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Volume-Induced Increase of K^+ and Cl^- Permeabilities in Ehrlich Ascites Tumor Cells. Role of Internal Ca^{2+}

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Summary. Ehrlich ascites tumor cells resuspended in hypotonic medium initially swell as nearly perfect osmometers, but subsequently recover their volume within 5 to 10 min with an associated KCl loss. 1. The regulatory volume decrease was unaffected when nitrate was substituted for Cl^-, and was insensitive to bumetanide and DIDS. 2. Quinine, an inhibitor of the Ca^{2+}-activated K^+ pathway, blocked the volume recovery. 3. The hypotonic response was augmented by addition of the Ca^{2+} ionophore A23187 in the presence of external Ca^{2+}, and also by a sudden increase in external Ca^{2+}. The volume response was accelerated at alkaline pH. 4. The anti-calmodulin drugs trifluoperazine, pimozide, flupentixol, and chlorpromazine blocked the volume response. 5. Depletion of intracellular Ca^{2+} stores inhibited the regulatory volume decrease. 6. Consistent with the low conductive Cl^- permeability of the cell membrane there was no change in cell volume or Cl^- content when the K^+ permeability was increased with valinomycin in isotonic medium. In contrast, addition of the Ca^{2+} ionophore A23187 in isotonic medium promoted Cl^- loss and cell shrinkage. During regulatory volume decrease valinomycin accelerated the net loss of KCl, indicating that the conductive Cl^- permeability was increased in parallel with and even more than the K^+ permeability. It is proposed that separate conductive K^+ and Cl^- channels are activated during regulatory volume decrease by release of Ca^{2+} from internal stores, and that the effect is mediated by calmodulin.

Key Words volume regulation · regulatory volume decrease · Ca^{2+}-dependent K^+ channel · Cl^- channel · Ca^{2+} ionophore A23187 · quinine · calmodulin · Ehrlich mouse ascites tumor cells

Introduction

A regulatory volume decrease in hypotonic media associated with a substantial net loss of KCl has been reported in Ehrlich ascites cells (Hendil & Hoffmann, 1974; Hoffmann, 1978) and in several other cell types investigated (for review *see* MacKnight & Leaf, 1977; Rorive & Gilles, 1979; Kregenow, 1981; Cala, 1983; Hoffmann, 1983). In addition to the KCl loss a selective loss of certain amino acids and taurine has been demonstrated during regulatory volume decrease in Ehrlich ascites cells (Hoffmann, 1980; Hoffmann & Lambert, 1983).

The K^+ loss observed during regulatory volume decrease has been demonstrated to reflect a volume-induced increase in apparent K^+ permeability of the cell membrane. This was initially shown by tracer flux measurements in duck red cells (Kregenow, 1971), frog oocytes (Sigler & Janácek, 1971), human red cells (Poznansky & Solomon, 1972), mouse lymphoblasts (Roti-Roti & Rothstein, 1973), Ehrlich ascites cells (Hendil & Hoffmann, 1974) and dog red cells (Parker & Hoffman, 1976).

Several transport pathways have been proposed to be activated during the regulatory volume decrease. An electro-neutral K^+, Cl^- cotransport has been suggested to be involved in the hypotonic response in duck red cells (*see* Kregenow, 1981), low K^+ sheep red cells (Lauf & Theg, 1980; Dunham & Ellory, 1981), fish erythrocytes (Lauf, 1982) and dog red cells (Parker, 1983). In those cell types the regulatory volume decrease was found to be Cl^--dependent and sensitive to furosemide or bumetanide which inhibit cotransport systems. A Cl^--dependent, furosemide-sensitive cotransport system which may play a role in cell volume regulation has also been reported in human red cells (Dunham, Stewart & Ellory, 1980; Chipperfield, 1980, 1981). Anion-dependent cation transport in erythrocytes has recently been reviewed by Ellory, Dunham, Logue and Stewart (1982).

A K^+/H^+ exchange functionally coupled to a Cl^-/HCO_3^- (or Cl^-/OH^-) exchange has been proposed as a model to account for the net loss of KCl observed during regulatory volume decrease in *Amphiuma* red cells (Cala, 1980; Kregenow, 1981).

The Ca^{2+}-dependent K^+ transport pathway

which has been demonstrated in a number of cell types (*see* review by Lew & Ferreira, 1978) has recently been demonstrated in Ehrlich ascites cells (Valdeolmillos, Garcia-Sancho & Herreros, 1982), and a role in cell volume regulation has recently been established in human lymphocytes by Grinstein, DuPre and Rothstein (1982).

The conductive Cl^- permeability in Ehrlich cells is low and of a magnitude similar to the K^+ and Na^+ permeabilities (Heinz, Geck & Pietrzyk, 1975; Simonsen, Hoffmann & Sjøholm, 1976; Hoffmann, Simonsen & Sjøholm, 1979). This means that an increase in conductive K^+ permeability alone cannot account for the observed net loss of KCl during regulatory volume decrease. An increase in conductive Cl^- permeability during regulatory volume decrease has previously been suggested, based on the low Cl^- conductance under steady-state conditions and on the finding of increased unidirectional Cl^- fluxes in swollen cells (Hoffmann, 1978; 1982). In human lymphocytes a Ca^{2+}-dependent activation of a separate Cl^- conductance pathway has recently been demonstrated to be an essential part of the mechanism of the regulatory volume decrease (Grinstein, Clarke & Rothstein, 1982; Grinstein, Clarke, DuPre & Rothstein, 1982).

The results reported in the present study of the regulatory volume decrease in Ehrlich ascites cells are consistent with a Ca^{2+}-dependent activation of separate conductive K^+ and Cl^- transport pathways induced by cell swelling. The findings suggest a release of Ca^{2+} from intracellular stores during the hypotonic volume response, and indicate that calmodulin may be involved in the Ca^{2+}-dependent increase in K^+ and Cl^- conductances. Preliminary results of this study have previously been presented (Hoffmann, 1982).

Materials and Methods

Cell Suspensions and Incubation Media

Ehrlich mouse ascites tumor cells (hyperdiploid strain) were maintained in white Theiler mice by weekly intraperitoneal transplantation and harvested 8 days after transplantation. The cells were washed twice by centrifugation (45 sec, $700 \times g$). 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. In the experiments at pH 8.2 TRICINE (N-tris (hydroxymethyl) methyl glycine) 10 mM was used as buffer.

Hypotonic 225 and 150 mOsm incubation media were prepared by diluting the standard incubation medium with distilled water containing the buffer alone. In nitrate media $NaNO_3$ was substituted for NaCl in equimolar amounts.

