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Separate, Ca^{2+}-Activated K^+ and Cl^- Transport Pathways in Ehrlich Ascites Tumor Cells

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Summary. The net loss of KCl observed in Ehrlich ascites cells during regulatory volume decrease (RVD) following hypotonic exposure involves activation of separate conductive K^+ and Cl^- transport pathways. RVD is accelerated when a parallel K^+ transport pathway is provided by addition of gramicidin, indicating that the K^+ conductance is rate limiting. Addition of ionophore A23187 plus Ca^{2+} also activates separate K^+ and Cl^- transport pathways, resulting in a hyperpolarization of the cell membrane. A calculation shows that the K^+ and Cl^- conductance is increased 14- and 10-fold, respectively. Gramicidin fails to accelerate the A23187-induced cell shrinkage, indicating that the Cl^- conductance is rate limiting. An A23187-induced activation of ^{42}K and ^{36}Cl tracer fluxes is directly demonstrated. RVD and the A23187-induced cell shrinkage both are: (i) inhibited by quinine which blocks the Ca^{2+}-activated K^+ channel. (ii) unaffected by substitution of NO_3^- or SCN^- for Cl^-, and (iii) inhibited by the anti-calmodulin drug pimozide. When the K^+ channel is blocked by quinine but bypassed by addition of gramicidin, the rate of cell shrinkage can be used to monitor the Cl^- conductance. The Cl^- conductance is increased about 60-fold during RVD. The volume-induced activation of the Cl^- transport pathway is transient, with inactivation within about 10 min. The activation induced by ionophore A23187 in Ca^{2+}-free media (probably by release of Ca^{2+} from internal stores) is also transient, whereas the activation is persistent in Ca^{2+}-containing media. In the latter case, addition of excess EGTA is followed by inactivation of the Cl^- transport pathway. These findings suggest that a transient increase in free cytosolic Ca^{2+} may account for the transient activation of the Cl^- transport pathway. The activated anion transport pathway is unselective, carrying both Cl^-, Br^-, NO_3^-, and SCN^-. The anti-calmodulin drug pimozide blocks the volume- or A23187-induced Cl^- transport pathway and also blocks the activation of the K^+ transport pathway. This is demonstrated directly by ^{42}K flux experiments and indirectly in media where the dominating anion (SCN^-) has a high ground permeability. A comparison of the A23187-induced K^+ conductance estimated from ^{42}K flux measurements at high external K^+, and from net K^+ flux measurements suggests single-file behavior of the Ca^{2+}-activated K^+ channel. The number of Ca^{2+}-activated K^+ channels is estimated at about 100 per cell.

Key Words volume regulation · regulatory volume decrease (RVD) · Ca^{2+}-activated K^+ channel · ^{42}K fluxes · single-file diffusion · K^+ conductance · Ca^{2+}-activated Cl^- channel · ^{36}Cl fluxes · Cl^- conductance · Ca^{2+} ionophore A23187 · quinine · calmodulin · anti-calmodulin drugs · pimozide · Ehrlich mouse ascites tumor cells

Introduction

In hypotonic media, vertebrate and invertebrate cells initially swell by osmotic water equilibration but subsequently regulate their volume (regulatory volume decrease) by a net loss of KCl, amino acids and associated loss of cell water [for references *see* recent reviews by Rorive & Gilles (1979), Kregenow (1981), Spring & Ericson (1982), Hoffmann (1983, 1985*a*,*b*), Cala (1983*a*, 1985), Grinstein et al. (1984) and Siebens (1985)].

Several transport mechanisms have been proposed to be activated during the regulatory volume decrease. In most cell types the KCl loss appears to be mediated via electroneutral ion transport mechanisms. An electroneutral K^+,Cl^- cotransport involved in regulatory volume decrease has been reported in red cells from duck (*see* McManus & Schmidt, 1978; Kregenow, 1981; McManus et al., 1985), dog (Parker, 1983), LK-sheep (Dunham & Ellory, 1981; Ellory et al., 1985*a*), and fish (Lauf, 1982). In human red cells a volume-sensitive K^+,Cl^- cotransport can be stimulated by high hydrostatic pressure (Ellory et al., 1985*a*,*b*). A Cl^--dependent K^+ transport can also be induced by N-ethylmaleimide (NEM) in LK-sheep and goat red cells (Lauf & Theg, 1980), human red cells (Wiater & Dunham, 1983; Lauf et al., 1984; Lauf, 1985) and Ehrlich ascites cells (Kramhøft et al., 1986), and moreover by Ca^{2+}-depletion in LK-sheep red cells (Lauf & Mangor-Jensen, 1984) and Ehrlich ascites cells (Kramhøft et al., 1986). In *Amphiuma* red cells the regulatory volume decrease involves electroneutral, functionally coupled exchange of K^+/H^+ and Cl^-/HCO_3^- (Cala, 1980, 1985; Kregenow, 1981; Siebens, 1985).

In contrast, separate conductive K^+ and Cl^- transport pathways have been reported to be activated during regulatory volume decrease in human lymphocytes (Grinstein et al., 1982*a*,*c*, 1984; Sarkadi et al., 1984*a*, 1985) and in Ehrlich ascites cells (Hoffman, 1978; Hoffmann et al., 1984*b*). A volume-sensitive conductive Cl^- transport pathway has been reported in the basolateral membrane of frog skin epithelial cells (MacRobbie & Ussing, 1961; Ussing, 1982; Kristensen & Ussing, 1985). A conductive K^+ transport pathway activated by cell swelling has been reported in the basolateral membrane of frog and toad urinary bladder epithelial cells (Davis & Finn, 1982), in liver cells (Kristensen & Folke, 1984) and in enterocytes (Lau et al., 1984; Schultz et al., 1985). In *Necturus* gallbladder epithelial cells activation of both K^+,Cl^- cotransport and K^+ conductance in the basolateral membrane during regulatory volume decrease has recently been suggested (Larson & Spring, 1984). In Ehrlich ascites cells at low pH or after Ca^{2+} depletion the combined activation during regulatory volume decrease of K^+,Cl^- cotransport and of conductive K^+ and Cl^- transport has also been reported (Kramhøft et al., 1986).

Ca^{2+} appears to play a key role in the regulatory volume decrease in *Amphiuma* red cells, human lymphocytes and in Ehrlich ascites cells (for references *see below*). In the two latter cell types calmodulin also seems to be involved. In the *Amphiuma* red cell Ca^{2+} seems to act as a modulator of K^+/H^+ exchange (Cala, 1983*b*). In lymphocytes and in Ehrlich ascites cells there is strong evidence that Ca^{2+} and calmodulin are involved in the activation of the K^+ transport pathway (Grinstein et al., 1982*c*, 1984; Hoffmann et al., 1984*b*; Sarkadi et al., 1984*a*, 1985), whereas the Cl^- transport pathway in lymphocytes seems to be essentially Ca^{2+} independent (Grinstein et al., 1982*c*; Sarkadi et al., 1984*a*). The precise role of Ca^{2+} in the regulatory volume decrease is, however, at present unclear. Release of Ca^{2+} from internal stores seems to play a role in lymphocytes (Grinstein et al., 1982*c*, 1984) and in Ehrlich ascites cells (Hoffman et al., 1984*b*). However, no change in cytosolic free Ca^{2+} concentration could be detected in human lymphocytes during regulatory volume decrease (Rink et al., 1983). Modulation of the Ca^{2+} sensitivity of the transport pathways has been suggested in relation to the pH dependence of the volume recovery during regulatory volume decrease in Ehrlich ascites cells (Hoffmann et al., 1984*b*).

The present study reports analogy between volume- and ionophore A23187 plus Ca^{2+}-induced responses in Ehrlich ascites cells, and demonstrates the activation of separate K^+ and Cl^- transport pathways. The data indicate that the Cl^- transport pathway in Ehrlich ascites cells is activated by Ca^{2+} and inhibited by the anti-calmodulin drug pimozide, similar to the findings for the K^+ transport pathway. The activation of the Cl^- transport pathway during regulatory volume decrease is transient, and the data suggest that this may reflect a transient increase in cytosolic free Ca^{2+}. A preliminary report of this study has been presented (Hoffmann et al., 1984*a*).

Materials and Methods

Cell Suspensions

Ehrlich mouse ascites tumor cells (hyperdiploid strain) were maintained in the peritoneal cavity of white female Theiler mice by weekly transplantation and harvested 8 days after transplantation. The ascites fluid was diluted in the standard incubation medium (*see below*) containing heparin (2.5 IU/ml), and centrifuged (700 × *g*, 45 sec). The packed cells were resuspended and washed by centrifugation, once with the standard incubation medium and once with the appropriate experimental incubation medium (*see below*). The cells were finally resuspended in the experimental incubation medium, the cytocrit adjusted to 8% and the cell suspension incubated in a shaking bath at 37°C. Where appropriate, [^{3}H]inulin (The Radiochemical Centre, Amersham, U.K.; 5×10^6 dpm/ml) was added as marker of the extracellular space in the cell pellets. In experiments with anion substitution the cells were pre-incubated in the experimental medium and, after 30 min, washed and resuspended in fresh incubation medium.

Incubation Media

The standard incubation medium (300 mOsm) had the following composition (mM): Na^+, 150; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 150; sulfate, 1; inorganic phosphate, 1; MOPS (morpholinopropane sulfonic acid), 3.3; TES (N-tris-(hydroxymethyl)methyl-2-amino-ethane sulfonic acid), 3.3; and HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid), 5; pH 7.40 (*cf.* Eagle, 1971). In media with pH 8.2 the MOPS, TES, and HEPES buffer was replaced by 5 mM TRICINE (N-tris(hydroxymethyl) methyl-glycine; N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-glycine) and 5 mM BICINE (N,N-bis(2-hydroxyethyl)-glycine). In the choline incubation medium choline chloride was substituted for NaCl in equimolar amounts; the K^+ concentration was the same as in the standard medium. Bromide, nitrate and thiocyanate media were prepared by substituting the Na^+ and K^+ salts of these anions for NaCl and KCl. Hypotonic incubation medium (150 mOsm) was prepared by diluting the isotonic incubation medium with one volume of a solution of MOPS, TES, and HEPES in distilled water in concentrations as above (pH 7.40), in order to lower both ion concentrations and total osmolarity.

