 2380) after 100-fold dilution of samples as well as standards with 1 mM CsCl. This dilution was performed in order to increase the linearity of the standard curves and to eliminate interference of Na^+ in K^+ measurements and vice versa. Cl^- was assessed by coulometric titration (CMT 10 chloride titrator, Radiometer, Denmark). ^3H and ^{14}C activities were measured in a liquid scintillation spectrometer (Packard TRI-CARP 460C Liq-

uid Scintillation System), using a 200 μl diluted sample in 2.5 ml scintillation fluid (Pico-Flour ™15, Packard).

The cellular concentrations were calculated as the concentration in cell water after correction for trapped incubation medium (^{3}H inulin space) in the cell pellets. Cell water content is calculated as mg cell water per mg dry wt.

Fluorometric Measurements

Membrane potential-sensitive fluorescence measurements were performed in polystyrene cuvettes at 37°C on a Perkin Elmer LS-5 luminescence spectrometer connected to a Perkin Elmer R 100A recorder and a Perkin Elmer CP 100 graphic printer. Excitation and emission wavelengths were 577 and 605 nm, respectively, and slit widths were 5 nm. The fluorescent dye, 1,1'-dipropyloxadicarbocyanine ($DiOC_3$-(5)), was added to cell suspensions with a cytocrit of 0.25% at a final concentration of 1.6 μM. All subsequent additions of ionophores and inhibitors to the cuvette were made after a steady level of fluorescence had been ensured.

Calibration of the membrane potential in fluorescence units was performed with cells suspended in Na^+-free K^+/choline$^+$ media by isosmotically varying the extracellular K^+ concentration and using a modified version of the valinomycin "null point" method, originally described by Laris et al. (1976). Measured intracellular and extracellular K^+ concentrations were used for the calculation of the Nernst equilibrium potential for each extracellular K^+ concentration. Gramicidin (0.5 μM) was added in order to ensure a high K^+ permeability. The fluorescence signal reached in all cases a new steady-state value within 1 to 2 min following addition of the cation ionophore. The calibration curve was obtained as a plot of the fluorescence after addition of gramicidin *vs.* the Nernst equilibrium potential for K^+. As gramicidin-induced K^+ conductance dominates the total conductance, the Nernst equilibrium potential for K^+ was taken to be identical to the membrane potential (*see* Results).

Cell Volume Measurements

Cell volumes were measured on cultures diluted 500 times with filtered media (Millipore, pore size 0.45 μm), resulting in a final cell density of 80,000-100,000 cells/ml (cytocrit $\approx$ 0.01%). Volumes were calculated as the median of volume distribution curves obtained with a Coulter counter model ZB equipped with a Coulter channellyzer C 1000. Tube orifice was 100 μm. Calibration was carried out with latex beads (diameter 13.5 μm, Coulter Electronics). Alternatively, cell volume was measured as mg cell water per mg dry wt as described above.

Chemicals

Stock solutions of valinomycin (0.6 mM), A23187 (2 mM), gramicidin (1 mM), quinine hydrochloride (1 M) (all from Sigma Chemicals, St. Louis, MO), $DiOC_3$-(5) iodide (1.2 mM) (Molecular Probes, Junction City, OR) and indacrinone, i.e., MK196 (300 mM) (Merck Sharp & Dohme, Rahway, NJ) were prepared in ethanol and kept at −22°C until use.

Statistical Evaluation

All values are expressed as the mean ± SEM with the number of experiments in brackets.

Calculations

Membrane Conductance and Transference Number

The current (I_j) carried across a biological membrane by the ion j is phenomenologically expressed as

$$I_j = G_j \cdot (V_m - E_j) \tag{1}$$

where G_j and E_j are the membrane conductance and the Nernst potential for j, respectively, and V_m is the membrane potential (for example, *see* Sten-Knudsen, 1978).

In the Ehrlich ascites tumor cell, the current through the plasma membrane is carried mainly by K^+, Na^+ and Cl^-, while any electrogenic contribution of the Na^+/K^+ pump seems to be negligible (*see* Gstrein et al., 1987). Accordingly, under steady-state conditions with constant potentials, i.e., where the total current across the membrane is zero we get

$$G_K \cdot (V_m - E_K) + G_{Na} \cdot (V_m - E_{Na}) + G_{Cl} \cdot (V_m - E_{Cl}) = 0 \tag{2}$$

which solved for V_m gives

$$V_m = \frac{G_K \cdot E_K + G_{Na} \cdot E_{Na} + G_{Cl} \cdot E_{Cl}}{G_K + G_{Na} + G_{Cl}}. \tag{3}$$

In media where the apparently nonpermeating choline$^+$ ion is substituted for Na^+ or where the permeating NO_3^- is substituted for Cl^-, Eq. (2) is transformed into

$$G_K \cdot (V_m - E_K) + G_{Cl} \cdot (V_m - E_{Cl}) = 0 \tag{4}$$

for choline media and

$$G_K \cdot (V_m - E_K) + G_{Na} \cdot (V_m - E_{Na}) + G_{NO_3} \cdot (V_m - E_{NO_3}) = 0 \tag{5}$$

for nitrate media.

Equations (2), (4) and (5) are in the present study used to calculate G_{Na}, G_{Cl} and G_{NO_3} relative to G_K, assuming that the conductances are unchanged by the experimental conditions.

The absolute membrane conductance for K^+ (G_K) can be calculated from the diffusional net flux of K^+ ($J_K^{net(d)}$) using the equations

$$G_K = F \cdot J_K^{net(d)}/(V_m - E_K) \tag{6}$$

$$J_K^{net(d)} = J_K^{o(d)} - J_K^{io} \tag{7}$$

where the passive component of the K^+-influx ($J_K^{o(d)}$) is calculated from the unidirectional K^+-efflux (J_K^{io}), under the assumption that the flux ratio equation (Ussing, 1949) is valid, i.e.

$$J_K^{in}/J_K^{out} = \exp\{(V_m - E_K) \cdot F/RT\}. \quad (8)$$

F, R and T are Faraday's constant, the gas constant and the absolute temperature, respectively. G_K can also be estimated from the unidirectional K^+-flux (J_K) measured near electrochemical equilibrium using the equation (*see* Läuger, 1980)

$$G_K = F^2 \cdot J_K/RT. \quad (9)$$

In the case of single-file diffusion, where the potassium ion is supposed to cross the membrane through narrow pores, which can only accommodate a single row of two or more particles, tracer and parent species will not cross the membrane independently. Hodgkin and Keynes (1955) have demonstrated that movement of tracer in the direction of its electrochemical potential difference is accelerated while flow in the opposite direction is retarded. According to Läuger (1980), Eqs. (8) and (9) then take the form

$$J_K^{in}/J_K^{out} = \exp\{n' \cdot (V_m - E_K) \cdot F/RT\} \quad (10)$$

$$G_K = n' \cdot F^2 \cdot J_K/RT \quad (11)$$

where n' is an expression for the deviation from free electrodiffusion. It is emphasized that single-file diffusion has previously been demonstrated for K^+ in the Ehrlich cells (Hoffman et al., 1986).

The fractional conductance or the transfer number for K^+ (t_K), Na^+ (t_{Na}) and for Cl^- (t_{Cl}) are calculated from the membrane conductances using the following equations (*see* Lassen, Pape & Vestergaard-Bogind, 1978)

$$t_K = G_K/G_m \quad (12)$$

$$t_{Na} = G_{Na}/G_m \quad (13)$$

$$t_{Cl} = G_{Cl}/G_m \quad (14)$$

$$G_m = G_K + G_{Na} + G_{Cl}. \quad (15)$$

Results

Calibration Curve for the Membrane Potential Measured with a Fluorescent Probe

In a Na^+-free K^+/choline$^+$ medium, the equilibrium potential (Nernst potential) for Cl^- (E_{Cl}) and for K^+ (E_K) have been calculated from the extracellular and the cellular concentrations of the ions and plotted as a function of the external K^+ concentration (K_o^+). From Fig. 1*A*, it is seen that the slope of E_K *vs.* K_o^+ is 27 mV per *e*-fold change or 62 mV per decade, whereas the slope in a E_{Cl} *vs.* K_o^+ plot is only 7 mV per *e*-fold change or 16 mV per decade. $E_{Cl} = E_K = -13$ mV at 119 mM K_o^+ as determined from the intersection of the two regression lines in Fig. 1*A*. It is assumed that Na^+ does not play any significant role in choline media and that the membrane potential, therefore, is determined by the distribution of Cl^-

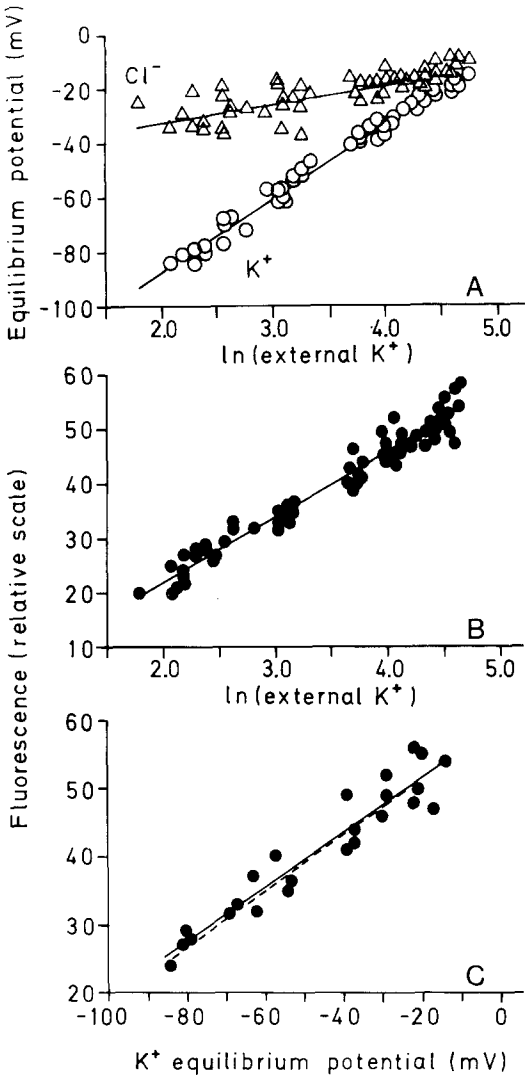

Fig. 1. Calibration curve for the membrane potential of Ehrlich cells as measured with a fluorescence probe. The media are Na^+-free K^+/choline$^+$ media where the sum of K^+ and choline$^+$ was kept constant at 155 mM. (*A*) Equilibrium potential for Cl^- and K^+ as a function of the external K^+ concentration. The distribution of Cl^- and K^+ across the membrane was measured at steady state (20 to 60 min after transfer to the experimental solution) and the equilibrium potential calculated as the Nernst potential. The values are from 12 different experiments. (*B*) The effect of variation in external K^+ concentration on the $DiOC_3$-(5) (1.6 μM) fluorescence of the cell suspension after addition of 0.5 μM gramicidin. The fluorescence is given in percent of the fluorescence of the dye in medium without cells. The values are from 14 different experiments. (*C*) Fluorescence of the cell suspension as a function of the K^+ equilibrium potential. The fluorescence is given in percent of the fluorescence of the dye in medium without cells after a steady fluorescence signal was obtained. The values are from four different experiments where the K^+ gradient and the fluorescence were measured in parallel. The *dashed line* is calculated from the regression lines for K^+ in Fig. 1*A* and the regression line in Fig. 1*B*. The *solid line* is as in all figures the regression line calculated from the experimental points