Reagents

All reagents were analytical grade. Quinine hydrochloride, valinomycin and the ionophore A23187 were obtained from Sigma, St. Louis, Mo. Valinomycin and A23187 were added to the cell suspension from a concentrated stock solution in ethanol. Pimozide, *cis*-flupentixol, *trans*-flupentixol, trifluoperazine and chlorpromazine were gifts from Lundbeck & Co., Copenhagen. Pimozide was added from a 10 mM stock solution in ethanol, and the other drugs from 10 mM stock solutions in distilled water. Bumetanide was a gift from Dr. Feit, Leo Pharmaceutical Products, Copenhagen. The drug was dissolved in the standard incubation medium at a concentration of 2.5 mM by addition of dilute NaOH and the pH was subsequently adjusted 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.

Cellular and Extracellular Ion Concentrations

These concentrations were measured as previously described (Hoffmann, Sjøholm & Simonsen, 1983). Briefly, duplicate samples of the cell suspension were centrifuged and the packed cells lysed in distilled water and deproteinized with perchloric acid. Cell water was determined by drying of a parallel sample. The cellular water and ion contents are corrected for trapped volume ([^{3}H]inulin space) in the cell pellets. K^+ and Na^+ were determined by emission flame photometry and Cl^- by coulometric titration.

Cell Volume Measurements

Cell volume measurements were obtained using a Coulter Counter model Z^B with a Coulter Channellyzer (c-1000) and a recorder (HR 2000). Orifice diameter was 100 μm to ensure linearity between cell volume and counter pulse height. An aliquot of the cell suspension was diluted to 50,000 cells per ml with medium filtered 3 times (0.45 μm Millipore filter). The mean cell volume (arbitrary units) was calculated as the median of the distribution curves. Absolute cell volumes were obtained using polystyrene latex beads of 19.0 and 13.5 μm diameter as standards. Control experiments with these polystyrene latex beads showed that the instrument readings were nearly independent of the ionic concentration of the media within the range used here. The pulse height was increased by about 3% in the 150 mOsm incubation medium; this small deviation was ignored. For calculation of volume recovery during regulatory volume decrease in hypotonic media *see* legend to Fig. 3.

Statistical Evaluation

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

Results

In hypotonic media Ehrlich cells swell initially as nearly perfect osmometers but subsequently regulate their volume (regulatory volume decrease) re-

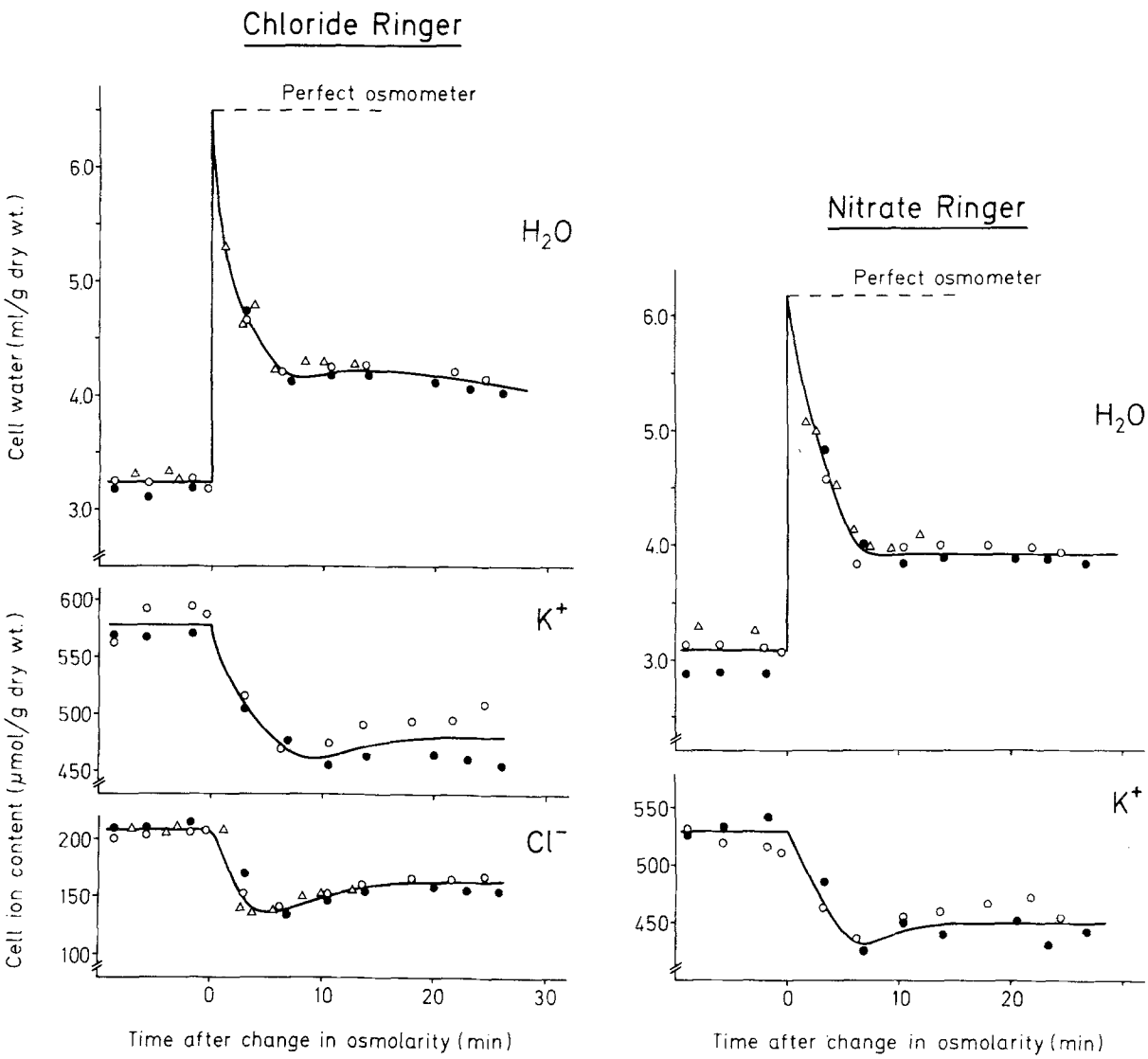

Fig. 1. Regulatory volume decrease in Ehrlich ascites cells after reduction of external osmolarity. The cells were pre-incubated in 300 mOsm chloride or nitrate medium for 40 to 50 min, and at zero time resuspended in 150 mOsm chloride medium (left frame) or nitrate medium (right frame). Cell water, K^+ and Cl^- content were followed with time after the reduction of external osmolarity. The cell water content calculated for a perfect osmometer is indicated by the broken line. The Figure shows measurements from three independent expts. (o, •, △), each including parallel groups in chloride and nitrate media. The results shown for cells in chloride medium are representative for a total of 11 expts. giving similar results. The K^+ data for one expt. (△) are not shown. The response was similar but the values were slightly higher