Reagents

All reagents were analytical grade. Ionophore A23187, quinine hydrochloride, valinomycin and gramicidin D were obtained from Sigma, St. Louis, Mo. Pimozide was a gift from Lundbeck & Co., Copenhagen. Ionophore A23187 was added to the cell suspension from a 2-mM stock solution in absolute ethanol, quinine from a 1-M stock solution in ethanol, and valinomycin, gramicidin D, and pimozide from 10-mM stock solutions in ethanol. Bumetanide, a gift from Dr. P.W. Feit, Leo Pharmaceutical Products, Copenhagen, was dissolved at a concentration of 2.5 mM in the standard incubation medium by addition of dilute NaOH and subsequent adjustment of pH to 8.0 with dilute HCl. DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) was a gift of Dr. J.O. Wieth, University of Copenhagen. EGTA (ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) was obtained from Sigma and added from a stock solution adjusted to pH 7.2 with Tris base (Tris(hydroxymethyl)aminomethane).

Cell Volume Measurements

Cell volume distribution curves were obtained using a Coulter counter model Z with Coulter channelyzer (C-1000) and recorder (HR 2000). The orifice diameter was 100 μm. For the measurements an aliquot of the cell suspension was diluted 500- to 1500-fold with incubation medium at 37°C (filtered 3 times, 0.45 μm filter) to give a final cell density of about 70,000 cells per ml. The mean cell volume (arbitrary units) was calculated as the median of the cell volume distribution curves, and absolute cell volumes were obtained using polystyrene latex beads (19.2 and 13.1 μm diameter) as standards. Control experiments with polystyrene latex beads have shown that the measured volumes are unaffected by changes in osmolarity and ionic composition within the range used here.

^{36}Cl and ^{42}K Flux Experiments

The ^{36}Cl influx was measured by adding ^{36}Cl to the external medium and monitoring the increase with time of cell ^{36}Cl activity (corrected for trapped volume in the pellet of packed cells) as previously described (Simonsen & Nielsen, 1971). ^{42}K fluxes were measured using the efflux technique as previously described (Hoffmann et al., 1979). ^{42}K was counted by Cerenkov radiation in a Packard liquid scintillation counter. ^{36}Cl and ^{42}K were obtained from Risø, Denmark.

In a previous study (Hoffmann et al., 1979) the fluxes were converted from units of μmol/g dry wt · min to units of pmol/cm^2 · sec by multiplication using the factor 0.818. In the present study a revised factor of 0.283 has been used. This factor is calculated from a cell density of 1.06 g/ml (Hendil & Hoffmann, 1974), measured values of cell water content (3.24 mg/g dry wt, or 0.764 ml/g wet wt) and cell volume (938 μm^3) in control cells (Hoffmann et al., 1984*b*), and an estimated surface area of 1383 μm^2 [derived from the assumption that the cells become spherical without microvilli at a volume 2.9 times the isotonic volume (*see* Hoffmann et al., 1986)]. Using these numbers 1 g cells (wet weight) is calculated to represent 1.01×10^9 cells with a total surface area of 1.39×10^4 cm^2, and 1 g cell solids calculated to represent 4.26×10^9 cells with a total surface area of 5.90×10^4 cm^2. Mean values are given as mean ± SEM with the number of independent experiments in brackets.

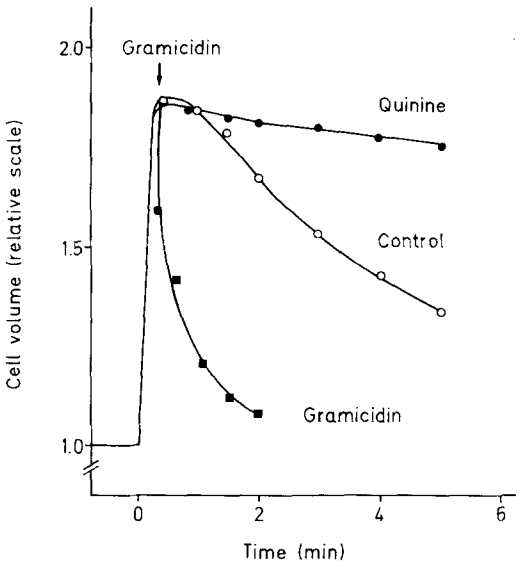

Fig. 1. Effect of quinine and gramicidin on regulatory volume decrease in Ehrlich ascites cells in Na^+-free choline medium. Ehrlich ascites cells were pre-incubated at 3 to 8% cytocrit in standard incubation medium containing Ca^{2+} (1 mM). At zero time a sample of the cell suspension was diluted 500- to 1500-fold (final cell density about 70,000 cells/ml) in hypotonic (150 mOsm) choline medium containing Ca^{2+} (0.5 mM), and the cell volume was followed with time using a Coulter counter. The initial cell volume was measured by dilution of a parallel sample of the cell suspension in standard incubation medium (mean value of two experiments). In one group quinine (1 mm) was present in the hypotonic choline medium (●). In one group gramicidin (5 μM) was added at the time indicated by the arrow (■). The experiment illustrated was performed at pH 8.2 but similar results were obtained in experiments at pH 7.2

Results

Regulatory Volume Decrease: Transient Activation of Separate K^+ and Cl^- Transport Pathways

Ehrlich cells swell in hypotonic media but subsequently regulate their volume (regulatory volume decrease) by a net loss of KCl, involving activation of a Ca^{2+}-dependent K^+ channel which can be blocked by quinine (Hoffmann et al., 1984*b*). Figure 1 shows a typical volume response in hypotonic choline medium, and its inhibition by quinine. The half-time for volume recovery during regulatory volume decrease in choline medium is 5.2 ± 1.0 min (n = 6), similar to the half-time observed in standard incubation medium (Hoffmann et al., 1984*b*). Furthermore, the experiment shows that the volume decrease is accelerated by addition of gramicidin which imposes a high cation permeability

(Pressman, 1976), indicating that the net K^+ permeability is rate limiting during regulatory volume decrease. This is in accord with the previous observation that the regulatory volume decrease can be accelerated by addition of valinomycin (Hoffmann et al., 1984*b*). Figure 2 shows the inhibited hypotonic volume response in the presence of quinine (labeled control). Gramicidin was added to impose a high cation permeability, and since the cells were suspended in Na^+-free choline medium the addition will result in a net cation and Cl^- loss, the rate of which will be limited by the Cl^- permeability. It can be seen that the fast cell shrinkage induced by addition of gramicidin is progressively reduced when gramicidin is added with increasing delay. These findings indicate that a separate Cl^- transport pathway is activated by the cell swelling but inactivated within the next 10 min.

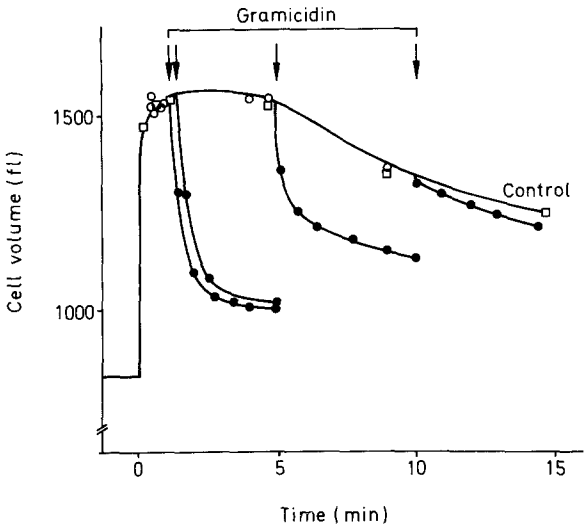

Fig. 2. Transient increase of Cl^- permeability of Ehrlich ascites cells after hypotonic cell swelling in choline medium containing quinine. Experimental protocol as in Fig. 1. Quinine (1 mM) was added to the hypotonic choline medium in order to block the Ca^{2+}-dependent K^+ channels (control, open symbols). At the times indicated by the arrows 0.5 μM gramicidin, (●) was added to impose a high cation permeability. The figure is representative of two independent experiments

Ionophore A23187 Plus Ca^{2+}-Induced Activation of Separate K^+ and Cl^- Transport Pathways

Addition of the Ca^{2+}-ionophore A23187 to a suspension of Ehrlich cells in steady state in the presence or absence of external Ca^{2+} induces a net loss of KCl with concomitant cell shrinkage (Hoffmann et al., 1984*b*). Figure 3 shows the ionophore A23187-induced volume response in the presence of 1 mM external Ca^{2+} (left panel) and in Ca^{2+}-free medium (middle panel). The initial cell shrinkage is unaffected by substitution of nitrate for Cl^- (right panel) but strongly inhibited by 1 mM quinine (left panel), or by the anti-calmodulin drug pimozide (10 μM)