and K^+. Thus, for all external K^+ values lower than 119 mM, an increase in Cl^- permeability will depolarize the resting membrane potential and an increase in the K^+ permeability will hyperpolarize the resting membrane potential. Since gramicidin is known to produce a substantial K^+ permeability (Pressman, 1976), addition of this ionophore to cells suspended in choline medium should bring their membrane potential close to E_K. Figure 1*B* shows the fluorescence intensity of the dye $DiOC_3$-(5) (1.6 μM) approximately 2 min after addition of gramicidin (0.5 μM) as a function of external K^+ in choline medium. A plot of E_K measured at different external K^+ concentrations without addition of gramicidin *vs.* E_K measured in the presence of gramicidin (3 to 5 μM) gave in four different experiments a slope of 0.96 ± 0.05, indicating that addition of gramicidin does not cause an immediate change in E_K in Ehrlich cells. Therefore, the change in the fluorescence signal following variation in K_o^+ in the range 5.5 to 90 mM may be proportional to the change in the K^+ equilibrium potential. Thus, a plot of the fluorescence intensity after addition of gramicidin as a function of the K^+ equilibrium potential may serve as a calibration curve in the range −15 to −80 mV. Figure 1*C* shows a calibration curve constructed from corresponding measurements of the fluorescence intensity and the calculated values of the K^+ equilibrium potential. The solid line, calculated from the experimental points in Fig. 1*C* and the dashed line, calculated from Fig. 1*A* and *B*, give essentially the same calibration curve. The slopes of the calibration curves are 0.403 (solid line) and 0.417 (dashed line), respectively.

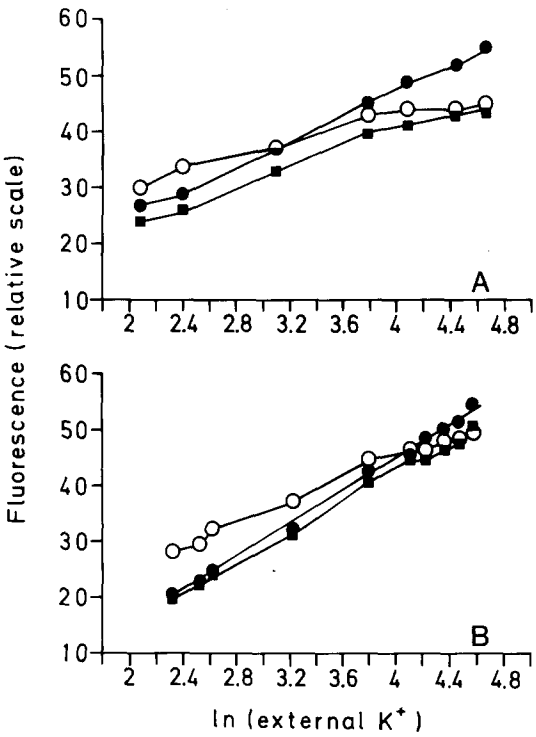

Fig. 2. The effects of K^+-ionophores and the Cl^--channel inhibitor indacrinone (MK196) on the fluorescence of the cell suspensions as a function of the external K^+. Cells were incubated in different K^+/choline$^+$ media as described in the legend to Fig. 1. (*A*) Fluorescence of the cell suspension as a function of external K^+ just before (○) and about 1 to 2 min after addition of 1.5 μM valinomycin (●). Indacrinone (1 mM) was added subsequent to the valinomycin and the fluorescence recorded 1 to 2 min later (■). (*B*) The experimental protocol is identical to the one in (*A*) but with addition of 0.5 μM gramicidin instead of valinomycin. The fluorescence is given in percent of the fluorescence of the dye in cell-free medium. The intersection of the lines with or without addition of valinomycin (*A*) or gramicidin (*B*) is measured at ln K = 3.34 ± 0.53 (n = 5) and ln K = 4.66 ± 0.24 (n = 4), respectively

Evaluation of the "Null Point" Methods Using Valinomycin or Gramicidin

In the valinomycin "null point" method previously used for the estimation of membrane potentials in Ehrlich cells by Laris et al. (1976) and by Philo and Eddy (1978), a concentration of K^+ in the Ringer solution was found where the addition of valinomycin caused no change in fluorescence. Under these conditions, it was assumed that K^+ was passively distributed, i.e., E_K was equal to V_m (*see* Freedman & Laris, 1981). Figure 2*A* shows how the fluorescence observed before and 1 to 2 min after the addition of valinomycin varies with ln (K_o^+) in a Na^+-free, choline medium. The fluorescence signal increases with external K^+ in the presence as well as in the absence of valinomycin, as previously shown by Burckhardt (1977) and by Philo and Eddy (1978). The intersection of the lines is taken to be the interpolated valinomycin "null point" (*see* Philo and Eddy, 1978). This "null point" is found at an external K^+ of 28 mM corresponding to an E_K = −55 mV (*see* legend to Fig. 2). However, from Fig. 2*A* and *B*, it is seen that it is possible to decrease the fluorescence signal further at the valinomycin "null point" (indicating a hyperpolarization of the membrane potential) either by adding MK196, which blocks Cl^- channels in Ehrlich cells (Aabin & Hoffmann, 1986) or by using gramicidin as the cation ionophore instead of valinomycin. Since K^+ and Cl^- are the only current carrying ions in this system and E_{Cl} is less negative than E_K (*see* Fig. 1*A*), it is precluded that V_m could be equal to E_K at the valinomycin "null point." In the presence of MK196,

Table 1. The effect of anion and cation substitution on cell volume, the Nernst equilibrium potential for K^+, Na^+, Cl^- and the membrane potential (V_m) after ion substitution and addition of ionophores[a]

	Standard medium	Choline medium	Nitrate medium
Cell volume, ml/g cell dry wt			
Control	3.37 ± 0.05 (32)	2.86 ± 0.04 (4)	3.10 ± 0.09 (8)
A23187	3.05 ± 0.08 (4)		
Equilibrium potentials, mV			
E_K	−97 ± 0.4 (17)	−81 ± 0.8 (12)	−100 ± 1.6 (8)
E_{Na}	+53 ± 1.9 (17)	—	+70 ± 5 (7)
E_{Cl}	−21 ± 0.5 (16)	−30 ± 3.0 (5)	[b]
Measured membrane potentials, mV			
Control	−62 ± 1.3 (65)	−79 ± 2.6 (9)	−47 ± 1.3 (17)
A23187	−79 ± 1.8 (10)	—	—
Valinomycin	−76 ± 2.7 (4)	—	—
Corrected membrane potentials, mV[c]			
Control	−61 ± 1	−78 ± 3	−46 ± 1

[a] Ehrlich cells, preincubated in isotonic standard NaCl medium or $NaNO_3$ medium for 30 min, were washed to standard NaCl medium, choline chloride medium or to $NaNO_3$ medium, respectively (for details, *see* Materials and Methods). Final cytocrit was 6–7% for ion determinations and 0.25% for estimation of the membrane potentials. Samples for determination of the cell volume, the cellular and extracellular concentrations of Na^+, K^+ and Cl^- were taken 10 to 30 min after transfer to the experimental medium. The membrane potential was determined from the steady fluorescence signal of $DiOC_3$-(5) (1.6 μM) and a standard curve similar to the one in Fig. 1*C*, performed in parallel on the experimental cell culture. In the case of A23187 (1.5 μM) and valinomycin (1.5 μM), stable signals were obtained within 2 min following the addition of the ionophore.

[b] E_{NO_3} in NO_3-medium is assumed to be equal to E_{Cl} in Cl-medium.

[c] Values are corrected for the effect of $DiOC_3$-(5) on the anion conductances (*see* text).

the "null point" is shifted to approximately 90 mM external K^+ ($E_K = -20$ mV) close to the K_o^+ concentration where E_K equals E_{Cl} (*see* Fig. 1*A*). Thus, apparently valinomycin can only be applied for the calibration of the probe in the presence of a Cl^- channel inhibitor.

Figure 2*B* shows that a similar value for the "null point" after addition of gramicidin in Na^+-free choline medium is found close to the concentration where $E_{Cl} = E_K$ and that addition of MK196 has a much smaller effect on the fluorescence signal. In the range of K^+ concentration from 55 to 6 mM, corresponding to −35 to −80 mV, the relation between fluorescence and $\ln K_o^+$ is not at all affected by the chloride channel inhibitor, whereas a slight hyperpolarization is found in the presence of MK196 when the equilibrium potential to K^+ is less negative than −35 mV. Thus, it is concluded that the curve in Fig. 1*C* can be used as a calibration curve in the interval of −35 to −80 mV.

Does Variation in Cell Volume Affect the Fluorescence Signal?