sulting in a new steady state being attained within 5 to 10 min with a cell volume only slightly above the original volume (Hendil & Hoffmann, 1974). The volume recovery in hypotonic medium can be seen in Fig. 1 (left frame) which shows the cell water content plotted against time after a decrease in external osmolarity. A similar volume recovery can be seen in experiments monitoring the cell volume using a Coulter counter (*see* Figs. 3, 4, 6, 7 and 9; control). The original cell volume was 938 ± 26.3 μm^3 ($n=12$), and the maximal volume measured after about 1 min was 1656 ± 51.6 μm^3 ($n=12$), close to the value calculated for a perfect osmometer (1701 ± 48.1 μm^3, $n=12$) (for details *see* legend to Figs. 3 and 9). From these values and the cell water content Ponder's R (Ponder, 1948) can be estimated at 0.972. After 5 min the cell volume was decreased to 1331 ± 37 μm^3 ($n=13$), corresponding to about 50% volume recovery ($49 \pm 2.4\%$ ($n=13$) of the initial swelling, measured with the perfect osmometer value as reference). The volume recovery is accompanied by a parallel net loss of KCl (*see* Hendil & Hoffmann, 1974). This is seen in the experiment shown in the left frame of Fig. 1 in which Cl^- is the predominant anion. The right frame shows that a similar re-

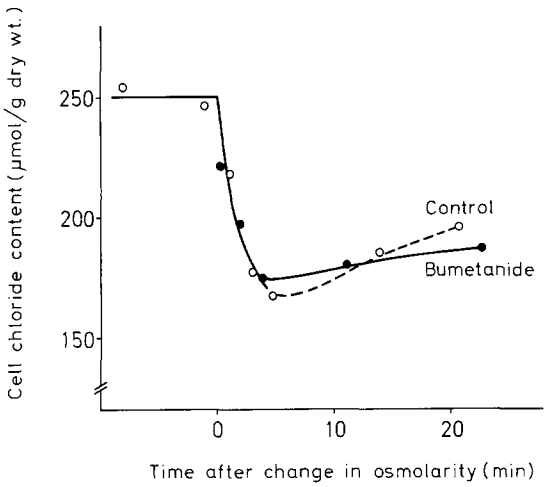

Fig. 2. Regulatory volume decrease in Ehrlich ascites cells in the presence and absence of bumetanide. Exptl. protocol as in Fig. 1. After pre-incubation in 300 mOsm chloride medium the cells were resuspended in 225 mOsm chloride medium in the presence (•) or absence (o) of bumetanide, 25 μM. Only the cell chloride content is shown on the Figure. The expt. is representative of three similar ones

sponse could be observed following replacement of cellular and extracellular Cl^- by nitrate (*see also* Fig. 4). Thus, the net loss of K^+ is independent of whether the accompanying anion is Cl^- or nitrate. Figure 2 shows that the net Cl^- loss during regulatory volume decrease was unaffected by the presence of bumetanide which inhibits the anion-cation cotransport previously reported in Ehrlich cells during regulatory volume increase (Hoffmann et al., 1983). The regulatory volume decrease in hypotonic medium was also found to be unaffected by DIDS (*see* Fig. 3) which inhibits anion exchange in Ehrlich cells (Levinson, 1978; Sjøholm & Hoffmann, 1984).

The K^+ permeability is substantially increased during regulatory volume decrease (Hendil & Hoffmann, 1974; Hoffmann, 1978) suggesting an activation of K^+ channels. As seen in Fig. 4 the volume recovery in hypotonic medium was inhibited by quinine and quinidine which inhibit the Ca^{2+}-dependent K^+ channel in red cells (*see* Lew

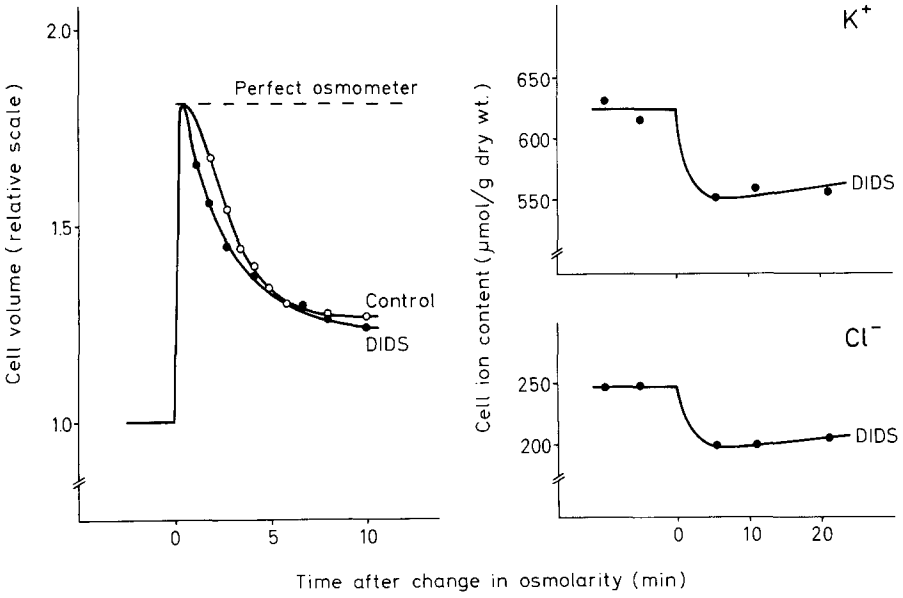

Fig. 3. Regulatory volume decrease in Ehrlich ascites cells in the presence and absence of DIDS. Left panel: After pre-incubation in 300 mOsm incubation medium for 15 min a sample of the cell suspension was diluted 1000 to 1500-fold with hypotonic (150 mOsm) medium, and the cell volume followed with time using a Coulter counter. In the experimental group DIDS, 100 μM (•) was present both during pre-incubation and during the regulatory volume decrease. The cell volumes are given relative to those measured for a parallel sample diluted in 300 mOsm medium. The perfect osmometer value (indicated by the broken line) is 1.81 × the original cell volume, calculated based on a cell water content of 3.24 ml/g dry wt (*see* Fig. 1), and a cell density of 1.06 g/ml, corresponding to 0.81 ml cell water/ml cells. The volume recovery after 5 min was calculated as: $(V_{max}-V_5)/(V_{max}-V_o)$, where V_{max} is the perfect osmometer value, V_o the initial cell volume, and V_5 the cell volume measured 5 min after the change in osmolarity. The expt. shown in the right panel demonstrates the net K^+ and Cl^- loss during regulatory volume decrease in the presence of irreversibly bound DIDS. The cells were pretreated with DIDS, 20 μM for 40 min in low chloride medium (15 mM Cl^-). After the DIDS treatment the cells were washed and resuspended in standard incubation medium (150 mM Cl^-) and pre-incubated for 25 min before the reduction of external osmolarity. For exptl. protocol *see* Fig. 1

& Ferreira, 1978) and in Ehrlich cells (Valdeolmillos et al., 1982). In the present experiments the external K^+ concentration was 4 mM which may have reduced quinine inhibition (*see* Reichstein & Rothstein, 1981).