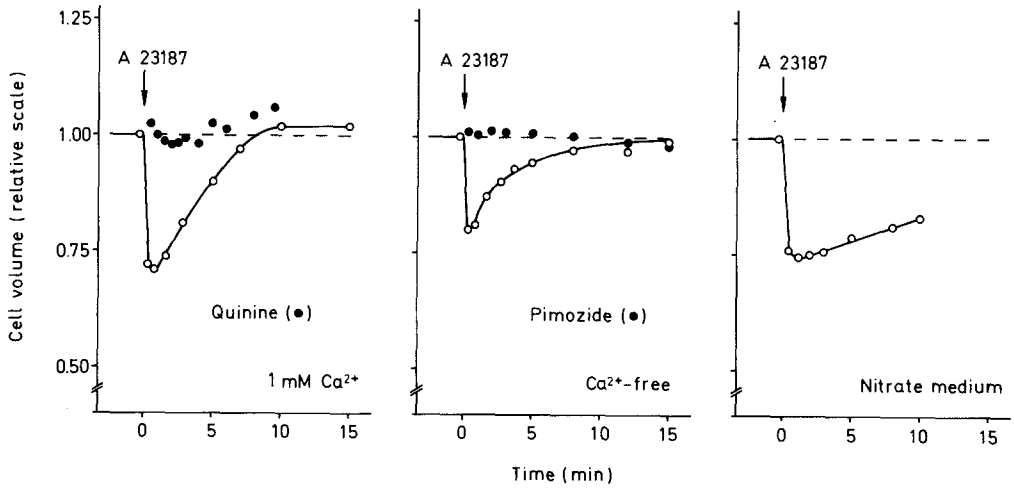

Fig. 3. Ionophore A23187-induced cell shrinkage under steady-state conditions in isotonic incubation media. The cells were preincubated for 10 to 30 min in standard incubation medium (left and middle frame) or about 30 min in nitrate medium (right frame). A sample of the cell suspension was diluted 1000-fold in the same incubation medium (containing Ca^{2+}, 1 mM) (left and right frame), or in nominally Ca^{2+}-free standard medium containing EGTA, 0.1 mM (middle frame). The diluting media all contained Mg^{2+}, 0.15 mM. In parallel groups the media contained, in addition, 1 mM quinine, which would block Ca^{2+}-dependent K^+ channels (left frame, closed symbols), or the anti-calmodulin drug pimozide, 10 μM (middle frame, closed symbols). At zero time 2 μM ionophore A23187, was added and the cell volume followed with time. The cell volume (ordinate) is given relative to that recorded before ionophore addition. The curves are representative of three experiments in the presence of quinine, and otherwise five or more independent experiments

(middle panel). These findings are consistent with the involvement of Ca^{2+}-dependent K^+ channels. It may be noted that the ionophore A23187-induced cell shrinkage is transient in NaCl media. The secondary volume recovery may well be caused by activation of a Na^+,Cl^- cotransport system (Hoffmann et al., 1984*b*; Lambert, 1984).

The above findings demonstrate parallels between the A23187-induced KCl loss and the KCl loss seen during regulatory volume decrease, which are both unaffected by nitrate substitution and inhibited by quinine or pimozide (Hoffmann et al., 1984*b*).

Figures 4 and 5 show that the A23187-induced cell shrinkage involves activation of a separate Cl^- transport pathway. The conductive Cl^- permeability of the cell membrane in Ehrlich cells is low (Hoffmann et al., 1979, 1984*b*), and consequently the cell volume changes only slowly when a high K^+ permeability is imposed by addition of valinomycin (Fig. 4, lower frame; *see also* Fig. 10, left frame) or gramicidin (Fig. 5, right frame). The observation that the cell shrinkage is slightly faster after addition of gramicidin than after addition of valinomycin can probably be accounted for by an increased driving force upon Cl^- efflux, because addition of gramicidin in Na^+-free choline medium (at 5 mM external K^+) results in a somewhat larger hyperpolarization than addition of valinomycin in standard incubation medium (I.H. Lambert & F. Jørgensen, *unpublished observation*). The cell volume is unaffected by addition of ionophore A23187 when the Ca^{2+}-dependent K^+ channels are blocked by quinine (Fig. 4, upper frame; Fig. 5, middle frame). Under these conditions, however, cell shrinkage could be induced by subsequent addition of valinomycin or gramicidin. The same result was obtained when the order of additions was reversed (Fig. 4, lower frame; Fig. 5, right frame). These findings demonstrate a high Cl^- permeability after A23187 addition.

The A23187-induced ionic permeability (P_j) was calculated under constant-field assumptions from the measured net flux (J_j^{net}) using the equation (*see* Sten-Knudsen, 1978):

$$J_j^{net} = P_j \frac{z_j V_m F/RT}{1 - e^{z_j V_m F/RT}} \left[(C)_0 - (C)_i \cdot e^{z_j V_m F/RT}\right]. \quad (1)$$

V_m is the membrane potential, $(C)_0$ and $(C)_i$ denote the ionic concentration in the external medium and in cell water, respectively, and F, R, and T have their usual meaning. In unperturbed cells V_m is about −30 mV (*see* Hoffmann et al., 1979; Hoffmann & Lambert, 1983). Addition of A23187 plus Ca^{2+} induces about 30 mV hyperpolarization of the cell membrane as estimated using a fluorescent probe (I.H. Lambert & F. Jørgensen, *unpublished observation*), and hence in the calculations V_m was taken at −60 mV. The results for K^+ and Cl^- are given in the Table (last column). It can be seen that addition of A23187 induces a 20-fold increase in P_K and a 12-fold increase in P_{Cl}.

The finding that the rate of cell shrinkage is similar in the presence and absence of gramicidin (*compare* left and middle frame in Fig. 5; *see also* the control curves with and without gramicidin in Fig. 9) indicates that the Cl^- conductance is rate limiting for the ionophore A23187-induced cell shrinkage. The ionic conductance (G_j) induced by ionophore A23187 for K^+ and Cl^- was calculated from the measured net flux (J_j^{net}) using the equation:

$$G_j = I_j/(V_m - E_j) = z_j F \cdot J_j^{net}/(V_m - E_j). \quad (2)$$

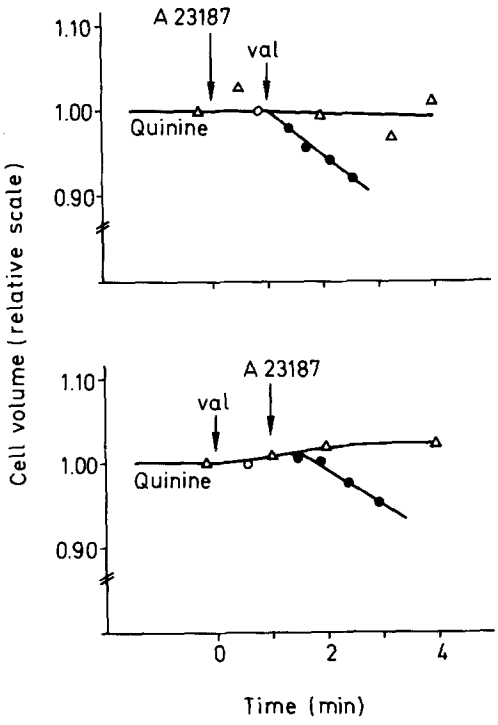

Fig. 4. Ionophore A23187-induced increase of Cl^- permeability under conditions where the Ca^{2+}-dependent K^+ channels are blocked by quinine. Valinomycin was added to impose a high K^+ permeability. The cells were pre-incubated for 40 to 75 min in standard incubation medium, and diluted 1000-fold in the same medium, containing quinine, 1 mM. In the experimental groups (●) 1 μM ionophore A23187 and 0.5 μM valinomycin were added as indicated by the arrows. The cell volume was followed with time, and given relative to that recorded before addition of valinomycin or A23187 (○). In parallel control groups (△) the cell volume was recorded with addition of A23187 only (upper frame) or valinomycin only (lower frame). The figures are representative of five and two independent experiments (upper and lower frame, respectively) giving similar results

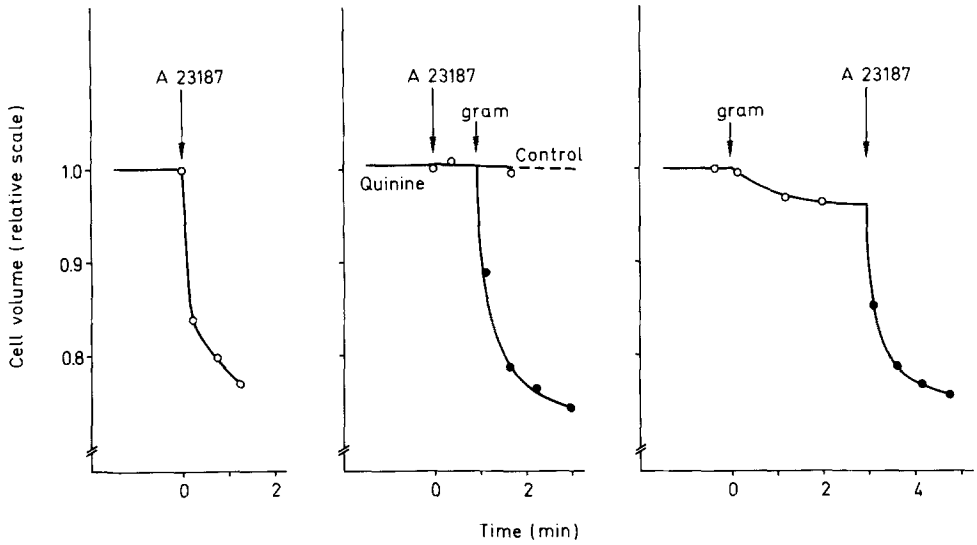

Fig. 5. Effect of ionophore A23187 on cell volume in choline medium in the presence and absence of quinine. Gramicidin was added to impose a high cation permeability. The cells were pre-incubated for 20 min in standard incubation medium, and diluted 1000-fold in choline medium containing 1 mM Ca^{2+} and 0.15 mM Mg^{2+}. Ionophore A23187 (2 μM) and gramicidin (0.5 μM) were added as indicated by the arrows. In the experiment shown in the middle frame the choline medium contained 1 mM quinine in order to block the Ca^{2+}-activated K^+ channels. In the control group (middle frame) the addition of gramicidin was omitted. The cell volume was followed with time and given relative to the initial value. The curves are representative of three independent experiments. The A23187 addition shown in the right frame (●) was, however, included in a single experiment only

Table 1. Estimate of ionophore A23187 plus Ca^{2+}-induced membrane conductance and net permeability to K^+ and Cl^- in Ehrlich ascites cells

	Conductance		Net permeability	
	Control[a]	A23187[b]	Control[a]	A23187[b]
	(μS/cm^2)		(10^{-8} cm/sec)	
K^+	5	72	3.6	75[c]
Cl^-	5	49	1.4	17

[a] Data from Hoffmann et al. (1979) corrected for previous underestimate of the area/volume ratio (*see* Materials and Methods).
[b] Calculated for (data at pH 7.4) $J_K^{net} = 89 \pm 9$ and $J_{Cl}^{net} = 63 \pm 9$ μmol/g dry wt · min (mean ± SEM, $n = 5$), corresponding to 25 and 18 pmol/cm^2 · sec, respectively, assuming $V_m = -60$ mV (*see* text). The ion concentrations were measured at $(K^+)_i = 175 \pm 6$ mM, $(K^+)_o = 5.2 \pm 0.1$ mM. $(Cl^-)_i = 58 \pm 4$ mM, and $(Cl^-)_o = 146 \pm 1$ mM. In one experiment at pH 8.2 the A23187-induced net K^+ efflux was measured at 175 μmol/g dry wt · min, corresponding to 50 pmol/cm^2 · sec, or about twice the value measured at pH 7.4.
[c] The application of the Goldman regime is probably not valid because there is evidence of single-file behavior in the case of A23187-induced K^+ flux (*see* Discussion).

z_j is the valency, I_j the ionic current, and E_j the equilibrium potential for the ion j. The results (given in the Table) confirm that the Cl^- conductance is rate limiting during A23187-induced cell shrinkage. This is in contrast to the findings during volume-induced cell shrinkage where, as discussed above, the K^+ conductance seems to be rate limiting.