It is clear that the fluorescence depends not only on V_m (which dictates the dye distribution across the membrane), but also on the relative compartment sizes of the cells and the medium (i.e., the larger the relative cellular compartment volume, the more dye uptake is needed to establish the distribution). Cells in standard medium are swollen compared to cells in Na^+-free choline medium with extracellular K^+ concentration below 6 mM (*see* Table 1). Thus, the same amount of cells in standard NaCl medium as in the calibration medium results in a larger relative cellular compartment and a resulting lower fluorescence signal. Thus, their membrane potential will be overestimated (regarded as more negative). As shown in the Appendix, it is possible to test a measured fluorescence signal for effects of differences in the relative cellular compartment and, thereby, separate this effect from effects related to V_m. The conclusion drawn from the data described in the Appendix is that volume effects are almost negligible because of a large intracellular concentration of a low affinity buffer for the dye. Figure 3 shows the cell water content for cells suspended in different calibration media. The standard curve from Fig. 2 is indicated as a dotted line. This figure is used to find differences in relative cellular compartments, which are used to evaluate the eventual correction of the fluorescence signal. Cells in standard medium have a 15% higher cell volume than the corresponding cell volume in the calibration medium, resulting in a decrease in the fluorescence signal, which is <1%

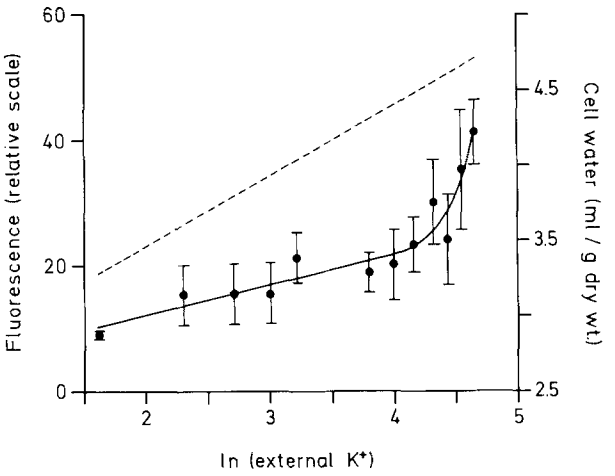

Fig. 3. The effect of variation in external K^+ concentration on cell volume and the $DiOC_3$-(5) fluorescence. Cell volume measurements from the experiments shown in Fig. 1 are grouped in 5-mM K^+ intervals from 2.5 to 47.5 mM and in 10-mM K^+ intervals from 50 to 110 mM with SEM indicated as bars. In the range of external K^+ concentration from 6 to 70 mM, the cell volume increased linearly with the slope = 0.19. In three series of experiments performed in Na^+/K^+ media (the sum of K^+ and Na^+ was kept constant at 155 mM), the cell volume (ml cell water/g dry wt) increased linearly with the slope = 0.25. The fluorescence values from Fig. 1 (*B*) are represented by the linear regression line (dotted)

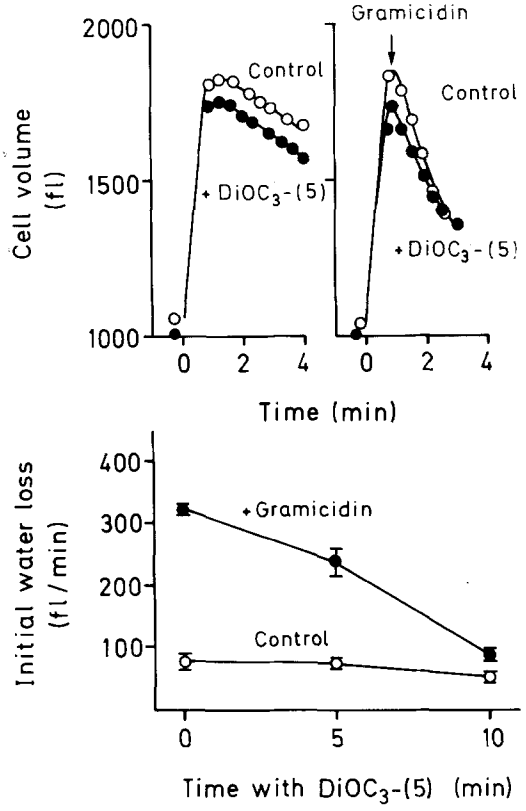

Fig. 4. The effect of $DiOC_3$-(5) on the regulatory volume decrease in Ehrlich cells in hypotonic, Na^+-free choline medium. *Upper panels:* Ehrlich cells were preincubated at 0.25% cytocrit in standard incubation medium. At zero time, a sample of the cell suspension was diluted 25-fold (final cell density about 90,000 cells/ml) in hypotonic (150 mOsm) choline medium, and the cell volume in fl (10^{-15} liter) was followed with time using a Coulter counter. $DiOC_3$-(5) (1.6 μM, closed symbols) was added to the preincubation solution 5 min prior to the hypotonic exposure. Control cells (open symbols) were not treated with $DiOC_3$-(5). Gramicidin (0.5 μM) was added to the cells, as indicated by the arrow in the upper right panel in order to ensure a high K^+ permeability. The cell volumes in swollen cells treated with $DiOC_3$-(5) are smaller than the cell volumes in untreated control cells because the pretreatment in general caused a reduction of the initial cell volume. *Lower panel:* The initial rate of volume recovery was in the absence of gramicidin calculated as the water loss within 1 and 3 min after the osmotic shock, and in the presence of gramicidin as the water loss within 1.5 min after addition of gramicidin. Note that control cells in the lower panel are cells not treated with gramicidin. Values are given as the mean of at least four independent measurements ± SEM

(*see* Appendix). Thus, no correction of the fluorescence signal is needed.

Does $DiOC_3$-(5) Affect the Ionic Conductances?

In order to examine the possible effects of $DiOC_3$-(5) on the membrane permeabilities to K^+ and Cl^-, the influence of $DiOC_3$-(5) on the regulatory volume decrease (RVD) following swelling in hypotonic medium was investigated. In the swollen cells, the Cl^- conductance exceeds that for K^+, so that the rate-limiting step in RVD is the K^+ conductance (Hoffmann et al., 1986). Since the rate of RVD in the absence of gramicidin is not significantly changed after 5 min preincubation with $DiOC_3$-(5) (Fig. 4, upper left panel and lower panel), it seems reasonable to conclude that the K^+ conductance is not significantly affected by 5-min incubation with the dye. The effect of addition of valinomycin or gramicidin to cells swollen in Na^+-free medium is an acceleration of RVD (Hoffmann et al., 1984, 1986). After addition of sufficient gramicidin, the cation permeability exceeds the anion permeability, so that the Cl^- permeability now becomes the rate-limiting step in RVD. It is assumed that any drug inhibiting RVD in Na^+-free choline medium in the presence of gramicidin, acts by blocking the anion pathway. The initial rate of RVD seen after addition of gramicidin to cells swollen in hypotonic, Na^+-free choline medium is reduced by 26 ± 5% (n = 4) and 72 ± 3% (n = 4) after 5- and 10-min preincubation with $DiOC_3$-(5), respectively (*see* Fig. 4, upper right panel, and lower panel). In cells where all ex-

tracellular and cellular Cl^- is replaced by NO_3^-, the RVD in hypotonic, Na^+-free N-methyl-D-glucamineNO_3 medium was reduced by 15 ± 2% (n = 3) after 5-min preincubation with $DiOC_3$-(5). Thus, this conductive anion permeability seems to be decreased by 15–26%, resulting in a slightly more negative potential after 5-min incubation with $DiOC_3$-(5) compared to potentials in cells not treated with the dye. This may indicate that a correction of the measured membrane potential values is necessary, provided that the chloride channels activated by cell swelling are similar to the conductive pathways of chloride in the unpertubated cells. It should be noted that although the K^+ conductance is rate limiting for RVD, a decreased Cl^- conductance and an unchanged K^+ conductance should still result in a weak inhibition of RVD caused by a smaller driving force for K^+ movement (*see* Eq. (6)). This could explain the weak inhibition of RVD seen after prolonged incubation with $DiOC_3$-(5) (Fig. 4, lower panel).

Can the Cl^- or the SCN^- Equilibrium Potential Be Used as a Measure of the Membrane Potential?

The equilibrium distribution of the lipophilic anion SCN^- might alternatively be used to measure the membrane potential, since Ehrlich cells have a 14 times higher conductance to SCN^- than to Cl^- (Hoffmann et al., 1986, Kramhøft et al., 1986). Ehrlich cells do, however, have a fast anion exchange system for which SCN^- has an even higher affinity than Cl^- (Hoffmann et al., 1979). Since the anion fluxes via the exchange system are much higher than the net fluxes (Hoffmann et al., 1979), the following relation will in steady state exist between the distribution of Cl^- and the distribution of SCN^-, HCO_3^-, NO_3^- and H^+ as well as for any anion that traverses the cell membrane by the anion exchange system (*see* Hoffmann, 1987)

$$\frac{[Cl^-]_i}{[Cl^-]_o} = \frac{[SCN^-]_i}{[SCN^-]_o} = \frac{[HCO_3^-]_i}{[HCO_3^-]_o} = \frac{[NO_3^-]_i}{[NO_3^-]_o} = \frac{[H^+]_o}{[H^+]_i}. \quad (16)$$

The membrane potential after addition of gramicidin in choline medium is close to E_K (*see above*). In order to investigate whether Cl^- or the lipophilic SCN^- are in electrochemical equilibrium in cholineCl medium 5 min after treatment with gramicidin, we have tested whether E_{Cl} or E_{SCN} were similar to E_K. A plot of E_{Cl} and E_{SCN} *vs.* E_K (*see* Fig. 5) gave slopes of 0.39 ± 0.07 (n = 3) and 0.50 ± 0.03 (n = 3), respectively, which are both significantly lower than one. Thus, after addition of gramicidin, Cl^- as well as SCN^- are far from electrochemical

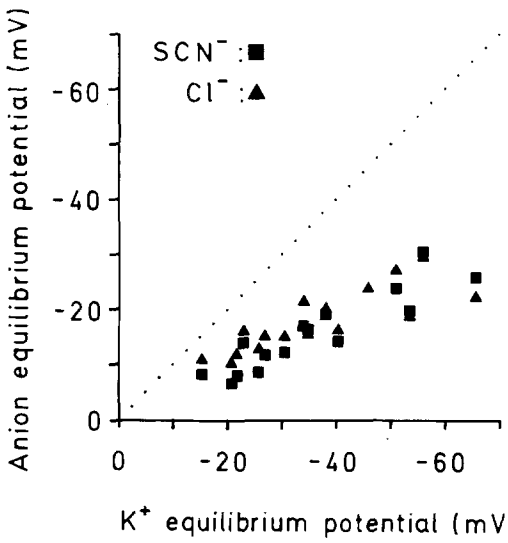

Fig. 5. The relationship between the Nernst equilibrium potential for K^+ (E_K) and for Cl^- (E_{Cl}) or ^{14}C-labeled SCN^- (E_{SCN}) in Ehrlich cells incubated in Cl^--medium where a high permeability for K^+ has been ensured by additions of gramicidin. Cells were prepared and incubated in different K^+/choline$^+$ media as described in the legend to Fig. 1. KSCN (^{14}C-labeled) was added at a final activity at 14 × 10^5 dpm/ml and the cells were incubated for 20 min. Gramicidin (3.5 μM) was added and samples for determination of cellular and extracellular K^+, Cl^- and SCN^- (^{14}C-labeled) activity were taken after another 5-min incubation. E_K, E_{Cl} and E_{SCN} were calculated from the concentrations of K^+ and Cl^- and the ^{14}C-activity of SCN^- in the cellular and the extracellular water phase. The values are from three independent experiments

equilibrium for at least 5 min. This means either that protons are not in equilibrium after 5 min or that the relation described in Eq. (16) has not yet been established. If only 40% of the cellular K^+ should behave as if it was in free solution, as suggested by Dawson and Smith (1986), the data points in Fig. 5 would all be shifted 15.5 mV to the left. This would mean that Cl^- and SCN^- could be closer to the equilibrium at membrane potentials between 0 and −20 mV, but still far from equilibrium at more negative potentials.