Consistent with the low conductive Cl^- permeability in Ehrlich cells (Heinz et al., 1975; Simonsen et al., 1976; Hoffmann et al., 1979), the valinomycin-induced net loss of cell K^+ in steady state as shown in Fig. 5 was balanced mainly by a net gain of Na^+ with only a minor net loss of cell Cl^- and a minor reduction of cell volume. Similar findings have recently been reported by Valdeolmillos et al. (1982) following propranolol-induced activation of the Ca^{2+}-dependent K^+ channel. During the regulatory volume decrease the unidirectional Cl^- fluxes are substantially increased (Hoffmann, 1978, 1982). The unidirectional ^{36}Cl efflux was increased about twofold (*data not shown*) in Ehrlich cells swollen in high K^+-Ringer's. The experiment of Fig. 6 shows that during regulatory volume decrease the volume recovery was accelerated by valinomycin. The contrast between the effect of valinomycin in steady state and during regulatory volume decrease shows that the conductive Cl^- permeability is increased during volume recovery in parallel with and even more than the K^+ permeability, resulting in the latter being rate limiting. Increasing the K^+ permeability by activation of the Ca^{2+}-dependent K^+ channel using the Ca^{2+} ionophore A23187 also accelerated the volume recovery during regulatory volume decrease (Fig. 7, left panel). A similar effect can be seen after a sudden rise in the external Ca^{2+} con-

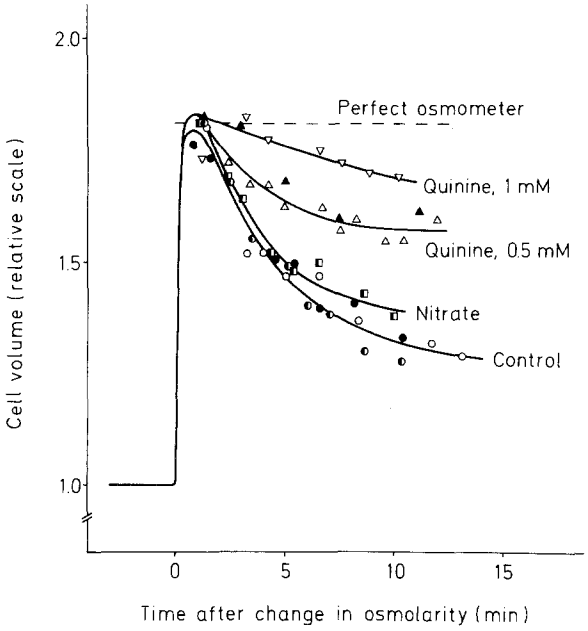

Fig. 4. Effect of quinine and anion substitution on regulatory volume decrease in Ehrlich ascites cells. The cells were preincubated in 300 mOsm chloride or nitrate medium for 40 min or more and diluted with hypotonic (150 mOsm) chloride (○, ◐, ●) or nitrate (◧) medium. The cell volume was followed with time using a Coulter counter (for details *see* legend to Fig. 3). In the expts. in the presence of quinine, 1 mM (▽) or 0.5 mM (△, ▲), the inhibitor was added to the 150 mOsm chloride medium. The Figure shows curves from three typical expts. marked individually (open, semiclosed or closed symbols, respectively). The cell volumes are given relative to those measured for a parallel sample diluted in 300 mOsm chloride or nitrate medium. Quinine in the range 0.05 to 0.25 mM showed a significant but somewhat reduced inhibition of the regulatory volume decrease. Quinidine, 1 mM, had a similar effect to quinine, 1 mM (*data not shown*)

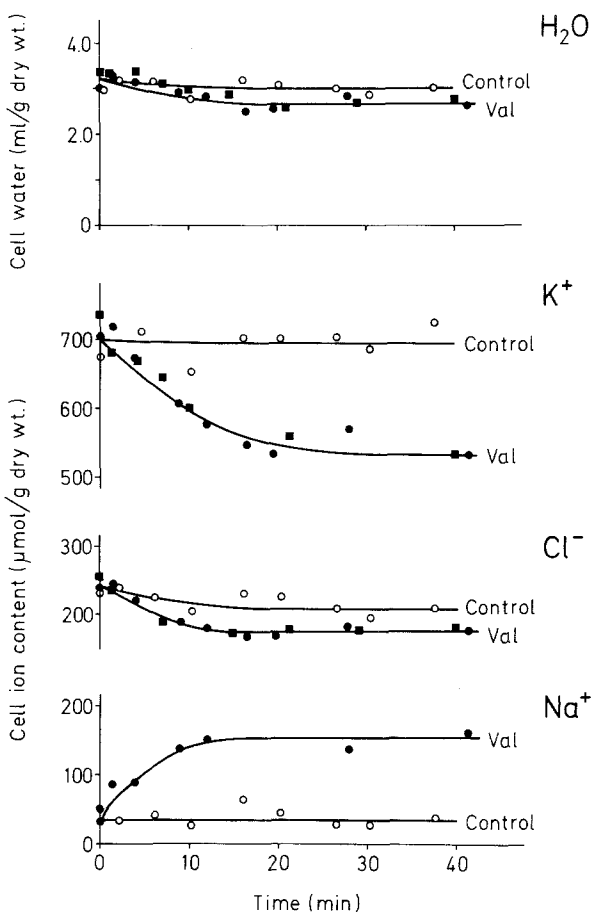

Fig. 5. Effect of valinomycin under steady-state conditions on cell water and ion content of Ehrlich ascites cells. After preincubation for 30 min the cell suspension was split into three parallel groups, with addition of valinomycin, 0.3 μM (●) or 1.5 μM (■) or without additions (control, ○), and the cell water and ion content was followed with time. The Na^+ data for the experiment with 1.5 μM valinomycin are not shown; the Na^+ uptake was similar but slightly slower. A similar net K^+ loss and Na^+ gain was observed after addition of 0.025 μM valinomycin (*data not shown*). The Figure is representative of three independent expts. each testing two valinomycin concentrations in the range 0.025to 2.3 μM