Sarkadi et al. (1985) has recently reported the puzzling observation that valinomycin inhibits the volume-induced anion transport pathway. In the present study a similar inhibition by valinomycin could be demonstrated in the case of the ionophore A23187-induced Cl^- transport pathway by comparing the initial rate of cell shrinkage in the absence and presence of valinomycin in a number of experiments similar to those of Fig. 3 (left frame) and Fig. 13 (upper left frame). The rate of fractional cell volume decrease induced by ionophore A23187 was 0.70 ± 0.04 min^{-1} ($n = 5$) in controls (minimum estimate, based on first data point only), and significantly lower, 0.38 ± 0.10 min^{-1} ($n = 4$), in the presence of valinomycin (0.5 to 1 μM) (mean ± SEM with the number of experiments given in brackets; $P = 0.025$ in Student's t-test). The inhibition by valinomycin could conceivably be caused by valinomycin interference with the cell metabolism which might somehow quench the response of the Cl^- transport system. The effect of ionophore A23187 plus Ca^{2+} is presumably not affected by the valinomycin-induced hyperpolarization because A23187 mediates an electroneutral $Ca^{2+}/2H^+$ exchange.

The rate of cell shrinkage induced by the diffusional K^+ carrier valinomycin (*see* Fig. 4) is, as expected, much slower than that induced by addition of the channel-forming ionophore gramicidin (*see*

Fig. 5, middle frame). The increase in K^+ permeability induced by valinomycin is also greatly exceeded by that induced by ionophore A23187 plus Ca^{2+}. This can be seen by comparing the rate of A23187-induced cell shrinkage seen in Fig. 13 (upper left frame) with that seen in Fig. 4 where the A23187-induced K^+ transport pathway is blocked by quinine but bypassed by valinomycin.

In order to explore the possible involvement of simultaneous Cl^-/HCO_3^- (or OH^-) and K^+/H^+ exchange in the A23187-induced volume response, the external pH was monitored in preliminary experiments with the cells suspended in HCO_3^--free, unbuffered medium in the presence of DIDS (200 μM). Addition of ionophore A23187 induced a small transient change in the slope of the curve monitoring external pH *vs.* time, lasting about 1 min. The change in external pH relative to control was unaffected by the presence or absence of DIDS, and amounted to 0.032 ± 0.004 pH units/min (n = 3), equivalent to a H^+ influx at 9.7 ± 0.7 μeq/g dry wt · min, or 2.8 ± 0.2 pmol/cm^2 · sec (n = 3). For comparison, the A23187-induced K^+ net efflux was substantially higher [89 μmol/g dry wt · min or 25 pmol/cm^2 · sec (*see* Table)].

An activation of Cl^- transport following addition of ionophore A23187 is directly demonstrated in Fig. 6. The acceleration of Cl^- tracer influx is most conspicuous when the influx is monitored in the presence of DIDS and bumetanide (*see* Fig. 6, lower panel) which would reduce Cl^- influx via the anion exchange system and via the Na^+, Cl^- cotransport system, respectively (Hoffmann, 1982; Hoffmann et al., 1983). The activation of the Cl^- transport is observed in the presence of quinine which blocks the A23187-induced K^+ transport, showing that the A23187-induced Cl^- and K^+ transport pathways are separate. It may be noted that addition of ionophore A23187 increases ^{36}Cl influx more in the absence of DIDS plus bumetanide (upper panel) than in their presence (lower panel). Thus, ionophore A23187 plus Ca^{2+} appears to activate also a flux component which is sensitive to DIDS plus bumetanide.

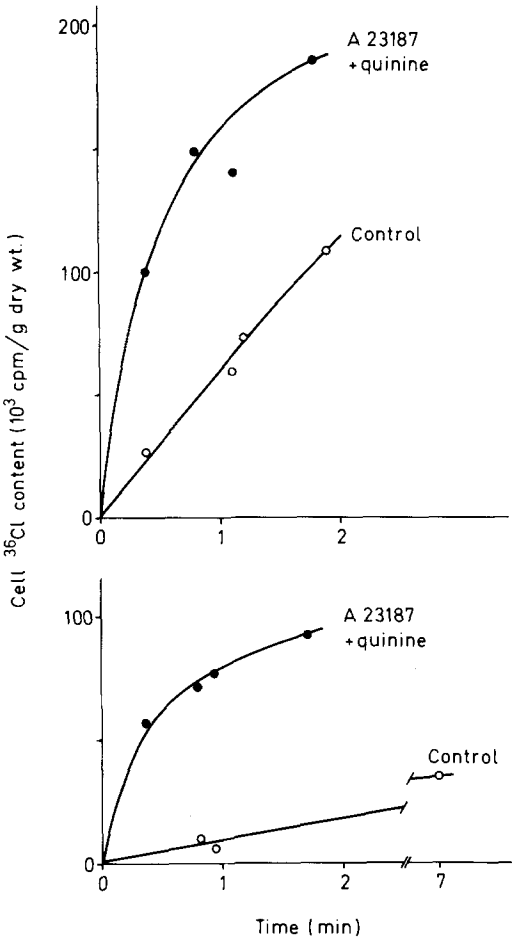

Fig. 6. Ionophore A23187-induced increase of ^{36}Cl influx in Ehrlich ascites cells. The cells were pre-incubated for 30 min at 8% cytocrit in standard incubation medium containing 1 mM Ca^{2+} and 0.15 mM Mg^{2+}. At zero time ^{36}Cl was added, and the ^{36}Cl content of the cells followed with time (control (○)). In a parallel group (●) 15 μM A23187 and 1 mM quinine were added with the tracer at zero time. In the groups represented by the curves in the lower frame the ^{36}Cl influx was monitored in the presence of 500 μM DIDS and 50 μM bumetanide, which would reduce ^{36}Cl influx via the anion exchange system and via the Na^+,Cl^- cotransport system, respectively. DIDS and bumetanide were added 2 min before the start of influx. The figure is representative of two independent experiments

Time Dependence of the A23187-Induced Activation of the Cl^- Transport Pathway. Role of Ca^{2+}

Figure 7 (upper panel) shows that the A23187-induced activation of the net Cl^- permeability is transient in Ca^{2+}-free media, and inactivated within about 5 min, reminiscent of the inactivation of the Cl^- permeability observed during regulatory volume decrease following hypotonic exposure (*see* Fig. 2). In contrast, in the presence of ionophore A23187 plus 1 mM Ca^{2+} the activation of the Cl^- transport pathway is persistent (Fig. 7, lower panel). Figure 8 shows that under these conditions the A23187-induced Cl^- transport pathway is inactivated when excess EGTA is added to chelate external Ca^{2+}. Under these conditions the cellular Ca^{2+} content will be reduced both via the Ca^{2+}-pump flux and via ionophore A23187-mediated efflux.

Inhibition by the Anti-Calmodulin Drug Pimozide of the Ionophore A23187- and Volume-Induced Cl^- and K^+ Transport Pathways

The anti-calmodulin drug pimozide, a diphenylbutyl piperidine neuroleptic, inhibits (Hoffmann et al.,

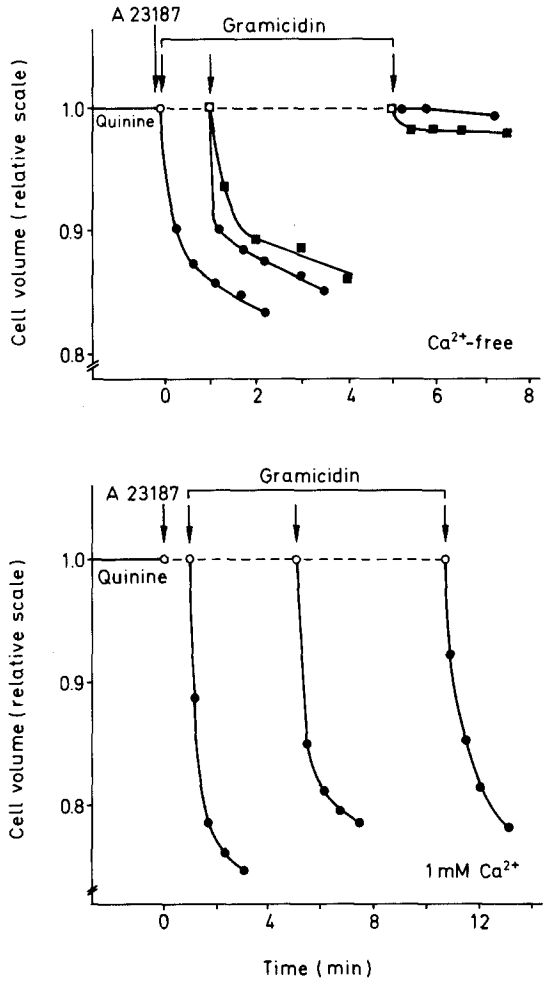

Fig. 7. Time dependence of Cl^- net permeability following addition of ionophore A23187 in Ca^{2+}-free and Ca^{2+}-containing choline medium. The media contained quinine to block the Ca^{2+}-dependent K^+ channels. Gramicidin was added at the times indicated by the arrows to impose a high cation permeability. Experimental protocol as in Fig. 5. The choline medium was nominally Ca^{2+}-free, containing 0.5 mM EGTA (upper frame), or contained 1 mM Ca^{2+} (lower frame). In both media 0.8 mM choline was replaced by Na^+ in order to provide an equilibrium potential for Na^+ near that for K^+. The experiment is representative of two and three experiments in Ca^{2+}-free and Ca^{2+}-containing medium, respectively. The cell volume is given relative to the value measured before addition of A23187 and gramicidin (open symbols). The curves shown in the upper frame are compiled from two experiments marked individually.