At steady state in standard medium, E_{Cl} is different from the resting potential. Blocking the K^+ channels in a Na^+-free medium either by addition of quinine (Figs. 8 and 9) or by addition of Ba^{2+} (Gstrein et al., 1987) produces a strong depolarization of the membrane potential. Since Na^+ has no influence on the membrane potential in nominantly Na^+-free medium, and E_K is more negative than the membrane potential (*see* Table 1), such depolarization can only be explained if E_{Cl} is less negative than V_m. As discussed by Wieth and Braham (1985) and by Hoffmann and Simonsen (1989), the cellular Cl^- concentration is in several cell types, including the

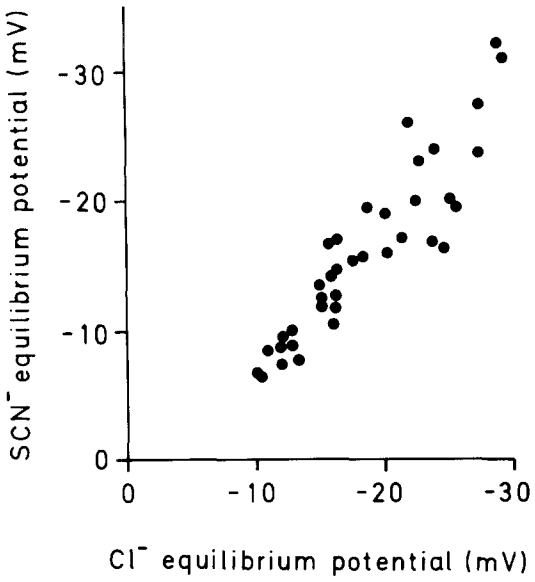

Fig. 6. The relationship between the Nernst equilibrium potential for Cl^- (E_{Cl}) and for ^{14}C-labeled SCN^- (E_{SCN}) in Ehrlich cells incubated in a Cl^--medium. Cells were incubated in different K^+/choline$^+$ media as described in the legend to Fig. 1. KSCN (^{14}C-labeled) was added at a final activity 14×10^5 dpm/ml and samples for determination of cellular and extracellular Cl^- and SCN^- (^{14}C-labeled) activity were taken within the next 15 to 20 min. E_{Cl} and E_{SCN} were calculated from Cl^- concentration and the ^{14}C-activity in the cellular and extracellular water phase. The values are from three different experiments

Ehrlich cells, maintained above electrochemical equilibrium by secondary active Cl^--influx via Na, Cl or Na, K, 2Cl cotransport systems. As seen from Table 1, Cl^- is also above equilibrium in Na^+-free choline medium. This can only be explained if protons are kept out of equilibrium by a H^+ extruding mechanism (like a H^+ pump), resulting in a high $[H^+]_o/[H^+]_i$ value and consequently a high $[Cl^-]_i/[Cl^-]_o$ value (*see* Eq. (16)). An ATP-fueled electrogenic H^+ efflux has been proposed in Ehrlich ascites cells (Heinz, Sachs & Schafter, 1981; Bowen & Lewinson, 1984).

From Fig. 6, it is seen that E_{SCN} (calculated from the distribution of ^{14}C-labeled SCN^- in the Cl^--medium) and E_{Cl} are almost identical in the range $E_{Cl} = -10$ mV to $E_{Cl} = -30$ mV, where E_{Cl} has been changed by varying external K^+ as described in the legend to Fig. 1*A*. Under these circumstances, the cells have been incubated with the ^{14}C-labeled SCN^- in the actual Cl^--medium for 15 to 20 min at 37°C. This is caused by a rapid exchange of ^{14}C-SCN^- with Cl^- via the anion exchanger (Hoffmann et al., 1979) resulting in the distribution shown in Eq. (16). Thus, SCN^- cannot be used for the determination of the membrane potential in Ehrlich cells.

The Influence of Anion and Cation Permeabilities on the Membrane Potential

The membrane potential in Ehrlich ascites cells suspended in standard medium is measured to be −62 mV in the presence of $DiOC_3$-(5) (Table 1). In choline medium, where Na^+ has no influence and where the permeability to choline is negligible, the membrane potential is found to be hyperpolarized to −79 mV (*see* Table 1). Substituting the impermeant cation N-methyl-D-glucammonium$^+$ for Na^+ resulted in a membrane potential of -83 ± 4 mV ($n = 3$). In contrast, the membrane potential in Ehrlich cells suspended in NO_3 medium is depolarized to −47 mV (Table 1). The values for the membrane potential in Table 1 are all measured within the first 5 min after addition of the dye.

We have previously estimated the membrane conductances to the permeating ions in Ehrlich ascites cells using a membrane potential of −27 mV, measured with microelectrodes (Hoffmann et al., 1979). If we now recalculate these values using the membrane potential in the *presence* of $DiOC_3$-(5), i.e., $V_m = -62$ mV, we get the following relative conductance values: $G_K = 1.4 \cdot G_{NO_3} = 3.5 \cdot G_{Na} = 24.5 \cdot G_{Cl}$ (*see* Materials and Methods, Eqs. (3) to (5)). Corrected for the influence of the dye (*see* Fig. 4), G_{Cl} and G_{NO_3} are 1.35 and 1.16 times higher in the absence than in the presence of the dye, while G_K is unaffected by the 5-min preincubation with $DiOC_3$-(5). Assuming that $DiOC_3$-(5) does not affect G_{Na}, we find the following relative conductances in the *absence* of the dye: $G_K = 1.2 \cdot G_{NO_3} = 3.5 \cdot G_{Na} = 18 \cdot G_{Cl}$. Inserting the relative conductances in Eqs. (3) to (5) (*see* Materials and Methods), we calculate the membrane potential in the *absence* of the dye to be −61, −78 and −46 mV in standard medium, choline medium and nitrate medium, respectively (the values are inserted in Table 1).

The absolute K^+ conductance can be calculated from either a unidirectional tracer flux measured near electrochemical equilibrium or from a conductive net flux (*see* Materials and Methods). The ionophore A23187-induced K^+ net flux and the equivalent unidirectional ^{42}K flux performed at pH 8.2 have previously been estimated at 50 and 20 pmol/cm^2 · sec, respectively (*see* Hoffmann et al., 1986). G_K in the presence of A23187 is calculated to be 268 μS/cm^2 (Eq. (6)), using a net flux of 50 pmol/cm^2 · sec, $V_m = -79$ mV and $E_K = -97$ mV (values are from Table 1). On the other hand, using Eq. (9) and the A23187-induced, unidirectional ^{42}K flux of 20 pmol/cm^2 · sec, G_K is calculated to be 72 μS/cm^2, i.e., G_K is at least 3.7 times underestimated when calculated from tracer flux experiments at high external K^+. As previously discussed (*see* Hoffmann

Table 2. Estimates of membrane conductances and transference numbers to K^+, Na^+, Cl^- and NO_3^- in Ehrlich ascites tumor cells[a]

Membrane conductances	(μS/cm^2)
G_K	10.4
G_{Na}	3.0
G_{Cl}	0.6
G_{NO_3}	8.7
Transference number	
t_K	0.74
t_{Na}	0.22
t_{Cl}	0.04

[a] G_K is calculated from a passive ^{42}K efflux = 3.92 pmol/cm^2 · sec (data recalculated from Hoffmann et al., 1979; *see* text), V_m = −61 mV, E_K = −97 mV, using Eqs. (6), (7) and (10) (*see* Materials and Methods). G_{Na}, G_{Cl} and G_{NO_3} are calculated from G_K (*see* text).

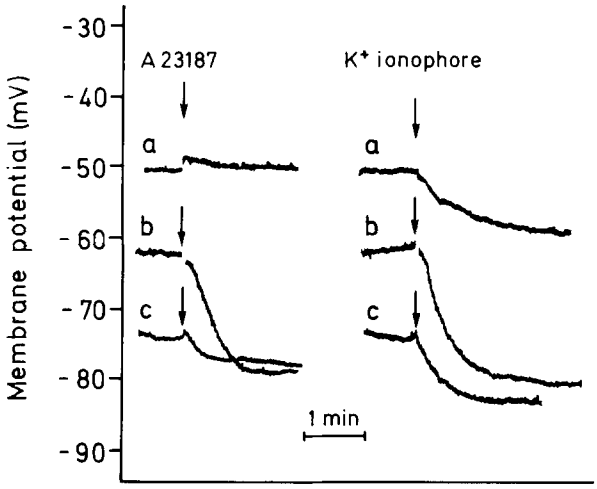

Fig. 7. The effects of anion and cation substitution on a Ca^{2+} ionophore-induced hyperpolarization, as well as on a K^+ ionophore-induced hyperpolarization. The cells were in $NaNO_3$ medium (trace *a*), a standard NaCl medium (trace *b*) or choline chloride medium (trace *c*). Samples for estimation of the membrane potentials were prepared as described in the legend to Table 1. The membrane potential was determined from the fluorescence signal of $DiOC_3$-(5) (1.6 μM) and a standard curve equivalent to the one in Fig. 1*C*. The Ca^{2+}-ionophore (1.5 μM A23187) or the K^+-ionophore (1.5 μM valinomycin, in trace *a* and *b* and 0.5 μM gramicidin in trace *c*) were added within 2 to 3 min after addition of the potential-sensitive dye, at which time stable fluorescence signals were obtained. The external Ca^{2+} was 0.15 mM in experiments with the Ca^{2+}-ionophore A23187

et al., 1986), this finding could be accounted for by assuming single-file diffusion for K^+ with the apparent number n' of positive charges of K^+ during its passage through the membrane equal to 3.7. The passive unidirectional $^{42}K^+$ efflux from Ehrlich cells in standard medium (pH 7.4) is 3.92 pmol/cm^2 · sec as recalculated from previously published data ((Hoffmann et al., 1979), using a correction for a previous underestimate of the area/volume ratio (*see* Hoffmann et al., 1986)). If it is suggested that the passive, unidirectional efflux of K^+ in steady state in standard medium is through the same type of K^+ channels as the net efflux after addition of A23187, then n' can be applied in Eq. (10). For n' = 3.7, V_m = −61 mV and E_K = −97 mV, the absolute K^+ conductance in untreated cells may be estimated to be 10.4 μS/cm^2 (using Eqs. (6), (7) and (10)). Table 2 summarizes the absolute values for conductances and transfer numbers. The NO_3^- conductance is seen to be much higher than the Cl^- conductance in Ehrlich cells in agreement with previous indirect estimations (Hoffmann et al., 1986; Kramhøft et al., 1986). It should be noted that in the human red cell, which is normally impermeable to cations, the Cl^- conductance has been estimated to be twice the NO_3^- conductance (Bennekou & Stampe, 1988).