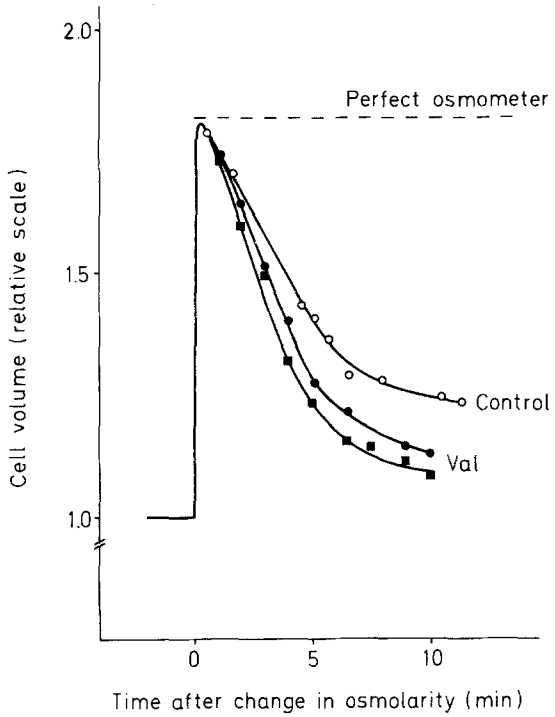

Fig. 6. Effect of valinomycin on regulatory volume decrease in Ehrlich ascites cells. Experimental protocol for control cells (o) as in Fig. 3. Valinomycin was added to the hypotonic (150 mOsm) chloride medium in a concentration of 1.6 μM at zero time (●), and in a concentration of 3 μM 1.4 min after the change in osmolarity (■). The perfect osmometer value was calculated based on a cell water content of 0.82 ml/ml cells (*see* legend to Fig. 9). The volume recovery in the presence of valinomycin is representative of six groups in three independent experiments. The volume recovery after 5 min (using the perfect osmometer value as reference) was significantly increased relative to control (1.31 ± 0.058, $n=6$). In two groups (in one expt.) no effect of valinomycin on regulatory volume decrease could be detected

centration during regulatory volume decrease (Fig. 7, right panel). In the experiments of Fig. 7, Ca^{2+} or ionophore A23187 was added near the time of maximal swelling, about 1 min after the change in osmolarity. The initial swelling was considerably reduced when the addition was made at the time of exposure to the hypotonic medium: When 1.5 to 5 μM A23187 was added at zero time the initial swelling was reduced to $35 \pm 3\%$ ($n=5$) of the initial swelling in control cells.

Addition of the ionophore A23187 to Ehrlich cells in steady state in the presence of external Ca^{2+} resulted as shown in Fig. 8 in a considerable cell shrinkage, in striking contrast to the effect of valinomycin in steady state (*see* Fig. 5). The addition of A23187 in the presence of extracellular Ca^{2+} has previously been shown in Ehrlich cells to induce a net loss of K^+ which was proposed to be balanced by uptake of Na^+, secondary to

Table 1. Net movements of water, K^+, Na^+, Cl^- across the membrane of Ehrlich ascites cells following addition of ionophore A23187[a]

	H_2O (ml/g dry wt)	K^+	Na^+	Cl^-
		μmol/g dry wt		
Control	3.42 ± 0.05[b]	611 ± 10	28 ± 4	191 ± 3
A23187	3.09 ± 0.08	531 ± 7	68 ± 4	151 ± 4
Net movement[c]	-0.33 ± 0.04	-80 ± 3	$+39 \pm 4$	-40 ± 1

[a] The cells were incubated in 300 mOsm saline with 1 mM Ca^{2+} and 0.15 mM Mg^{2+}. At zero time 7 to 15 μM ionophore A23187 was added. Cell water and electrolytes were measured before ionophore addition (control), and again between 1.0 and 1.4 min after ionophore A23187 addition.
[b] The values are given as mean ± SEM from four independent experiments.
[c] Paired analysis.

the membrane hyperpolarization induced by the increase in K^+ permeability (Valdeolmillos et al., 1982). As seen in Table 1 only about half the net loss of cell K^+ was balanced by Na^+ uptake, and a definite net loss of KCl followed by water could be demonstrated. The observed cell shrinkage (*see* Fig. 8) therefore represents a net loss of KCl, indicating a Ca^{2+}-dependent increase of Cl^- permeability in parallel with the increase in K^+ permeability. A similar cell shrinkage following addition of ionophore A23187 to Ehrlich cells under steady-state conditions was observed in Ca^{2+}-free media with 0.1 mM EGTA (*data not shown*). This effect was blocked by pimozide, 10 μM (*cf.* Table 4).

The cell shrinkage induced by ionophore A23187 was transient (*see* Fig. 8). A fast and transient net loss of cell K^+ induced by A23187 has previously been reported by Valdeolmillos et al. (1982) in Ehrlich ascites cells. Cell shrinkage has recently been proposed to activate a bumetanide-sensitive Na^+, Cl^- cotransport system with subsequent replacement of Na^+ by K^+ via the Na^+/K^+ pump (Hoffmann et al., 1983). The more persistent cell shrinkage observed in the presence of bumetanide (*see* Fig. 8) could well point to the involvement of the Na^+, Cl^- cotransport system in the transient response.

The absence of external Ca^{2+} during regulatory volume decrease had no effect on the volume recovery. After 5 min the volume recovery was 0.46 ± 0.02 ($n=20$) in the presence of 0.5 mM Ca^{2+} and 0.56 ± 0.03 ($n=8$) in Ca^{2+}-free medium (0.5 mM EGTA). This might indicate that the Ca^{2+} which is presumed to be involved in the regulatory volume decrease is released from internal stores. The experiments of Table 2 were designed to explore the effect of depletion of internal Ca^{2+}

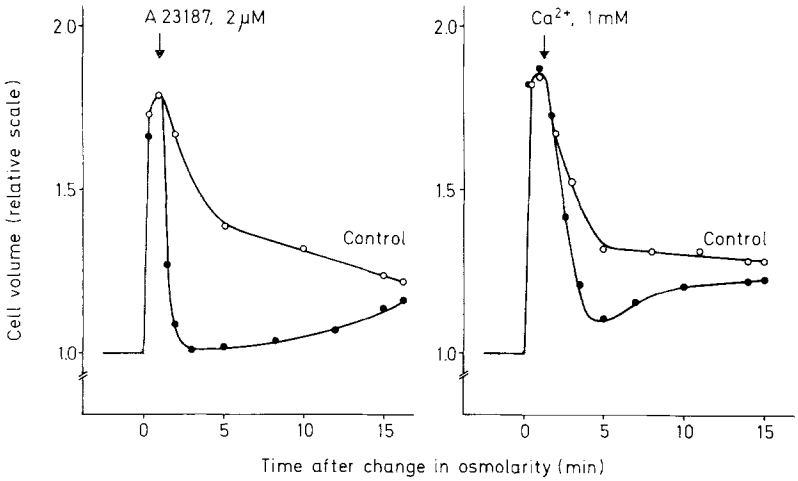

Fig. 7. Effect of the Ca^{2+} ionophore A23187 and of external Ca^{2+} on regulatory volume decrease in Ehrlich ascites cells. Experimental protocol as in Fig. 3. Left frame: The cells were pre-incubated in 300 mOsm medium with 1 mM Ca^{2+} and 0.1 mM Mg^{2+}, and transferred to 150 mOsm medium with half the Ca^{2+} and Mg^{2+} concentration. In the experimental group the ionophore A23187 (2 μM) was added to the hypotonic medium 1 min after the transfer (•). Right frame: The cells were preincubated in 300 mOsm medium with 0.1 mM EGTA and 0.15 mM Mg^{2+}, and transferred to 150 mOsm medium with half the Mg^{2+} concentration, without EGTA. In the experimental group Ca^{2+}, 1 mM was added to the hypotonic medium 1.2 min after the transfer (•). The experiments are representative of 15 experiments with addition of A23187 and four with addition of external Ca^{2+}

stores. Following pre-incubation in excess EGTA plus ionophore A23187 a slight inhibition of volume recovery was observed. Stronger inhibition was achieved by repeating the regulatory volume response in hypotonic medium, expecially when no external Ca^{2+} was present. In the latter case the second response was reduced to 57%, suggesting depletion of internal Ca^{2+} stores during the first response. During the second response in the presence of Ca^{2+}, addition of ionophore A23187 was found in a single experiment to accelerate the volume recovery similar to the effect observed during the first response (*cf.* Fig. 7).