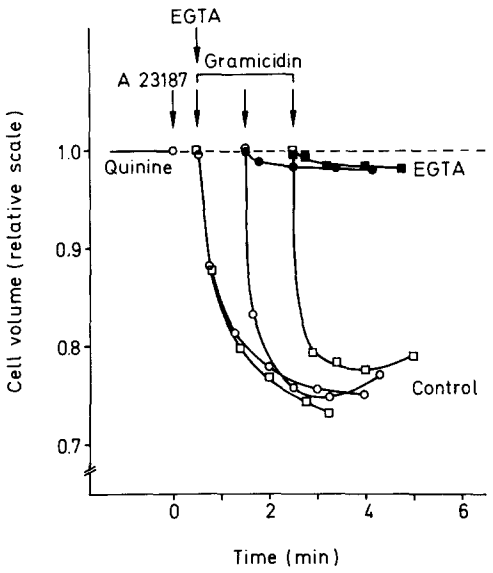

Fig. 8. Inactivation of the A23187-induced increase of Cl^- permeability in Ca^{2+}-containing media by subsequent addition of excess EGTA. Experimental protocol for control groups (open symbols) as in Fig. 7, lower frame, except that the Ca^{2+} concentration was reduced to 0.25 mM. In the experimental groups (closed symbols) 1 mM EGTA was added (arrow) 0.5 min after addition of ionophore A23187. The figure is representative of two experiments giving similar results

1984*b*) the regulatory volume decrease following hypotonic exposure, and the A23187-induced KCl loss (*see* Fig. 3 (middle frame) and Fig. 9). The inhibition by pimozide of the A23187-induced cell shrinkage is dependent upon the Ca^{2+} concentration. The inhibition is nearly complete in Ca^{2+}-free medium (Fig. 3) and at 0.15 mM Ca^{2+} (*see* Fig. 9), but strongly reduced at 1 mM Ca^{2+} (*data not shown*). The experiments in Figs. 9, 10 and 11 were designed to explore whether pimozide affects the Cl^- transport pathway, the K^+ transport pathway, or both. Figure 9 shows that pimozide blocks the A23187-induced volume response also when a high K^+ permeability is ensured by the presence of gramicidin (open and closed squares), demonstrating that the Cl^- transport pathway activated by ionophore A23187 is inhibited by pimozide.

The experiment presented in Fig. 10, left frame, shows that addition of valinomycin induces a cell shrinkage when the dominating cellular and extracellular anion is nitrate or thiocyanate, whereas the cell volume is essentially unaffected in Cl^- medium. The rate of cell shrinkage was also very low in bromide medium (*not illustrated*). These findings demonstrate a low net permeability of the cell membrane for Cl^- and Br^- but a higher net permeability for nitrate and in particular thiocyanate. In the case of thiocyanate the apparent net permeability (ground permeability) is a minimum estimate because the valinomycin-induced K^+ permeability might conceivably be rate limiting. A high conductive permeability to nitrate in Ehrlich cells has also been demonstrated by Thornhill and Laris (1984), and in human lymphocytes the conductive permeability to thiocyanate is higher than that to Cl^- (Grinstein et al., 1982*a*). Figure 10, right frame, shows

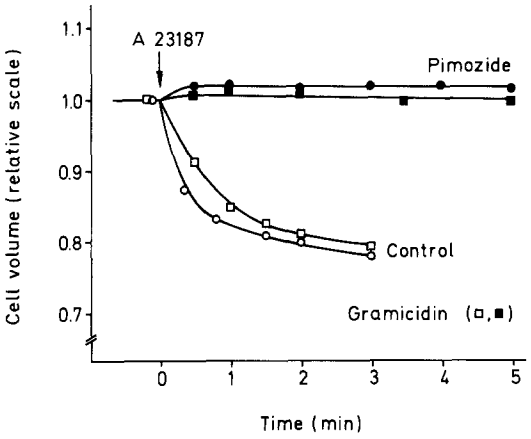

Fig. 9. Inhibition by pimozide of ionophore A23187-induced increase of Cl^- net permeability. A high K^+ permeability was imposed by addition of gramicidin. The cell volume was followed after dilution in choline medium containing 0.15 mM Ca^{2+} and 0.15 mM Mg^{2+}. In the experimental groups that medium contained, in addition, 15 μM pimozide (closed symbols). Ionophore A23187 (2 μM) was added at zero time. Gramicidin (0.5 μM) was added immediately before A23187 to two of the groups (□,■); for comparison gramicidin was omitted in two parallel groups (○,●). Other experimental details as in Fig. 5. The curves are representative of three independent experiments; the control group with gramicidin included (□) was, however, omitted in one experiment

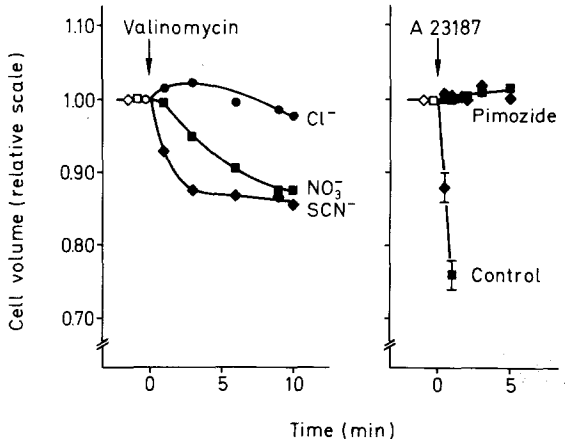

Fig. 10. Inhibition by pimozide of ionophore A23187-induced increase of K^+ permeability in media where Cl^- is replaced by the more permeant anions nitrate and thiocyanate. The cells were pre-incubated for 35 min in Cl^- medium (standard incubation medium) (○,●), in nitrate medium (□,■), or in thiocyanate medium (◇,◆), all containing 0.15 mM Ca^{2+} and 0.15 mM Mg^{2+}. The media were shifted once after 15-min pre-incubation. The cell suspension was diluted 1000-fold in the corresponding medium, and the cell volume followed with time. In the experiment shown in the left frame 1 μM valinomycin was added at zero time (closed symbols). In the experiment shown in the right frame 2 μM ionophore A23187.was added at zero time (closed symbols). Pimozide (15 μM) was present in the experimental groups. The curves shown in the left and right frame are representative of 5 and 2 independent experiments, respectively. The data points with error bars (right frame, control) give the mean ± SEM for four independent experiments

that pimozide blocks the A23187 plus Ca^+-induced volume response also when the cellular and extracellular Cl^- is replaced by the more permeable anions nitrate or thiocyanate. Control experiments (*not illustrated*) showed that the thiocyanate net permeability (ground permeability) is only slightly reduced in the presence of pimozide. The findings in Fig. 10 (right frame) therefore demonstrate that the K^+ transport pathway activated by ionophore A23187 is blocked by pimozide.

The inhibitory effect of pimozide on the ionophore A23187 plus Ca^{2+}-activated K^+ transport was directly demonstrated in a single ^{42}K flux experiment with parallel groups in duplicate to quadruplicate. In this experiment the ^{42}K efflux was monitored at pH 8.2 and 0.3% cytocrit. The efflux medium contained 62 mM K^+, 8 mM Na^+, and 78 mM choline, with the aim of approaching electrochemical equilibrium for K^+, Na^+ and Cl^-, and measuring equilibrium K^+ exchange in the absence of substantial net fluxes. Ionophore A23187 (5 μM) induced an increase in the unidirectional K^+ efflux from 27 ± 5 μmol/g dry wt · min (mean ± SEM, n = 4) (control) to 98 ± 13 μmol/g dry wt · min (n = 3). In the presence of pimozide (50 μM) plus A23187 the unidirectional K^+ efflux was only 29 ± 10 μmol/g dry wt · min (n = 2). These tracer flux measurements confirm that the K^+ transport pathway activated by ionophore A23187 plus Ca^{2+} can be blocked by pimozide.

The experiment presented in Fig. 11 explores whether the volume-induced transport pathways for Cl^- and K^+ can also be blocked by pimozide, similar to the findings for the ionophore A23187 plus Ca^{2+}-induced transport pathways. The experiment was performed at pH 8.2 because at this pH the KCl loss during regulatory volume decrease proceeds via separate Cl^- and K^+ transport pathways only, without the Cl^--dependent component of the volume recovery (K^+,Cl^- cotransport) which is observed at lower pH values (Kramhøft et al., 1986). At pH 8.2 pimozide was noted to be (when compared at identical cytocrits, *see below*) three- to fourfold more effective as inhibitor of the volume recovery than at pH 7.4. A comparison of the control curves in Cl^- and thiocyanate media (Fig. 11, left and right frames) shows that the volume response induced by cell swelling is unaffected by substitution of thiocyanate for Cl^-. Pimozide was found to block the volume recovery also in the presence of gramicidin (Fig. 11, middle frame), in thiocyanate medium (Fig. 11, right frame), and in