Effect of Ionophores on the Membrane Potential

We have previously shown that the Ca^{2+} ionophore A23187 in Ehrlich cells increases the conductance to both K^+ and Cl^- and that the increase in G_K exceeds the increase in G_{Cl} (Hoffmann et al., 1986). Table 1, Figs. 7 (left frame, trace *b*), 8 and 9 show that addition of the Ca^{2+} ionophore A23187 hyperpolarizes the cell. However, if Cl^- is substituted with the more permeating anion NO_3^-, the resting membrane potential is depolarized. This depolarization is expected if the cellular concentration of NO_3^- is higher than that corresponding to electrochemical equilibrium in unpertubated cells and if the permeability to NO_3^- is larger than the permeability to Cl^-. Addition of the Ca^{2+} ionophore (Fig. 7, trace *a*) results in an unchanged or a slightly depolarized membrane potential, suggesting that $I_K + I_{NO_3} \approx 0$. Increasing the K^+ permeability alone, by adding either valinomycin (Table 1; right frame in Fig. 7, traces *a* and *b*) or gramicidin (right frame in Fig. 7, trace *c*), results in a hyperpolarization of the membrane potential in all media. The hyperpolarization is more pronounced in Cl^- medium compared to NO_3^- medium.

The previously published values for K^+ and Cl^- conductances in the presence of A23187 (*see* Hoffmann et al., 1986) are recalculated using a V_m equal to −79 mV as measured in the present report (*see* Table 1). From the corrected values, which are

Table 3. Estimates of membrane conductances to K^+ and Cl^- in Ehrlich ascites cells after swelling in hypotonic medium (150 mOsm) and after treatment with the Ca^+-ionophore A23187

	G_K	G_{Cl}
	($\mu S/cm^2$)	
Control[a]	10	0.6
After cell swelling[b]	19	41
After addition of A23187[c]	134	30

[a] Values are from Table 2.
[b] Calculated from Eq. (6) using $J_K^{net} = 11 \pm 1$ pmol/cm² · sec and $J_{Cl}^{net} = 8.5 \pm 0.8$ pmol/cm · sec, each measured in six separate experiments as the loss in cellular content of K^+ and Cl^- during the first four minutes after the cells were transferred to hypotonic (150 mOsm) medium. V_m was estimated at −41 mV, assuming a 20-mV depolarization of the membrane potential (*see* Fig. 13). E_K and E_{Cl} used in Eq. (6) were equal to their values in isotonic NaCl solutions (*see* Table 1), because the Ehrlich cells initially swell as nearly perfect osmometers (*see* Hoffmann et al., 1984).
[c] Calculated from Eq. (6), using $J_K^{net} = 25$ pmol/cm² · sec, $J_{Cl}^{net} = 18$ pmol/cm² · sec (data from Hoffmann et al., 1986) and $V_m = -79$ mV (*see* Table 1).

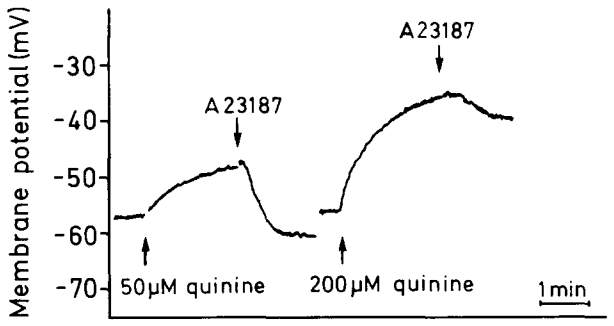

Fig. 8. Effect of quinine on the Ca^{2+} plus A23187-induced hyperpolarization. Ehrlich cells, preincubated in isotonic NaCl medium for 30 min were washed to isotonic choline medium 20 min before the start of the experiment. The Ca^{2+} concentration was 1 mM and the cell density was 0.25%. The potential-sensitive dye $DiOC_3$-(5) was added at a final concentration of 1.6 μM. Quinine (50 or 200 μM) was added 2 min after the dye, at which time the fluorescence signal had become stable. The Ca^{2+}-ionophore A23187 (1.5 μM) was added 2 min after quinine. Absolute potentials were obtained from arbitrary fluorescence values and a calibration curve similar to the one in Fig. 1*C* made on cells from the experimental batch

inserted in Table 3, it is seen that G_K exceeds G_{Cl} in A23187-treated cells by a factor of 4.

Effect of Quinine and External Ca^{2+}

Quinine, an inhibitor of the Ca^{2+}-activated K^+ pathway in Ehrlich cells (Valdeolmillos, Garcia-Sancho & Herreros, 1982; Hoffmann et al., 1984), depolarizes the resting membrane potential in a dose-de-

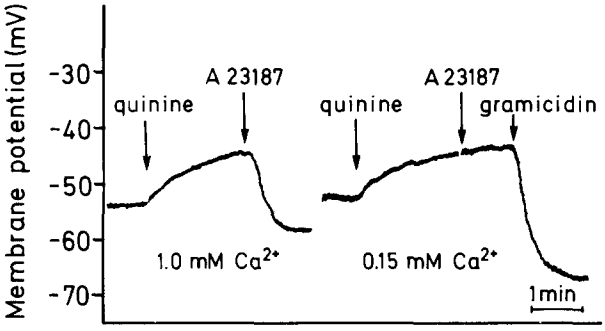

Fig. 9. Effect of the external Ca^{2+} concentration on the quinine inhibition of the Ca^{2+}-activated K^+ channel. Ehrlich cells were pretreated as described in the legend to Fig. 8. The Ca^{2+} concentration was either 1 mM (left trace) or 0.15 mM (right trace). The cell density was 0.25%. Quinine (50 μM) was added 3 min after $DiOC_3$-(5) (1.6 μM), at which time the fluorescence signal had become stable. The Ca^{2+}-ionophore A23187 (1.5 μM) was added 2 min after quinine. Gramicidin (0.5 μM) was added in order to test that a steep K^+ gradient across the cell membrane was still present as seen by the induced hyperpolarization. For a control curve without addition of quinine similar to the right trace, *see* Fig. 7 (left, trace *c*). Absolute potentials were obtained from arbitrary fluorescence values and a standard curve equivalent to the one in Fig. 1*C* made on cells from the same experimental batch

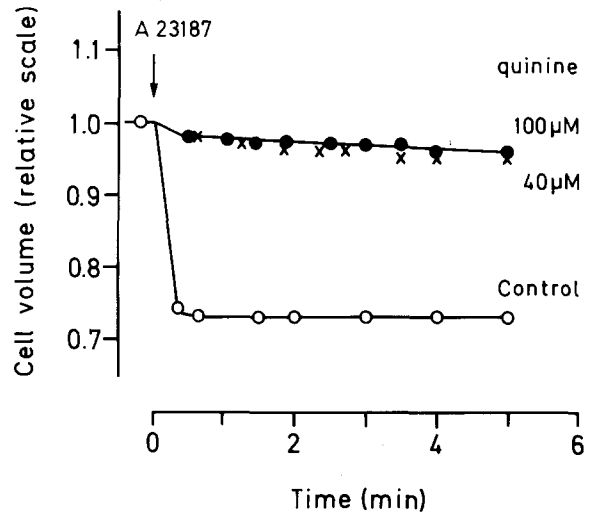

Fig. 10. Ionophore A23187-induced cell shrinkage in isotonic choline chloride medium. The cells were preincubated for 30 to 50 min in isotonic standard NaCl medium followed by a 500-fold dilution in isotonic Ca^{2+}-free choline chloride medium. The final cell density was 0.008%. Quinine 40 μM (×) or 100 μM (●) was added in order to block the Ca^{2+}-dependent K^+ channels. Control cells (○) contained no quinine. At zero time, 1.5 μM A23187 was added and the cell volume was followed with time. The cell volume (ordinate) is given relative to the cell volume recorded before the ionophore addition