The Ca^{2+}-induced increase in K^+ permeability has in *Amphiuma* and human red cells been found to be pH-sensitive (Lassen, Lew, Pape & Simonsen, 1977; Pape, 1982). Table 3 shows that the volume response in Ehrlich cells was also pH-dependent, being accelerated at alkaline pH and inhibited at low pH. The pH effect was of similar magnitude in the presence and absence of external Ca^+ during the hypotonic exposure (*see* Table 3).

Figures 9, 10 and Table 4 show the inhibition of the regulatory volume decrease by a number of antipsychotic drugs reported to inactivate the Ca^{2+}-binding protein calmodulin (Weiss et al., 1980). As seen in Table 4 the inhibitory effect of the anti-calmodulin drugs tested was roughly parallel to the inhibitory effect of the drugs on the calmodulin-activated phosphodiesterase reported by Weiss et al. (1980). The inhibitory effect of flu-

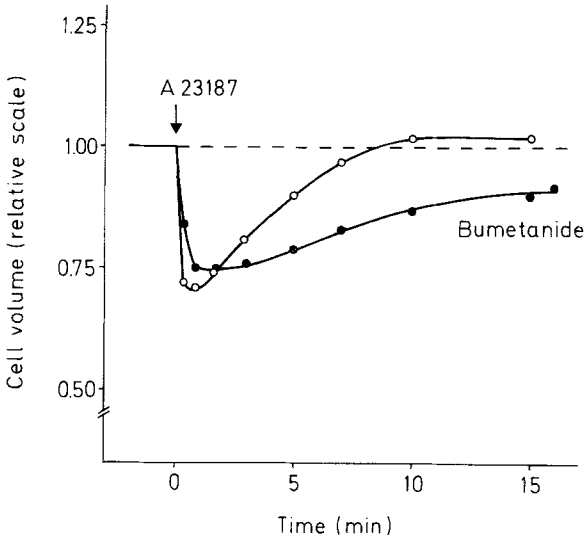

Fig. 8. Effect of the ionophore A23187 on the volume of Ehrlich ascites cells under steady-state conditions in the presence of external Ca^{2+}. After pre-incubation for 30 min in 300 mOsm saline with 1.0 mM Ca^{2+} and 0.15 mM Mg^{2+} a sample of the cell suspension was diluted 1000-fold in the same incubation medium and the cell volume recorded using a Coulter counter. At time zero the ionophore A23187, 2 μM was added to the diluted cell suspension and the cell volume followed with time. The cell volume (ordinate) is given relative to the cell volume recorded before addition of ionophore. The curve (o) is representative of five experiments, showing an initial cell shrinkage of $27 \pm 1.2\%$ ($n = 5$). In a parallel group (•) bumetanide, 50 μM was present during 10 min pre-incubation and after diluting the cell suspension in the Coulter counter. In this group the cell volume was reduced by about 5% during the 10 min pre-incubation before addition of ionophore. The curve (•) is representative of four experiments giving similar results

Table 2. Inhibitory effect of Ca^{2+} depletion on volume recovery in Ehrlich ascites cells during regulatory volume decrease in hypotonic saline[a]

Pretreatment	5-min volume recovery (relative to control)
Isotonic pretreatment in the presence of:	
Ca^{2+} (control)	1.0
EGTA plus A23187	0.84±0.05 (4)[b]
Hypotonic pretreatment in the presence of:	
Ca^{2+}	0.72±0.004 (3)
EGTA plus A23187	0.57±0.05 (7)

[a] A suspension of Ehrlich cells was split into four parallel groups and exposed to hypotonic or isotonic pretreatment in the presence of either 0.5 to 1 mM Ca^{2+} or 0.5 mM EGTA plus 2 μM A23187. Two groups were exposed to hypotonic pretreatment by incubation in 150 mM chloride medium for 15 min followed by incubation in 300 mOsm chloride medium for 15 min. The two other groups were incubated in 300 mOsm chloride medium only, but in most experiments carried through an equal number of washes.

After the hypotonic or isotonic pretreatment a sample of cell suspension was transferred to 150 mOsm chloride medium with either Ca^{2+} or EGTA as indicated, and the volume recovery monitored in a Coulter counter. The volume recovery after 5 min was read from curves similar to those of Figs. 3 and 4, using the curve peak value as reference. The values are given relative the 5-min volume recovery in the parallel group of cells pretreated in isotonic saline with Ca^{2+} (control).

[b] The values are given as mean ± SEM with the number of independent experiments in brackets.

Table 3. Effect of pH on volume recovery in Ehrlich ascites cells during regulatory volume decrease in hypotonic saline in the presence and absence of external Ca^{2+} [a]

pH	3-min volume recovery in hypotonic saline	
	with Ca^{2+}	Ca^{2+} free
6.6	0.13±0.01 (4)[b]	0.17±0.01 (3)
7.4	0.34±0.05 (4)	0.37±0.07 (3)
8.2	0.54±0.02 (4)	0.58±0.02 (3)

[a] The cells were pre-incubated in 300 mOsm saline with 1 mM Ca^{2+}, and exposed to hypotonic (150 mOsm) saline with or without Ca^{2+} (0.5 m Ca^{2+} or 0.1 mM EGTA, respectively) at the pH values indicated. The Mg^{2+} concentration in both groups was 0.15 mM during pre-incubation, and 0.075 mM during hypotonic exposure. The cell volume was monitored in a Coulter counter and the volume recovery measured after 3 min from curves similar to those of Figs. 3 and 4, using the curve peak value as reference.