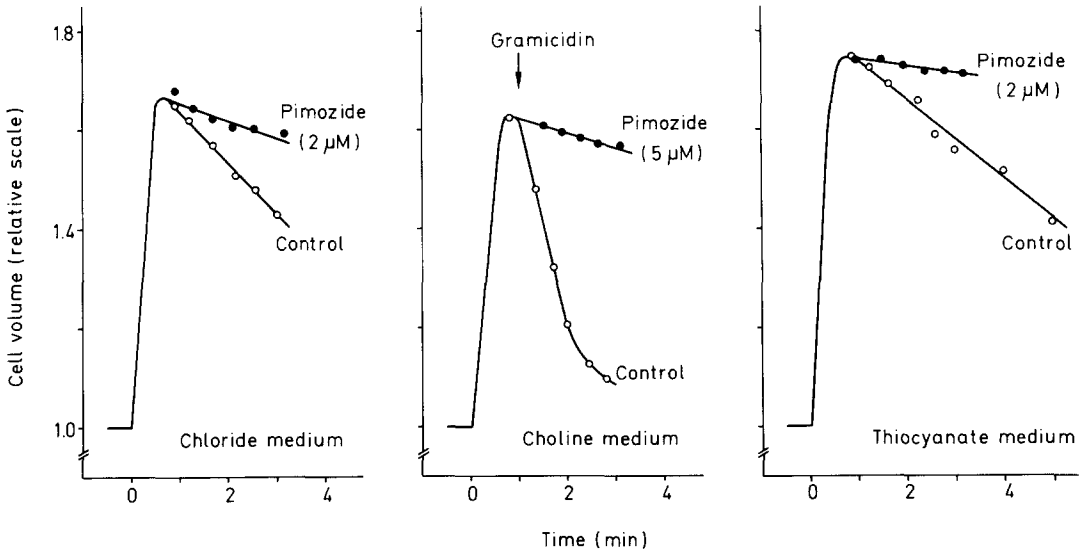

Fig. 11. Inhibition by pimozide of the increase in Cl^- and K^+ net permeabilities induced by cell swelling. Parallel groups of cells were pre-incubated at 4% cytocrit for 25 min in Cl^- medium (standard incubation medium), choline medium, or thiocyanate medium (left, middle and right panel, respectively) with shift of the medium once after 15-min pre-incubation. The media were buffered at pH 8.2 and contained 0.15 mM Ca^{2+} and 0.15 mM Mg^{2+}. At zero time the cell suspensions were diluted 1000-fold in the corresponding media with the tonicity reduced to 150 mOsm, and the cell volume followed with time. Pimozide was added to the experimental groups (closed symbols) in the concentrations indicated. In the experiment in choline medium (middle frame) gramicidin (0.5 μM) was added at $t = 1$ min to control and experimental groups in order to impose a high K^+ permeability. The cell volume is given relative to the initial cell volume, measured by dilution of a parallel sample of the cell suspension in the corresponding isotonic incubation medium. Similar results were obtained in nitrate medium (*data not illustrated*). The data for a total of five pimozide concentrations tested in two independent experiments are summarized in Fig. 12

nitrate medium (*not illustrated*). At pH 7.4 similar inhibition of the volume recovery by pimozide (8 to 10 μM) was demonstrated in three experiments (*not illustrated*). These findings demonstrate that the volume-induced transport pathways for Cl^- and K^+ are also blocked by pimozide.

Analysis of the concentration dependence of the pimozide inhibition of the hypotonic volume response (Fig. 12) shows a similar inhibition in Cl^-, nitrate, and thiocyanate media but a significantly weaker inhibition in the presence of gramicidin. This finding suggests that the volume-activated Cl^- transport pathway is somewhat less sensitive to pimozide than the K^+ transport pathway. A similar although more pronounced difference in the sensitivities of the volume-induced Cl^- and K^+ transport pathways has recently been reported in human lymphocytes where, based on a comparison of the inhibition in the absence and presence of gramicidin, pimozide and R24571 were found to be at least 10-fold more effective as inhibitors of volume-activated K^+ transport than of Cl^- transport (Sarkadi et al., 1985). In the present experiments it was observed that the inhibitory effect of pimozide is critically dependent upon the cytocrit, indicating that a substantial amount of the drug becomes cell associated. This implies that a comparison of the inhibitor concentration added at 50% inhibition in different systems [*see* e.g., Hoffmann et al., 1984*b* (Table 4)] is only valid at very low cytocrits.

Selectivity of the Anion Transport Pathway

The finding that pimozide inhibits the anion transport pathway activated by A23187 plus Ca^{2+} (*see* Fig. 9) is used in Fig. 13 to explore the anion selectivity of this anion transport pathway. In this experiment valinomycin was added to ensure a high K^+ permeability, and the cell volume following A23187 addition was monitored in the presence and absence of pimozide, with Cl^-, Br^-, NO_3^- or SCN^- as the dominating cellular and extracellular anion. The volume response was nearly blocked by pimozide (15 μM) in chloride and bromide medium, and inhibited about 90 and 65% in nitrate and thiocyanate medium, respectively, showing that these anions all permeate via an anion transport pathway which is blocked by pimozide. The residual cell shrinkage in thiocyanate medium in the presence of pimozide probably reflects the comparably high ground permeability to this anion (*cf.* Fig. 10, left panel).

The rate of pimozide-sensitive net anion efflux

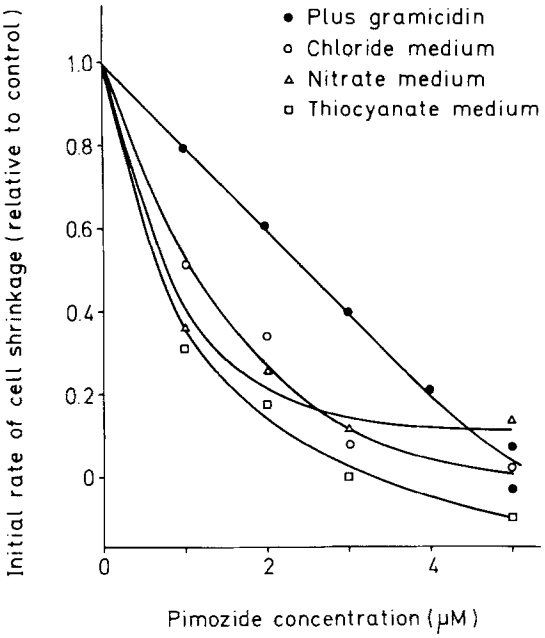

Fig. 12. Inhibitory effect of pimozide on the increase in Cl^- and K^+ net permeabilities induced by cell swelling. The initial rate of volume recovery was read from curves similar to those of Fig. 11 using lines fitted to the values taken between 1 and 2 to 3 min after the change in osmolarity. The ordinate shows the initial rate of volume recovery at varying pimozide concentrations (abscissa) given relative to control. The data are from two independent experiments

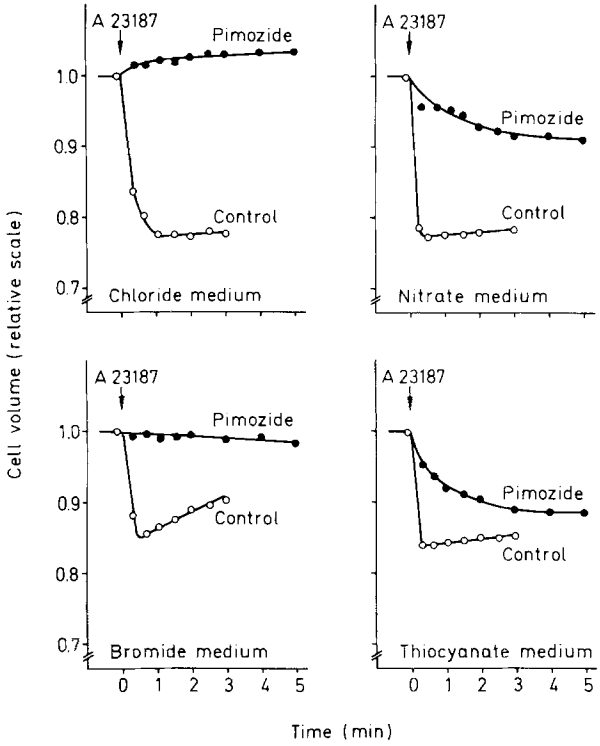

Fig. 13. Selectivity of the ionophore A23187-induced, pimozide-sensitive anion transport pathway. A high K^+ permeability was imposed by addition of valinomycin. The cells were pre-incubated for 35 min in Cl^- medium (standard incubation medium), bromide, nitrate, or thiocyanate medium as indicated, all containing 0.15 mM Ca^{2+} and 0.15 mM Mg^{2+}. The media were shifted once after 15-min pre-incubation. The cell suspension was diluted 1000-fold in the corresponding medium, and the cell volume followed with time. Pimozide (10 μM) was present in the experimental groups (closed symbols). Valinomycin (1 μM) was added to all groups immediately before A23187 (2 μM) which was added at zero time. The cell volume (ordinate) is given relative to that recorded immediately before A23187 addition. The curves are representative of three independent experiments in Cl^- medium and otherwise two independent experiments

induced by A23187 plus Ca^{2+} appears to be of similar magnitude with Cl^-, Br^-, and NO_3^- as the dominating anion. In the case of thiocyanate the pimozide-sensitive component may appear to be smaller (*see* Fig. 13), but probably is of similar magnitude because the curve drawn in the control group probably underestimates the rate of cell shrinkage. It may be noted that the absolute rates of pimozide-sensitive net anion fluxes for all the anions tested tend to be underestimated because valinomycin, as discussed above, inhibits the A23187-induced anion transport pathway.

Discussion

Activation of Separate K^+ and Cl^- Transport Pathways by Cell Swelling and by Addition of Ionophore A23187

During regulatory volume decrease the apparent K^+ permeability is increased (Hendil & Hoffmann, 1974; Hoffmann, 1978; Hoffmann et al., 1984*b*). A concomitant volume-induced increase in Cl^- net permeability is demonstrated in Fig. 2. Under conditions where the Ca^{2+}-sensitive K^+ channel is blocked by quinine, the addition of gramicidin, which imposes a high K^+ permeability, induces a fast net efflux of KCl, demonstrating a high Cl^- net permeability which greatly exceeds the low Cl^- net permeability found in unperturbed cells (Hoffmann et al., 1984*b*; *see also* Fig. 10, left panel).