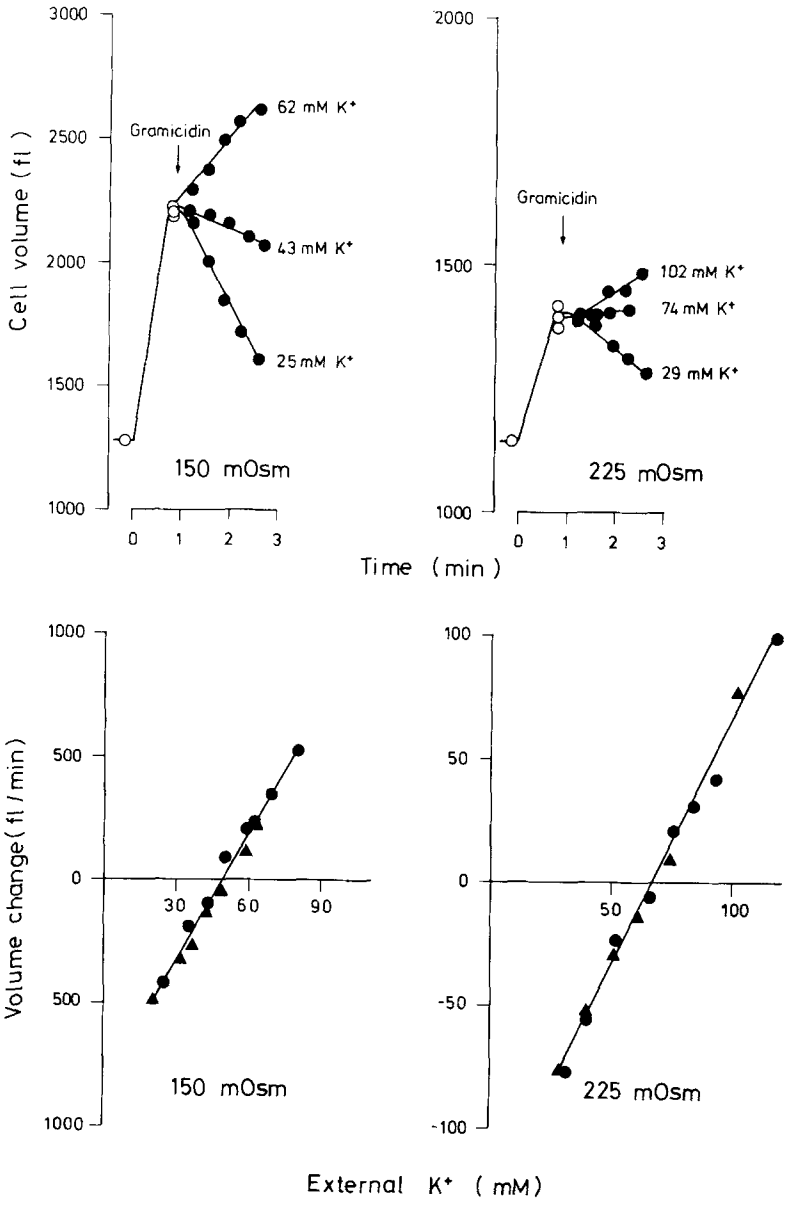

Fig. 11. Determination of the external K^+ concentration at which K^+ ions are in equilibrium across the cell membrane for cells in hypotonic media. This is estimated by measuring the effect of gramicidin on regulatory volume decrease in hypotonic, K^+/choline$^+$ media with different extracellular K^+ concentrations. Ehrlich cells were preincubated at cytocrit 4% in standard NaCl medium for 30 to 50 min. At zero time, a sample of the cell suspension was diluted 500 times in hypotonic, K^+/choline$^+$ media with varying extracellular K^+ concentrations. The sum of K^+ and choline$^+$ was kept constant at either 75 mM (150 mOsm medium, *left panels*) or at 113 mM (225-mOsm medium, *right panels*). The curves in the *upper panels* show the cell volume in fl (10^{-15} liter) followed with time after transfer to hypotonic solutions. Gramicidin (0.5 μM) was added at the time of maximal swelling as indicated by arrows. The curves in the *lower panels* show the initial rate of volume change after gramicidin treatment (ordinate) at varying extracellular K^+ concentration (abscissa). The initial rate of volume recovery (fl/min) was obtained from curves similar to those in the upper panels, using lines fitted to the initial five to six values, measured within the first 2 min. The intercept with the abscissa gives the extracellular K^+ concentration at which gramicidin does not induce any net movements of osmotic material and cell water. For cells transferred to 150 and 225-mOsm media, the K^+ is in equilibrium at 52 ± 2 mM and 68 ± 1 mM extracellular K^+, respectively. The number of independent experiments were three, two of which are shown in the figure (●, ▲)

pendent manner (Fig. 8). Addition of the Ca^{2+} ionophore A23187 still results in hyperpolarization in the presence of 50 μM quinine, whereas this hyperpolarization is almost abolished in the presence of 200 μM quinine (Fig. 8). As shown in Fig. 9, however, 50 μM quinine is sufficient to block the Ca^{2+}-activated K^+ channels if the external Ca^{2+} concentration is reduced from 1 to 0.15 mM. Addition of gramicidin under these conditions causes a hyperpolarization in the Na^+-free medium, indicating that the K^+ gradient is present (Fig. 9, right panel). In agreement with the inhibitory effect of 50 μM quinine upon the A23187 effect on the membrane potential in low Ca^{2+} medium, we find that 40 μM quinine completely blocks the cell shrinkage seen after addition of A23187 in Ca^{2+}-free medium (Fig. 10).

Depolarization During Regulatory Volume Decrease (RVD)

It has previously been shown that Ehrlich cells swell in hypotonic media, but subsequently regulate the volume by a net loss of KCl (RVD). The volume response is accelerated by addition of the cation-ionophore gramicidin in Na^+-free medium (*see* Hoffmann et al., 1986 and compare control curves in Fig. 4, upper panels). Figure 11 (upper frame) shows that RVD in the presence of gramicidin is dependent upon external K^+. Raising the external K^+ and thereby reversing the electrochemical gradient for K^+ results in an influx of water. From experiments like the ones in Fig. 11 (upper frame), it is possible to determine an external K^+ concentration

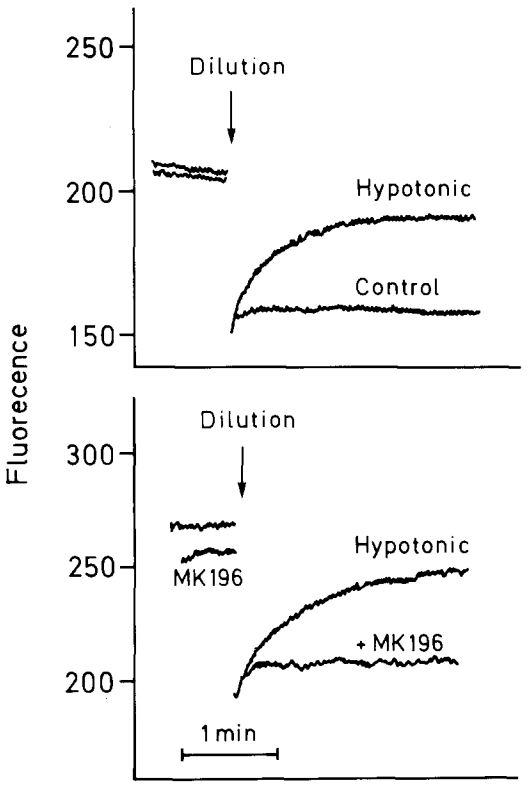

Fig. 12. Effect on the fluorescence signal of $DiOC_3$-(5) by isotonic or hypotonic dilution of Ehrlich cells in standard medium. Ehrlich cells were equilibrated with the fluorescent dye $DiOC_3$-(5) (1.6 μM, cytocrit 0.25%) for 2 min in isotonic standard medium. *Upper panel:* At the time indicated by the arrow, the cell suspension was diluted with an equal volume "dye-free" isotonic medium (control) or with buffered water (hypotonic), and the fluorescence followed with time (upper frame). *Lower panel:* Cells were diluted with dye-free buffered water as described above. One part of the cell suspension was treated with indacrinone (MK195, 2 mM) for 1 min before hypotonic dilution

where the volume change is zero. At this concentration $E_K = E_{Cl} = V_m$ after cell swelling. These K_o^+ values are found to be 52 ± 2 mM and 68 ± 1 mM in 150- and 225-mOsm Ringers, respectively (Fig. 11, lower panels). In such hypotonic equilibrium media, E_K and E_{Cl} have been calculated from measured extracellular and intracellular concentrations. In 150-mOsm Ringer, $E_K = E_{Cl} = -15$ mV ($n = 2$) and in 225-mOsm Ringer, E_K and E_{Cl} are calculated to be −17 and −20 mV, respectively ($n = 2$). In isotonic, Na^+-free choline media with 52 and 68 mM extracellular K^+, the corresponding potentials are estimated at −37 ± 1 mV ($n = 10$) and −33 ± 1 mV ($n = 10$), respectively, using the fluorescent dye technique. Thus, the membrane potential is depolarized from −37 to −15 mV, i.e., 22 mV after transfer to 150-mOsm Ringer with 52 mM external K^+ and from −33 to −17–20 mV, i.e., about 15 mV after transfer to 225-mOsm Ringer with 68 mM external K^+. The 15 mV found after transfer to 225-mOsm Ringer with 68 mM external K^+ (*see above*) is in reasonable agreement with the 10 mV depolarization found with conventional microelectrodes during RVD in 225-mOsm Ringer with normal external K^+ (Lang et al., 1987). However, using this "volume-method," the depolarization can be estimated only at one external K^+ concentration for each type of osmotic shock.

Figure 12 demonstrates the changes in membrane potential during RVD at normal external K^+. Dilution of the cell suspension with an equal volume of either isotonic medium or buffered water initially reduces the fluorescence to 50%, followed by a redistribution of the dye, which is so fast that we are not always able to follow it (*see* Fig. 14). If the cell swelling introduced by dilution with buffered water is not accompanied by any change in membrane potential, the resulting external fluorescence is slightly lower than in the suspension diluted with isotonic medium (*see* Fig. 14). RVD at normal external K^+ is, on the other hand, accompanied by a large increase in fluorescence, which reaches a new steady-state value within 1 to 2 min, indicating a depolarization of the cell membrane. The increase in fluorescence following hypotonic exposure is inhibited by the Cl^- channel blocker MK196 (Fig. 12, lower panel). It is concluded that the depolarization seen during RVD reflects an increase in the Cl^- conductance. We have quantified the difference in fluorescence between isotonic and hypotonic cells using the slope of a calibration curve in which the samples were diluted with an equal amount of "dye-free" isotonic solution. This is legalized by the fact that the stable fluorescence seen after dilution with buffered water and with isotonic medium is identical when no potential changes take place. Figure 13 shows that the corresponding depolarization during RVD in 150-mOsm medium is 20 mV, which is close to the 22 mV found by the "volume-method" in 150-mOsm Ringer with 52 mM external K^+ (*see above*).

The initial net K^+ efflux and net Cl^- efflux following transfer to hypotonic NaCl medium with half osmolarity of the standard medium were in six independent experiments estimated at 38 ± 3 and 30 ± 3 μmol/g cell dry wt · min, respectively. These net fluxes are, according to Hoffmann et al. (1986), equivalent to 11 and 8.5 pmol/cm² · sec, respectively. Since the cells depolarize 20 mV (*see* Fig. 13), the calculated K^+ and Cl^- conductances in hypotonically swollen cells are 19 and 41 μS/cm² · sec, respectively (*see* Table 3), i.e., the Cl^- is increased about 68-fold by the hypotonic swelling, while the K^+ conductance is only increased by twofold. We have previously estimated the increase in Cl^- con-

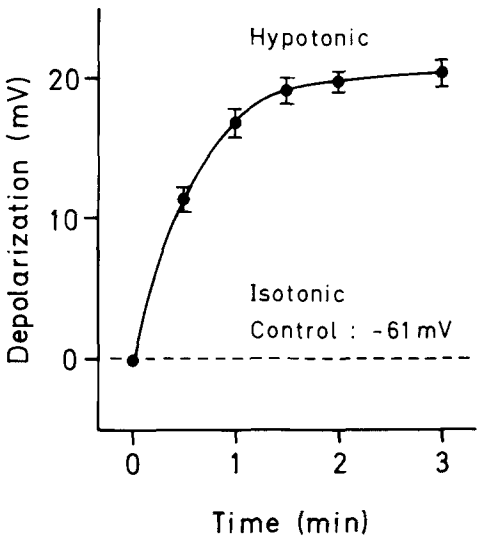

Fig. 13. Estimation of the depolarization induced by hypotonic dilution. From the slope of a standard curve in which all samples were diluted to 50% with an identical but "dye-free" solution, the difference in fluorescence between the parallel groups of cells in isotonic and hypotonic solutions (*see* Fig. 12, upper panel) was quantified. Values are given as the mean ± SEM of four independent sets of experiments

ductance indirectly by electronic cell sizing to be about 60-fold during RVD (Hoffmann et al., 1986).