[b] The values are given as mean ± SEM with the number of independent experiments in brackets.

pentixol on the regulatory volume decrease in Ehrlich cells showed, however, a definite stereospecificity (*see* Table 4), in striking contrast to the inhibitory effect on the calmodulin-activated phosphodiesterase and to the binding of flupentixol to calmodulin (Weiss et al., 1980). Pimozide also blocked

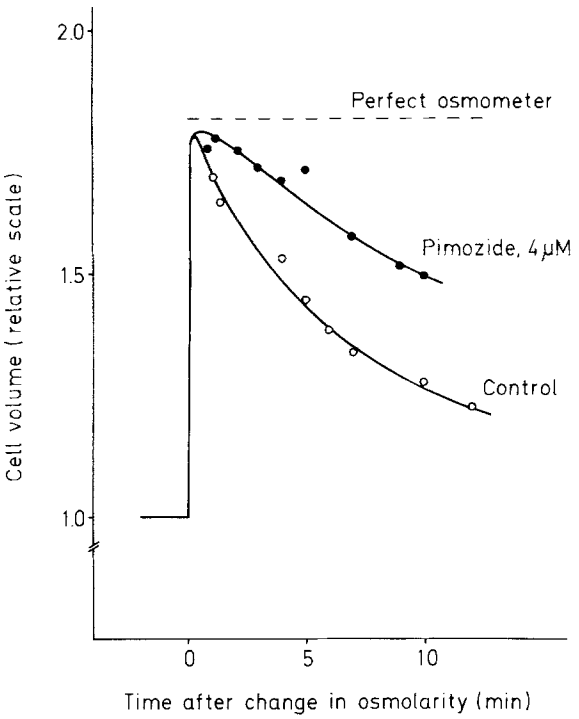

Fig. 9. Inhibition by pimozide of regulatory volume decrease in Ehrlich ascites cells. Experimental protocol for control cells (○) as in Fig. 3. In the expt. in the presence of pimozide (●) the inhibitor was added at a concentration of 4 μM to the hypotonic (150 mOsm) chloride medium. The perfect osmometer value (indicated by the broken line) is 1.82× the original cell volume, calculated based on the measured cell water content of 3.38 ml/g dry wt, corresponding to 0.82 ml/ml cells. The data for a total of six pimozide concentrations tested in two independent experiments are summarized in Fig. 10

(*data not shown*) the effect of a sudden increase of external Ca^{2+} during regulatory volume decrease (*cf.* Fig. 7).

Discussion

The regulatory volume decrease in Ehrlich ascites cells was in the present experiments found to be unaffected by substitution of nitrate for Cl^- (*see* Figs. 1 and 4), and also insensitive to bumetanide (Fig. 2). This is in sharp contrast with the Cl^- requirement and the sensitivity to cotransport inhibitors found for the anion-dependent cation transport in red cells from various species (for refs. *see* Introduction) and the finding argues against the involvement of K^+, Cl^- cotransport in the regulatory volume decrease in Ehrlich ascites cells. The insensitivity to bumetanide and the lack of anion selectivity are also in contrast to the properties of the K^+, Cl^- cotransport system reported in Ehrlich ascites cells by Aull (1981, 1982), the K^+, Na^+, 2 Cl^- cotransport system reported by Geck et al.

Table 4. Inhibitory effect of anti-calmodulin drugs on volume recovery during regulatory volume decrease in Ehrlich ascites cells, compared to the effect on calmodulin-activated phosphodiesterase

Inhibitor	*n*	IC_{50} for volume recovery in Ehrlich cells[a]	IC_{50} for calmodulin-activated phosphodiesterase[b]
Pimozide	6	3.4	7
Cis-flupentixol	8	8	25
Trans-flupentixol	4	>20[c]	15
Trifluoperazine	7	10	10
Chlorpromazine	5	40	42

[a] For each inhibitor the concentration required for 50% inhibition of the 5-min volume recovery (IC_{50}) was read from curves similar to those of Fig. 10. *n* is the number of determinations of volume recovery at varying inhibitor concentrations.
[b] Data from Weiss et al. (1980). IC_{50} gives the inhibitor concentration required for 50% inhibition.
[c] At the highest concentration tested (20 μM) the inhibition was 20 to 35%.

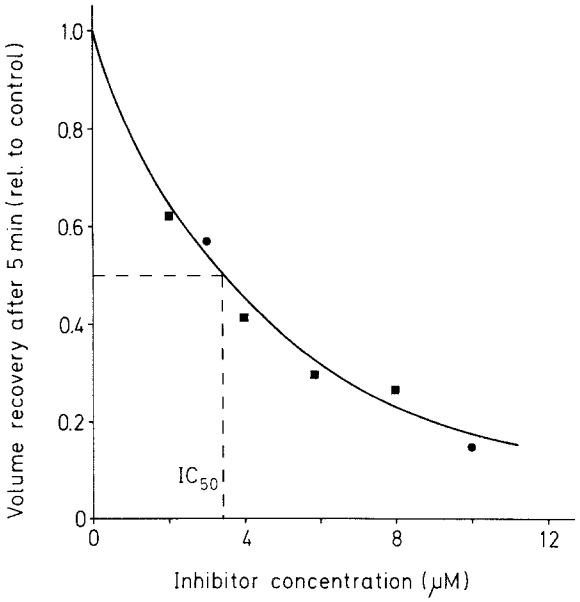

Fig. 10. Inhibitory effect of pimozide on regulatory volume decrease in Ehrlich ascites cells. The volume recovery after 5 min for the experimental group and for the control group was read from curves similar to those of Fig. 9 using the perfect osmometer value as reference. The ordinate shows the 5-min volume recovery at varying pimozide concentrations (abcissa), given relative to control. In the control group the 5-min volume recovery was about half the initial osmotic swelling (49±2% (n=13)). IC_{50} was read from the curve as the inhibitor concentration required for 50% inhibition of volume recovery. Data from two independent experiments, marked individually

(1980), and the Na^+, Cl^- cotransport system recently proposed to be involved in the regulatory volume increase in these cells (Hoffmann et al., 1983). The lack of discrimination between Cl^- and nitrate during regulatory volume decrease in Ehrlich ascites cells is, however, in line with the recent finding of a rather unselective anion pathway during regulatory volume decrease in human lymphocytes (Grinstein et al., 1982*a*).

A simultaneous operation of a K^+/H^+ exchange and a Cl^-/HCO_3^- (or Cl^-/OH^-) exchange as found in *Amphiuma* red cells (Cala, 1980; Kregenow, 1981) seems to be unlikely in Ehrlich ascites cells because the hypotonic volume response was unaffected by inhibition of anion exchange by DIDS (*see* Fig. 3), in contrast to the finding in *Amphiuma* red cells (Cala, 1980).

The inhibition of the volume recovery of Ehrlich ascites cells in hypotonic media by quinine and quinidine (Fig. 4) as well as the acceleration of the volume response by addition of ionophore A23187 in the presence of external Ca^{2+} (Fig. 7), suggest the involvement of the Ca^{2+}-dependent K^+ channel, consistent with the findings in human lymphocytes recently reported by Grinstein et al. (1982*c*). Bui and Wiley (1981), however, have reported the increase in K^+ permeability observed during regulatory volume decrease in human lymphocytes to be quinine-insensitive, and have proposed the involvement of a carrier-mediated process rather than the Ca^{2+}-dependent K^+ channel, based upon deviation of the observed K^+ flux ratio from the value predicted by the Ussing flux ratio equation. The volume-dependent K^+, Cl^- cotransport in LK sheep red cells is not Ca^{2+}-dependent and is quinine-insensitive (Ellory & Dunham, 1980).