The ionophore A23187, when added to a suspension of Ehrlich ascites cells in steady state, induces a net loss of KCl with associated cell shrinkage (Hoffmann et al., 1984*b*; *see* Fig. 3). A calculation of the net permeabilities to K^+ and Cl^- under constant-field assumptions (*see* Table) shows a substantial concomitant increase in net K^+ and Cl^- permeabilities. Addition of ionophore A23187

also induces a substantial activation of the unidirectional ^{42}K fluxes (*see* Results) and ^{36}Cl fluxes (*see* Fig. 6).

The KCl loss induced by ionophore A23187 shows, in several respects, parallels to the KCl loss observed during regulatory volume decrease: (i) The volume response is in both cases unaffected by substitution of nitrate or thiocyanate for Cl^- (*see* Figs. 3 and 11) which provides evidence against KCl loss via a volume-sensitive, Cl^--dependent cotransport system, similar to that reported in erythrocytes of a variety of species (for references *see* Ellory et al., 1982, 1985*b*; Hoffmann et al., 1984*b*; Hoffmann, 1985*a*; Siebens, 1985). (ii) The KCl loss is in both cases inhibited by quinine (*see* Figs. 1 and 3), suggesting the involvement of the Ca^{2+}-dependent K^+ transport pathway found in several cell types (*see* reviews by Lew & Ferreira, 1978; Schwartz & Passow, 1983) and also demonstrated in Ehrlich ascites cells (Valdeolmillos et al., 1982). (iii) The anti-calmodulin drug pimozide inhibits the KCl loss in both cases (*see* Figs. 9 and 11), suggesting that calmodulin may play a role in the activation of the K^+ and Cl^- transport pathways. (iv) The KCl loss can in both cases be observed in Ca^{2+}-containing as well as in Ca^{2+}-free media (containing excess EGTA) (*see* Fig. 3 and Hoffmann et al., 1984*b*), suggesting that release of Ca^{2+} from internal stores or perhaps modulation of Ca^{2+} sensitivity may play a role in the activation of the transport pathways.

Quinine blocks the net loss of KCl induced by cell swelling or by ionophore A23187, but the quinine inhibition is overcome when the blocked K^+ conductance is bypassed by addition of valinomycin (Fig. 4) or gramicidin (Figs. 2 and 5). These findings demonstrate that quinine blocks a separate K^+ transport pathway activated in parallel with the Cl^- transport pathway. An increased $^{36}Cl^-$ flux induced by ionophore A23187 in the presence of quinine is directly demonstrated in Fig. 6. The activation of the Cl^- flux was also observed in the presence of DIDS and bumetanide which would inhibit Cl^- flux via the anion exchange system (Hoffmann et al., 1979; Sjøholm et al., 1981) and via the cotransport system (Hoffmann et al., 1983), respectively, consistent with the interpretation that the activated Cl^- flux is effected via a conductive Cl^- transport pathway.

Quinine has been reported to be a weak inhibitor also of the volume-induced anion transport pathway in lymphocytes (Sarkadi et al., 1985). The findings in the present study show, however, that any inhibition by quinine of the anion transport pahway is not substantial, because the rate of volume- or A23187-induced cell shrinkage in the presence of gramicidin is, given the time-resolution of these curves, essentially unaffected by the presence or absence of quinine [*cf.* middle and right frames in Fig. 5; and Figs. 2 and 11 (middle frame); note that the volume response would, if anything, be accelerated at the more alkaline pH of Fig. 11].

In human lymphocytes convincing evidence for activation of independent K^+ and Cl^- conductance pathways during regulatory volume decrease has been presented (for refs. *see* Introduction). In these cells ionophore A23187 plus Ca^{2+} also induces a cell shrinkage which, however, follows a time course much slower than that observed in the present study. In lymphocytes the KCl loss was only inhibited about 50% by quinine, and moreover, no cell shrinkage was observed following addition of A23187 in the absence of external Ca^{2+} (Grinstein et al., 1982*c*).

In *Amphiuma* red cells the net loss of KCl induced by cell swelling or by ionophore A23187 has been demonstrated to be effected mainly via a K^+/H^+ exchange functionally coupled to a Cl^-/HCO_3^- exchange (Cala, 1980; 1983*b*; *see* recent reviews by Cala, 1983*a*, 1985). In those studies it was suggested that Ca^{2+} is involved in the activation of the K^+/H^+ exchange system, with the Ca^{2+}-activated conductive K^+ flux playing only a minor role for the observed KCl loss. In the present experiments there is evidence of A23187 plus Ca^{2+}-induced activation of K^+/H^+ exchange, detectable as H^+ influx (*see* Results), and of activation of the anion exchange system (*see* Fig. 6). Electroneutral, coupled exchanges seem, however, to play only a minor role in the net KCl loss observed in Ehrlich cells, because the A23187-induced pH shift in DIDS-treated cells in HCO_3^--free, unbuffered medium was equivalent to a H^+ influx of only about 3 pmol/cm^2 · sec, whereas the A23187-induced K^+ net efflux amounted to 25 pmol/cm^2 · sec.

In Ehrlich cells Thornhill and Laris (1984) reported a Cl^--dependent and quinine-insensitive net loss of KCl induced by cell swelling or by addition of ionophore A23187 plus Ca^{2+}, which was concluded to occur via an electroneutral K^+,Cl^- cotransport mechanism. The results reported by Thornhill and Laris (1984) deviate in several respects from those found in the present study. In their study the rate of KCl loss during regulatory volume decrease (Thornhill & Laris, 1984, Fig. 2) is about 20-fold slower than that found in our laboratory (Hoffmann et al., 1984*b*, Fig. 2). Ca^{2+}-depletion has been demonstrated to inhibit the regulatory volume decrease in Ehrlich cells (Hoffmann et al., 1984*b*) and in lymphocytes (Grinstein et al., 1982*c*), and the prolonged pre-incubation in Ca^{2+}-free media in the protocol of Laris and coworkers may perhaps account for the slow volume response. K^+,Cl^- cotransport has recently been found to be stimulated by Ca^{2+} depletion in LK-sheep red cells (Lauf

& Mangor-Jensen, 1984) and by swelling in Ca^{2+}-depleted Ehrlich cells (Kramhøft et al., 1986), and such an activation would explain the Cl^--dependent KCl transport observed by Laris and co-workers. In the study of Thornhill and Laris (1984) the cell shrinkage induced by ionophore A23187 plus Ca^{2+} was small and observed in only a quarter of the cases. In this context, it may be noted that the A23187-induced cell shrinkage is transient (Hoffmann et al., 1984*b*; *see* Fig. 3), and the cell volume would be expected to be essentially restored within the 30-min period before the first data point given by Thornhill and Laris (1984, Fig. 2).

Activation and Inactivation of the Cl^- Transport Pathway: Time-dependence and Role of Ca^{2+}

The Cl^- transport pathway is strongly activated during regulatory volume decrease (*see* Fig. 2). The increase in Cl^- net flux is estimated at about 60-fold, comparing the rate of cell shrinkage in the presence of gramicidin 1 min after hypotonic exposure (Figs. 1 and 2) with that observed for isotonic cells (Fig. 5, right frame). The activation of the Cl^- net permeability during regulatory volume decrease exceeds that of the K^+ permeability as seen by the acceleration of the volume response by gramicidin (*see* Fig. 1). In human lymphocytes the K^+ permeability is also rate limiting during regulatory volume decrease (Grinstein et al., 1982*a*; Sarkadi et al., 1984*a,b*). After addition of ionophore A23187 the increase in net Cl^- permeability is estimated at 12-fold (*see* Table), calculated from net Cl^- flux measurements. The A23187-induced increase in DIDS- and bumetanide-insensitive ^{36}Cl influx (Fig. 6, lower panel) is estimated at about 20-fold. These findings demonstrate a substantial A23187-induced activation of the Cl^- transport pathway which, however, is smaller than that induced by cell swelling. During ionophore A23187-induced cell shrinkage the Cl^- permeability is rate limiting, as gramicidin fails to accelerate the volume response (*see* Fig. 9), and the calculated K^+ conductance exceeds the Cl^- conductance (*see* Table).

Figure 2 shows a time-dependent inactivation within 5 to 10 min of the Cl^- transport pathway. A similar time-dependent inactivation has been demonstrated in human lymphocytes (Sarkadi et al., 1984*b*). The inactivation of the Cl^- transport pathway seen in Fig. 2 may, rather than a time-dependence *per se*, reflect a transient increase in cytosolic free Ca^{2+}. This notion is supported by the finding in Figs. 7 and 8 that the ionophore A23187-induced activation of the Cl^- transport pathway is transient in the absence of external Ca^{2+} but persistent in the presence of 1 mM external Ca^{2+}. In the latter case the Ca^{2+} pump is probably swamped by the A23187-induced Ca^{2+} leak across the cell membrane, although the Ca^{2+} pump in Ehrlich cells has been reported to be extremely powerful (Cittadini et al., 1982; Klaven et al., 1983). In Ca^{2+}-free media A23187-induced release of Ca^{2+} from internal stores (*see* Arslan et al., 1985) and a subsequent hysteretic activation of the Ca^{2+} pump (*see* Scharff et al., 1983) is likely to produce a transient increase in cytosolic Ca^{2+}. A similar transient increase in cytosolic Ca^{2+} could conceivably be produced by volume-induced release of Ca^{2+} from internal stores or by Ca^{2+} entry across the cell membrane. An increase in cytosolic free Ca^{2+} induced by cell swelling has recently been demonstrated in toad bladder epithelial cells using the intracellular Ca^{2+} indicator Quin-2 (Chase & Wong, 1985).