Discussion

Evaluation of the Fluorescense Method and the Thiocyanate Distribution for the Determination of the Membrane Potential

The results with $DiOC_3$-(5) indicate that Ehrlich cells in standard Ringer have a resting potential of about −61 mV (Table 1) and that the membrane is predominantly K^+ selective (t_K = 0.7, Table 2). Several side effects of the dye have, however, been under discussion (for example, *see* Johnstone et al., 1982; Smith, 1982). The major points investigated have been, the effect of the dye-induced inhibition of the respiration, and an eventual change in the K^+ permeability caused by the dye. In addition, the use of valinomycin and the "null point" method has been questioned.

Carbocyanine Dyes and Cell Metabolism

Several studies have shown that the carbocyanine dyes inhibit oxidative energy metabolism (Waggoner, 1976; Smith, Herlihy & Robinson, 1981) which in the absence of glucose results in ATP depletion (Laris et al., 1978; Smith et al., 1981). This would ultimately lead to an inhibition of active ion transport. However, in Ehrlich cells, no alteration in cellular cation levels are apparent until 10 to 20 min after exposure to the dye $DiSC_3$-(5). Indeed, direct measurements of V_m carried out during the first 10 min after addition of the dye show no indication of depolarization related to reduced active cation transport (Smith & Robinson, 1980). It seems therefore, likely that for short-term studies (less than 10-min exposure) the effect of ATP-depletion caused by $DiOSC_3$-(5) and most probably also by $DiOC_3$-(5) on V_m are negligible. All measurements used for determination of the absolute values in Table 1 are all obtained within the first 5 min after addition of the dye.

Effect of Carbocyanine Dyes on the Membrane Permeability to Cations and Anions

Addition of the membrane-sensitive probe $DiOC_3$-(5) has been demonstrated to hyperpolarize the cell membrane in Ehrlich cells (Smith & Robinson, 1980), to depolarize the membrane potential in lymphocytes (Rink et al., 1980) and to leave the membrane potential unchanged also in Ehrlich cells (*see* Johnstone et al., 1982). In the present report, we have demonstrated a slight effect of $DiOC_3$-(5) on the swelling-induced anion permeability (*see* Fig. 4) and we have estimated the resulting hyperpolarization caused by the dye to be less than 2 mV, assuming that the Cl^- conductance in unpertubated cells is comparable to the Cl^- conductance in pertubated cells. The corrected membrane potential of −61 mV (*see* Table 1) is in accordance with the −56.7 mV measured in Ehrlich cells by a conventional microelectrode by Gstrein et al. (1987). It should be noted that if only 40% of cellular K^+ should behave as if it was in free solution, as proposed by Dawson and Smith (1986), the dye method used in the present report would overestimate the true value by 15.5 mV, resulting in a membrane potential of −45.5 mV, which is significantly lower than the value measured by Gstrein et al. (1987).

The Use of Cation Ionophores and the "Null Point" Methods

From the results in Fig. 2*A*, it was concluded that valinomycin "null point" method does not give correct membrane potentials. Addition of an anion channel inhibitor (MK196), hyperpolarizes the cell even at the "null point," indicating that V_m is different from E_K. This explains why K^+ loss could still

be demonstrated at the "null point" (Smith, 1982). Using the chloride channel inhibitor (MK196) together with valinomycin allows a better determination of the membrane potential, since the ionophore now changes the membrane into a K^+-selective membrane. If gramicidin in Na^+-free choline medium is added as a K^+-ionophore, the K^+ permeability apparently is high enough to determine the membrane potential and no chloride channel inhibitor is necessary to make a true calibration curve in the interval of −35 to −80 mV (*see* Fig. 2*B*). Furthermore, addition of gramicidin does not cause a change in E_K within the period used for the membrane potential recordings (*see* Results). Thus, the use of gramicidin in choline medium is preferable to the use of valinomycin. Conclusively, it is possible to calibrate the dye fluorescence response using the gramicidin method and to obtain a calibration curve in the range of −35 to −80 mV.

It should be noted that the proper use of this method demands in principle that size of the cellular compartments, i.e., cytocrits, are matched in the suspensions used for calibration and for the experimental tests. This is a problem because cells in choline medium shrink compared to cells in standard medium and because the addition of ionophores, which affect membrane permeabilities often result in cell volume changes (*see* Table 1). The fluorescence depends not only on V_m, but also on the relative compartment sizes of the cells and the medium (i.e., the larger the cellular compartment or volume, the more dye is removed from the medium to establish the distribution). If the intracellular amounts of free and quenched dye are proportional, large volume effects on the extracellular fluorescence would be expected. If, however, the quenched intracellular dye is bound to a low affinity buffer with high capacity very little volume effect is expected (*see* Appendix). Since it is complicated experimentally to avoid volume differences between the cellular compartments in test solution and in calibration solution, we have experimentally evaluated the effect of volume changes on the fluorescence signal (*see* Appendix). From these results it is concluded that the Ehrlich cells can be described as having a large intracellular buffer capacity for the dye and that the effect from cell volume changes reported in this paper can be neglected.

In conclusion, Ehrlich ascites tumor cells have a membrane potential in the range of −56 to −61 mV, as estimated by conventional microelectrodes (Gstrein et al., 1987), or by the modified fluorescence method described in the present paper. Previously estimation of the membrane potential from our laboratory are probably all underestimated either due to leakage caused by the microelectrode impalement (Hoffmann et al., 1979) or from misinterpretation of the valinomycin "null point" (Hoffmann & Lambert, 1983).

Membrane Conductances

The present study presents evidence for a high potassium conductance of the Ehrlich ascites tumor cell membrane as previously suggested by Hoffman et al. (1979, 1986) and by Gstrein et al. (1987). The K^+ conductance is in the present report calculated to be at 10.4 $\mu S/cm^3$ (*see* Table 2), compared to previously determined values of 14 $\mu S/cm^2$ (Hoffmann et al., 1979) or 5 $\mu S/cm^2$ (Hoffmann et al., 1986). The difference between the present conductance value and the former published values is due to the difference in the measured membrane potentials and the difference in the surface area/cell volume ratio (*see* Hoffmann et al., 1986). Gstrein et al. (1987) estimated that the potassium conductance contributes more than 50% to the overall conductance of the cell membrane and that the contribution from the sodium and chloride conductance must be relatively smaller. In agreement with this, the transference numbers in Table 2 show that the potassium, the sodium and the chloride conductances contribute to the overall conductance by 74, 22 and 4%, respectively. That the chloride conductance is relatively small is also seen from the minor effect of the Cl^- channel blocker indacrinone (MK196; Distefano et al., 1985; Aabin & Hoffmann, 1986; Durr & Larsen, 1986) on the fluorescence signal, i.e., the membrane potential (*see* Fig. 2). If the potassium conductance, on the other hand, is inhibited by quinine, the membrane potential depolarizes by more than 20 mV (*see* Fig. 8). A similar depolarization is observed in Ehrlich cells when the potassium channels are inhibited by addition of Ba^{2+} (Gstrein et al., 1987; Smith & Robinson, 1989).

We have previously presented evidence of single-file behavior of the K^+ channels in Ehrlich cells (Hoffmann et al., 1986). Using the membrane potentials given in the present report, the apparent number (n') of positive charges of K^+ during its passage through the membrane can be estimated at 3.7. Single-file diffusion with about 2.5 K^+ ions in a single file has been demonstrated in the apical membrane of the frog skin (Eskesen & Ussing, 1986), while Cox and Helman (1986) have shown that K^+ transport in the basolateral membrane proceeds through single-file channels with an n' equal to 2.9. In human red cells, Vestergaard-Bogind, Stampe and Christophersen (1985) find an n' equal to 2.7 for the Ca^{2+}-activated K^+ channel.

The ionophore A23187, when added to a sus-

pension of Ehrlich cells in steady state, induces a net loss of KCl with associated cell shrinkage (*see* Fig. 10 and Hoffmann et al., 1984, 1986). This is concommitant with a hyperpolarization of the cell membrane (Fig. 7 and Table 2). Because of the cell shrinkage induced by A23187, this hyperpolarization is slightly underestimated, although the effect of the volume changes is probably small. Membrane hyperpolarization in Ehrlich cells is similarly seen after addition of propranolol, which is known to activate Ca^{2+}-dependent K^+ channels (Laris et al., 1976; Valdeolmillos, Garcia-Sancho & Herreros, 1986; Smith & Robinson, 1989). It was previously demonstrated that A23187 activated separate, conductive K^+ and Cl^- pathways (Hoffmann et al., 1986) and we find in the present report that the increase in G_K and G_{Cl} are 13 and 50 times, respectively, after addition of A23187 (*see* Table 3). Thus, although A23187 increases the Cl^- conductance more than the K^+ conductance, the resulting conductance for K^+ is still dominating. It should be noted that no hyperpolarization can be seen after addition of A23187 when all cellular and extracellular Cl^- is substituted with NO_3^- (Fig. 7, left frame, trace *a*) because the NO_3^- conductance is higher than the Cl^- conductance (*see* Table 2 and Hoffmann et al., 1986; Kramhøft et al., 1986). The A23187-activated K^+ channel is inhibited by quinine at a concentration of 200 μM in the presence of 1 mM extracellular Ca^{2+} and at a concentration of 50 μM in the presence of 0.15 mM extracellular Ca^{2+} as evidenced by potential measurements (Figs. 8 and 9) and by electronic cell sizing (Fig. 10).

Depolarization Induced by Cell Swelling

The depolarization (20 mV, *see* Fig. 13) observed during RVD after transfer to 150-mOsm Ringer in the present report is qualitatively in agreement with the 10 mV depolarization found during RVD in 225-mOsm Ringer using conventional microelectrodes (Lang et al., 1987). Lang et al. (1987) also observe a decrease in the transference number for K^+ (t_K) during RVD and they suggest that this reflects a decrease in G_K. We have measured a twofold increase in G_K during RVD which is, however, small compared to the 68-fold increase in G_{Cl} (Table 3). Provided that G_{Na} is unchanged during RVD (*see* Hoffmann, 1978), t_K and t_{Cl} can be estimated at 0.30 and 0.66, respectively, compared to 0.74 and 0.04 under isotonic conditions (*see* Table 2). Thus, it is apparent that there is a decrease in t_K, but an increase in G_K during RVD in Ehrlich cells. This strongly supports previous findings that osmotic swelling leads to a parallel activation of potassium and anion-selective channels, with the increase in anion conductance being the dominating one (Hoffmann et al., 1984, 1986). It may be noted that the depolarization will increase the driving force for K^+ efflux, and hence accounts for at least a part of the net K^+ loss. The K^+ loss is, however, still the rate-limiting factor during RVD as evidenced by the faster RVD after addition of the cation-ionophore gramicidin in Na^+-free choline medium (*see* Fig. 4). It may also be emphasized that the fast Cl^- efflux observed after addition of gramicidin to hypotonically swollen cells is partly accounted for by the gramicidin-induced hyperpolarization, which increases the driving force for Cl^- efflux. The modest increase in G_K during RVD is in contrast to the much more pronounced increase in G_K seen after addition of the Ca^{2+}-ionophore A23187 (*see* Table 3). The increase in G_{Cl} is, however, of similar magnitude during RVD and after addition of A23187 (*see* Table 3).