A sudden increase in the external Ca^{2+} concentration has been reported to induce a transient increase in the K^+ permeability in *Amphiuma* and human red cells (Lassen et al., 1977; Pape, 1982). In the present experiments a sudden increase of the external Ca^{2+} concentration was found to accelerate the regulatory volume decrease (*see* Fig. 7). The volume response in hypotonic medium was inhibited at low pH and accelerated at high pH (*see* Table 3). A similar pH-dependence has been reported for the increase in K^+ permeabilty induced by external Ca^{2+} in *Amphiuma* and human red blood cells (Lassen et al., 1977; Pape, 1982), and for the Ca^{2+}-induced K^+ efflux from ATP-depleted red cells (Hoffman et al., 1980). The effect in the present experiments of a sudden increase in external Ca^{2+} and the pH-dependence add circumstantial evidence that the Ca^{2+}-dependent K^+ channel is involved in the volume recovery. The finding that the pH effect is of similar magnitude both in the presence and in the absence

of external Ca^{2+} (*see* Table 3) suggests that it reflects modulation of the Ca^{2+}-sensitivity of the Ca^{2+} dependent K^+ channel rather than changes in the passive influx of Ca^{2+}.

The finding that the volume recovery is unaffected by the absence of external Ca^{2+} but slightly inhibited following prolonged pre-incubation in Ca^{2+}-free, EGTA plus ionophore A23187 containing media (*see* Table 2) suggests the involvement of intracellular Ca^{2+} stores. Similar results have recently been reported in human lymphocytes by Grinstein et al. (1982*c*). The significant reduction of the second regulatory volume response, in particular following a first response in Ca^{2+}-free medium (*see* Table 2), also suggests a release of internal Ca^{2+} during the first period of regulatory volume decrease, followed by a reduction of the cell Ca^{2+} content via the Ca^{2+} pump. Addition of ionophore A23187 induced a net loss of KCl even in the absence of external Ca^{2+}, at variance with the findings in human lymphocytes (Grinstein et al., 1982*c*). This suggests that Ca^{2+} from internal stores can be released in Ehrlich cells by the ionophore as well as by cell swelling. The finding that a sudden increase in external Ca^{2+} concentration accelerates the regulatory volume decrease (*see* Fig. 7) indicates, however, that Ca^{2+} movements through the cell membrane in experiments where external Ca^{2+} is present should not be entirely ruled out.

The volume recovery in hypotonic media was inhibited by anti-calmodulin drugs (pimozide, cis-flupentixol, trifluoperazine and chlorpromazine) with the inhibitory potency correlating with their inhibitory effect on the calmodulin-activated phosphodiesterase (Figs. 9, 10 and Table 4). This finding suggests the involvement of calmodulin in the regulatory volume decrease. Similar findings have recently been reported by Grinstein et al. (1982*c*) for human lymphocytes, although in their experiments pimozide and chlorpromazine were far more effective as inhibitors of the regulatory volume decrease than as calmodulin antagonists. The involvement of calmodulin in the regulation of the Ca^{2+}-dependent K^+ channel in red cells has been proposed by Lassen, Pape and Vestergaard-Bogind (1980). Conflicting results have been reported, however, concerning the effect of anti-calmodulin drugs on the Ca^{2+}-dependent K^+ channel in red cells. Hoffman et al. (1980) and Lackington and Orrego (1981) reported marked inhibition of Ca^{2+}-dependent K^+ efflux, whereas Plishker, Appel, Dedman and Means (1980) reported stimulation of Ca^{2+}-dependent K^+ efflux by phenothiazines. Lew, Muallem and Seymour (1982) and Garcia-Sancho, Sanchez and Herreros (1982) reported no effect of calmodulin on Ca^{2+} activated ^{86}Rb transport in inside-out vesicles from red cells.

The finding of stereospecificity in flupentixol inhibition of the regulatory volume decrease (*see* Table 4) is in contrast to the lack of stereospecificity in the inhibitory effect on calmodulin-activated phosphodiesterase and in the binding of flupentixol to calmodulin (Weiss et al., 1980). Stereospecificity has been demonstrated, however, for the binding of flupentixol to dopamine receptors and to dopamine-stimulated adenylate cyclase (Hyttel, 1978). The finding of stereospecificity in flupentixol inhibition of the volume response in the present experiments may suggest the involvement of adenylate cyclase in the regulatory volume decrease in Ehrlich cells. Activation of adenylate cyclase resulting in release of internal Ca^{2+} and in subsequent activation of the Ca^{2+}-dependent K^+ channel has recently been proposed to be involved in the synaptic hyperpolarization in *Helix* neurons (Christoffersen & Simonsen, 1983).

Consistent with the low conductive Cl^- permeability in Ehrlich ascites cells, the addition of valinomycin under steady-state conditions failed to induce a significant net loss of KCl and cell water (*see* Fig. 5). Under similar conditions addition of valinomycin caused a marked reduction of cellular volume in human red cells (Knauf, Fuhrmann, Rothstein & Rothstein, 1977). The finding that addition of valinomycin during regulatory volume decrease accelerates the volume recovery (*see* Fig. 6) indicates that the net loss of KCl is limited by the K^+ permeability. This means that during the regulatory volume decrease the Cl^- permeability is increased in parallel with and even more than the K^+ permeability. An increase in unidirectional Cl^- fluxes in hypotonic media has recently been reported in human lymphocytes by Grinstein et al. (1982*a*, *b*).

The addition of ionophore A23187 in the presence of external Ca^{2+} during the regulatory volume increase accelerates the volume recovery (*see* Fig. 7), consistent with the involvement of the Ca^{2+}-dependent K^+ channel. Moreover, under steady-state conditions the addition of A23187 (*see* Fig. 8) induces a net loss of KCl, in contrast to the effect of valinomycin, implying that an increase in intracellular Ca^{2+} concentration increases anion permeability as well as the K^+ permeability. Evidence of an increase in anion permeability brought about by an increased intracellular Ca^{2+} concentration has also been presented for human lymphocytes (Grinstein et al., 1982*a*, *b*). The water permeability of the cell membrane of Ehrlich ascites cells

is high (Hempling, 1960). Therefore, the reduction of the initial swelling in hypotonic media, observed when ionophore A23187 plus Ca^{2+} are added at the time of exposure to hypotonic conditions points to a very substantial increase in K^+ and Cl^- permeabilities during regulatory volume decrease.

In summary the present findings suggest that the net loss of KCl during regulatory volume decrease takes place via separate K^+ and Cl^- channels which are activated by release of Ca^{2+} from internal stores. There is some evidence that the effect of Ca^{2+} is mediated by calmodulin, but the mechanism by which cell volume changes trigger internal Ca^{2+} release is unknown.

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