In human lymphocytes an increased ^{45}Ca efflux during regulatory volume decrease has been demonstrated, suggesting a redistribution of Ca^{2+} inside the cell with a transient increase in cytosolic Ca^{2+} concentration (Grinstein et al., 1982*c*). Moreover, the KCl loss during regulatory volume decrease was reduced following cellular Ca^{2+} depletion in Ehrlich cells (Hoffmann et al., 1984*b*) and in human lymphocytes (*see* recent review by Grinstein et al., 1984). On the other hand, in experiments where the cytoplasmic free Ca^{2+} concentration was directly monitored in these cells using Quin-2, no changes could be demonstrated during regulatory volume decrease (Rink et al., 1983). As discussed by Grinstein et al. (1984) this finding can be explained by assuming either a 'local' increase in cytoplasmic free Ca^{2+}, or a Ca^{2+}-independent activation of K^+ channels by cell swelling. The absence of a detectable increase in cytosolic free Ca^{2+} during regulatory volume decrease and the inhibitory effect of cellular Ca^{2+} depletion could, however, also be accounted for by modulation of the Ca^{2+} sensitivity of the transport pathways, as previously suggested in relation to the pH dependence of the volume recovery in hypotonic media which was found to be similar in the presence and absence of external Ca^{2+} (Hoffmann et al., 1984*b*).

The finding that Ca^{2+} is involved in the activation of the Cl^- transport pathway appears to be at variance with the findings in human lymphocytes where the increase in Cl^- net permeability induced by A23187 plus Ca^{2+} is significant (Grinstein et al., 1982*b*) but not appreciable compared to that induced by cell swelling (Sarkadi et al., 1984*a,b*). A Ca^{2+}-activated Cl^- transport pathway has recently been reported in the luminal membrane of salivary gland epithelial cells (Nauntofte & Poulsen, 1984).

Selectivity of the Anion Transport Pathway

In the absence of activation of the anion transport pathway the net permeability of the cell membrane is substantially higher to nitrate and in particular thiocyanate than to Cl^-, as seen by comparing the rate of cell shrinkage induced by valinomycin in these media (*see* Fig. 10, left panel). Following ionophore A23187-induced activation of the anion transport pathway the rate of cell shrinkage in the presence of valinomycin is substantial both in Cl^-, Br^-, NO_3^- and SCN^- media (*see* Fig. 13). The absolute increase in the rate of cell shrinkage following addition of A23187 is of similar magnitude in the four media, indicating the activation by Ca^{2+} of a rather unselective anion transport pathway. The pimozide-sensitive net anion efflux induced by A23187 also appears to be of similar magnitude in the four media (*see* Fig. 13). The volume-induced anion transport pathway in human lymphocytes was also found to be rather unselective (Grinstein et al., 1982*a*).

Role of Calmodulin in the Activation of K^+ and Cl^- Transport Pathways

A number of drugs reported to inactivate the Ca^{2+}-binding protein calmodulin (Weiss et al., 1980), among others the diphenylbutyl piperidine neuroleptic pimozide, has previously been demonstrated to inhibit the KCl loss induced by cell swelling or by A23187 in lymphocytes (Grinstein et al., 1982*c*) and in Ehrlich cells (Hoffmann et al., 1984*b*; *see also* Fig. 3 in the present study). It may be noted that the inhibition by pimozide of the A23187-induced KCl loss is strongly reduced at high external Ca^{2+} concentration. Unless pimozide is partially inactivated by the formation of Ca^{2+} complexes, this finding indicates that Ca^{2+} is involved in the process which is inhibited by pimozide. In the *Amphiuma* red blood cell the phenothiazines promethazine and chlorpromazine have been reported to block the Ca^{2+}-induced increase in K^+ permeability and associated hyperpolarization (Gárdos et al., 1976), and also in this case the inhibition could be (partially) overcome by exposure to high concentrations of A23187 plus Ca^{2+}. The Ca^{2+}-activated K^+ channel in red cells is sensitive to anti-calmodulin drugs (Lackington & Orrego, 1981; Yingst & Hoffman, 1984), and the inhibition of the Ca^{2+}-induced hyperpolarization by phenothiazines may well have been caused by their anti-calmodulin activity (*see* Weiss et al., 1980) although at the time it was ascribed to their properties as local anesthetics (Gárdos et al., 1976). It should be noted, however, that the involvement of calmodulin in the activation of the Ca^{2+}-dependent K^+ channel is still an open question. Pape and Kristensen (1984) have presented direct evidence supporting the involvement of calmodulin, whereas negative results have been reported by Lew et al. (1982) and by Plishker (1984).

In the present study pimozide is found to block the volume- or A23187 plus Ca^{2+}-induced KCl loss also in the presence of anions with a high conductive permeability (nitrate or thiocyanate, *see* Figs. 10, 11, and 12), and also when a high K^+ net permeability is ensured by addition of gramicidin (*see* Figs. 9, 11 and 12). These findings demonstrate that the volume- or A23187 plus Ca^{2+}-activated K^+ and Cl^- transport pathways are both inhibited by pimozide. The inhibition by pimozide of the A23187-activated K^+ transport pathway is, moreover, directly demonstrated by ^{42}K flux experiments. Although pimozide is not selective as an anti-calmodulin drug, the findings suggest that calmodulin is involved in the activation of both the K^+ and the Cl^- transport pathway in Ehrlich cells.

In human lymphocytes a number of anti-calmodulin drugs have been demonstrated to inhibit both volume-induced K^+ and Cl^- transport, although as previously mentioned (*see* Results) with a weaker effect on the Cl^- transport pathway in the case of pimozide and R24571 (Grinstein et al., 1982*c*, 1983; Sarkadi et al., 1985). The findings in human lymphocytes are taken to indicate the involvement of Ca^{2+} and calmodulin in the volume-induced activation of the K^+ transport pathway, whereas the involvement of Ca^{2+} and calmodulin in the activation of the Cl^- transport pathway appears to be unlikely (Sarkadi et al., 1984*a*,*b*).

Conductance of the Ca^{2+}-Activated K^+ Channel

The A23187-activated ^{42}K flux is inhibited by pimozide as discussed above. When measured at pH 8.2 and near electrochemical equilibrium for K^+, the A23187-induced, pimozide-sensitive unidirectional ^{42}K flux amounts to 69 μmol/g dry wt · min (*see* Results), corresponding to 20 pmol/cm^2 · sec. Since the unidirectional flux (J_K) was measured near electrochemical equilibrium, the K^+ conductance (G_K) can be estimated from the equation:

$$G_K = \frac{F^2}{RT} \cdot J_K \qquad (3)$$

assuming that the pimozide-sensitive flux represents the flux via the conductive K^+ transport path-

way, and assuming independence of unidirectional influx and efflux (*see* Hodgkin, 1951; Ussing & Zerahn, 1951). Under these assumptions the A23187-induced, pimozide-sensitive K^+ conductance can be calculated at 71 $\mu S/cm^2$.

The ionophore A23187-induced K^+ conductance can also be calculated from the observed net K^+ efflux (J_K^{net}) in standard medium, using Eq. (2). The A23187-induced, pimozide-sensitive net K^+ efflux at pH 8.2 was measured at 50 pmol/cm^2 · sec (*see* Table). Using this value (taken to represent the conductive net K^+ flux) and taking V_m at −60 mV and E_K at −93 mV, G_K can be calculated at 147 $\mu S/cm^2$, or twice the value of G_K estimated from tracer flux measurements under equilibrium conditions. The ionic conductance of the membrane will change with the ion concentration and the membrane potential. A calculation shows that in the Goldman regime G_K will be lower under the conditions of the net flux experiments with low external K^+ concentration and hyperpolarized membrane potential (*see* Sten-Knudsen, 1978, Eqs. 258, 261). This means that G_K is at least twofold underestimated when calculated from tracer flux measurements at high external K^+ using Eq. (3). This finding could be accounted for by assuming single-file diffusion (*see* Ussing, 1978). In the single-file case, according to Hodgkin and Keynes (1955), the tracer flux-conductance relation becomes:

$$G_K = \frac{n' \cdot F^2}{RT} \cdot J_K \qquad (4)$$

with n' being the apparent number of positive charges of K^+ during its passage through the membrane. Single-file diffusion has been demonstrated in K^+ channels in giant axons (Hodgkin & Keynes, 1955; Bergenisich & Smith, 1984) and in frog striated muscle (Horowicz et al., 1968), and recently for the Ca^{2+}-activated K^+ channel in human red cells (Vestergaard-Bogind et al., 1985). The present findings could indicate single-file diffusion in the Ca^{2+}-activated K^+ channels of Ehrlich ascites cells. The single-channel conductance of Ca^{2+}-activated K^+ channels ranges from 20 to about 200 pS (Latorre & Miller, 1983). In the case of the Ehrlich ascites cell the findings suggesting single-filing would argue against a 'maxi channel.' Assuming, therefore, a single-channel conductance of 20 pS similar to that reported for human red cells (Grygorczyk et al., 1984) the number of activated K^+ channels in the Ehrlich ascites cell can be estimated at 7×10^6 cm^{-2}, or about 100 per cell. In human red cells (with a membrane area about 10-fold smaller than that of the Ehrlich cell) the number of K^+ channels has been estimated at 100 to 200 per cell (Lew et al., 1982), whereas Grygorczyk et al. (1984) report only a few Ca^{2+}-activated K^+ channels per cell in the main fraction of the cell population.

Conclusions

The present findings demonstrate that separate transport pathways for K^+ and Cl^- can be activated in Ehrlich cells by cell swelling in hypotonic media or by addition of the Ca^{2+}-ionophore A23187. The anion transport pathway seems to be rather unselective, in sharp contrast to the Cl^--dependent cotransport systems. The activation of the Cl^- transport pathway in hypotonic media is transient which may reflect a transient increase in cytosolic free Ca^{2+} concentration, as suggested by the marked difference in the time dependence of the ionophore A23187-induced response in the presence and absence of external Ca^{2+}. Calmodulin appears to be involved in the activation of both the K^+ and the Cl^- transport pathway. The volume- or ionophore A23187-induced K^+ transport seems to be mediated via the Ca^{2+}-activated K^+ channels reported in red cells and in several other cell types (*see* review by Schwartz & Passow, 1983), including Ehrlich ascites cells (Valdeolmillos et al., 1982). There is evidence of single-file behavior of the K^+ channels in Ehrlich cells similar to that recently demonstrated in red cells (Vestergaard-Bogind et al., 1985). The nature of the volume- or A23187 plus Ca^{2+}-induced anion transport system and its possible relation to other specific anion transport systems, e.g., the volume-dependent, potential-gated anion channels in epithelial cell basolateral membranes (*see* Ussing, 1986), remains to be explored.

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