Activation of Cl^- channels in hypotonically swollen Ehrlich cells has recently been demonstrated directly in studies using the patch-clamp technique (Hudson & Schultz, 1988). Depolarization in hypotonically swollen cells (measured as an increase in the fluorescence signal of a membrane potential-sensitive probe) has also been demonstrated in lymphocytes (Grinstein et al., 1982), hamster ovary cells (Sarkadi et al., 1984) and cerebrovascular endothelial cells (Kempski et al., 1985). A quantitative estimate of the depolarization has in addition to the Ehrlich cells been carried out on MDCK cells (Lang et al., 1987) and in the basolateral membrane of proximal tubule cells (Völkl & Lang, 1989).

Conclusion

The present study demonstrates that the potassium conductance under steady-state conditions 74% to the overall conductance of the Ehrlich cell membrane and that the contribution from the sodium and chloride conductances are much smaller, i.e., 22 and 4%, respectively. The anion conductive pathway is found to have a 14.5 times higher conductance to NO_3^- than to Cl^-. The potassium conductance can be blocked by quinine and Ba^{2+} resulting in a strong depolarization of the cells. Both the K^+ and the Cl^- conductances are increased after cell swelling and after increase of intracellular free Ca^{2+}, induced by addition of A23187. During RVD, the Cl^- conductance is increased 68 times and now exceeds the K^+ conductance by a factor of 2.2. The increase in Cl^- conductance is of similar magnitude after addition of A23187, but under these conditions

the K^+ conductance is also dramatically increased and now exceeds the Cl^- conductance by a factor of 4. As a consequence, the membrane potential is depolarized during RVD and hyperpolarized after addition of A23187 plus Ca^{2+}.

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Appendix

Correction for Cell Volume Changes

The purpose of this Appendix is to analyze the influence of cell volume changes (and cell density) on the extracellular amount of $DiOC_3$-(5). The effect on the fluorescence signal of dilution of the cell suspension in the cuvette with one volume of isotonic medium or buffered water is shown in Fig. 14 for an experimental solution where $V_m = E_K = E_{Cl} = E_{Na} = -13$ mV. In this medium, no change in membrane potential is expected after cell swelling. It can be seen that the initial decrease in the fluorescence signal after dilution is followed by a secondary increase representing redistribution of the dye. This redistribution takes place within the first min. For a definite amount of added dye Q_T

$$Q_T = V_i \cdot C_i + V_o \cdot C_o \tag{1}$$

where V_i and V_o refer to the size of the cellular and extracellular compartment and C_i and C_o to the respective dye concentrations.

$$V_T = V_{\text{total}} = V_i + V_o \tag{2}$$

$$C_i = C_{i(\text{total})} = C_{i(\text{free})} + C_{i(\text{bound})}. \tag{3}$$

Assuming that the membrane potential controls the equilibrium distribution ratio (r) between internal and external dye activity, we get

$$C_{i(\text{free})} = r \cdot C_o. \tag{4}$$

Assuming intracellular binding of the dye to a low affinity, large capacity buffer

$$C_{i(\text{bound})} = C_B \cdot \frac{C_{i(\text{free})}}{C_{i(\text{free})} + K_B} \tag{5}$$

where C_B is the intracellular "buffer" concentration and K_B the Michaelis constant for the buffer.

If:

$C_{i(\text{free})} \ll K_B$

$$C_{i(\text{bound})} \approx \frac{C_B}{K_B} \cdot C_{i(\text{free})} \tag{6}$$

using Eq. (3)

$$C_i = C_{i(\text{free})} + \frac{C_B}{K_B} C_{i(\text{free})} = C_{i(\text{free})} \cdot \left(1 + \frac{C_B}{K_B}\right). \tag{7}$$

Equation (A1) combined with Eqs. (2), (4) and (7) gives

$$C_o = \frac{Q_T}{V_i \cdot \left(1 + \frac{C_B}{K_B}\right) \cdot r + (V_T - V_i)} = \frac{Q_T/V_T}{\frac{V_i}{V_T} \cdot r + \frac{V_i}{V_T} \cdot \frac{C_B}{K_B} \cdot r + 1 - \frac{V_i}{V_T}}. \tag{8}$$

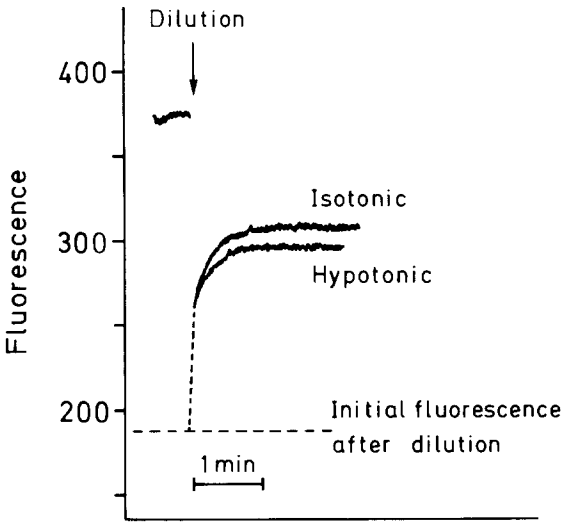

Fig. 14. Effect on the fluorescence signal of $DiOC_3$-(5) of Ehrlich cells in equilibrium medium. Ehrlich cells were equilibrated in isotonic choline medium with 120 mM extracellular K^+ and 2 mM Na^+. Since $V_m = E_K = E_{Cl} = E_{Na} = -13$ mV in this medium (*see* Fig. 1*A* and Results) and the Ehrlich cells behave as perfect osmometers, dilution with buffered water should not lead to any change in the membrane potential. $DiOC_3$-(5) (1.6 μM) was added to the cells (cytocrit 0.5%) and the fluorescence signal followed with time. At the time indicated by the arrow, the cell suspension was diluted with an equal volume "dye-free" isotonic medium (control) or buffered water (hypotonic trace)

Example 1

If the *cells swell or shrink* (assuming an unchanged membrane potential and hence an unchanged r)

$$C_o^{(1)} = \frac{Q_T/V_T}{\frac{V_i^{(1)}}{V_T} \cdot \left(1 + \frac{C_B^{(1)}}{K_B}\right) \cdot r + \left(1 - \frac{V_i^{(1)}}{V_T}\right)} \tag{9}$$

where $V_i^{(1)}$ and $C_B^{(1)}$ are the cell volume and intracellular buffer concentration, respectively, in the new condition, and $C_o^{(1)}$ is the extracellular dye concentration when redistribution of the dye has been completed.

Since $V_i^{(1)} \cdot C_B^{(1)} = V_i \cdot C_B$,

$$C_B^{(1)} = \frac{V_i}{V_i^{(1)}} \cdot C_B$$

can be inserted in Eq. (9) to give

$$C_o^{(1)} = \frac{Q_T/V_T}{\frac{V_i^{(1)}}{V_T} \cdot r + \frac{V_i}{V_T} \cdot \frac{C_B}{K_B} \cdot r + 1 - \frac{V_i^{(1)}}{V_T}}. \tag{9a}$$

Example 2

If the suspension is simply *diluted x times with an isotonic solution* (assuming an unchanged r)

$$C_o^{(2)} = \frac{Q_T/(x \cdot V_T)}{\frac{V_i}{x \cdot V_T} \cdot \left(1 + \frac{C_B}{K_B}\right) \cdot r + 1 - \frac{V_i}{x \cdot V_T}}$$

$$= \frac{Q_T/V_T}{\frac{V_i}{V_T} \cdot r + \frac{V_i}{V_T} \cdot \frac{C_B}{K_B} \cdot r + x - \frac{V_i}{V_T}} \tag{10}$$

where $C_o^{(2)}$ is the extracellular concentration of the dye after isotonic dilution and redistribution of the dye.

Example 3

If the suspension is *diluted x times with buffered water* and the cells are regarded as perfect osmometers (i.e., cells swell x times and the intracellular buffer concentration C_B is reduced x times).

$$C_o^{(3)} = \frac{Q_T/(x \cdot V_T)}{\frac{V_i}{V_T} \cdot \left(1 + \frac{C_B/x}{K_B}\right) \cdot r + 1 - \frac{V_i}{V_T}}$$

$$= \frac{Q_T/V_T}{x \cdot \frac{V_i}{V_T} \cdot r + \frac{V_i}{V_T} \cdot \frac{C_B}{K_B} \cdot r + x - x \cdot \frac{V_i}{V_T}}. \tag{11}$$

Using the measured values for initial fluorescence (C_o) and fluorescence after isotonic twofold dilution ($C_o^{(2)}$, isotonic) from figures like Fig. 14, and the known values for V_i/V_T and r, then Q_T/V_T and C_B/K_B can be estimated from Eqs. (8) and (10). Introducing these values in Eq. (A11) gives the calculated fluorescence after twofold dilution with buffered water ($C_o^{(3)}$, hypotonic) as predicted from the model.

From experiments like the one shown in Fig. 14 with the following parameters: $C_o = 1$ (all values are taken relative to C_o), $C_o^{(2)} = 0.848 \pm 0.028$ ($n = 5$), $x = 2$, $V_i/V_T = 0.005$, $r = 1.6247$ (equilibrium distribution ratio (cell to medium) for a monovalent cation, *see* legend to Fig. 1), we predict $C_o^{(3)}$ to be = 0.8475. Thus, under conditions where the membrane potential is unchanged, no significant difference between the measured fluorescence for cells in isotonic and hypotonic media is predicted. The actual $C_o^{(3)}$ value found in this hypotonic medium was 0.792 ± 0.01 ($n = 5$), which differs only slightly from the predicted value.

If we, in contrast, had assumed that a constant fraction (α) of the total intracellular dye is free, $C_{i(\text{free})} = \alpha \cdot C_i$, the predicted $C_o^{(3)}$ value after twofold hypotonic dilution would have been ≈ 0.50, which is far from the experimental value.

In consequence, we have decided to treat the results according to the model where the dye is bound intracellularly to a low affinity, high capacity buffer, and hence to omit corrections of the fluorescence readings for cell volume changes *per se*.