J. Membrane Biol. 102, 35–48 (1988)

The Journal of
Membrane Biology

Na^+/H^+ Exchange in Ehrlich Ascites Tumor Cells: Activation by Cytoplasmic Acidification and by Treatment with Cupric Sulphate

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Summary. Exposure of Ehrlich cells to isotonic Na^+-propionate medium induces a rapid cell swelling. This treatment is likely to impose an acid load on the cells. Cell swelling is absent in K^+-propionate medium but may be induced by the ionophore nigericin, which mediates K^+/H^+ exchange. Cell swelling in Na^+-propionate medium is blocked by amiloride, but an alternative pathway is introduced by addition of the ionophore monensin, which mediates Na^+/H^+ exchange. Consequently, swelling of Ehrlich cells in Na^+-propionate medium is due to the operation of an amiloride-sensitive, Na^+-specific mechanism. It is concluded that this mechanism is a Na^+/H^+ exchange system, activated by cytoplasmic acidification. We have previously demonstrated that the heavy metal salt $CuSO_4$ in micromolar concentrations inhibits regulatory volume decrease (RVD) of Ehrlich cells following hypotonic swelling. The present work shows that $CuSO_4$ inhibits RVD as a result of a net uptake of sodium, of which the major part is sensitive to amiloride. Measurements of intracellular pH show that $CuSO_4$ causes significant cytoplasmic alkalinization, which is abolished by amiloride. Concomitantly, $CuSO_4$ causes an amiloride-sensitive net proton efflux from the cells. The combined results confirm that a Na^+/H^+ exchange system exists in Ehrlich cells and demonstrate that the heavy metal salt $CuSO_4$ activates this Na^+/H^+ exchange system.

Key Words Ehrlich ascites tumor cells · pH regulation · Na^+/H^+ exchange · volume regulation · hypotonic shock · cupric sulphate · cytoplasmic acidification · phorbol ester · TPA.

Introduction

It has been demonstrated that an electroneutral Na^+-H^+ antiport system or Na^+/H^+ exchange is present in the plasma membrane of virtually all vertebrate cells and plays a central role in cell homeostasis. The Na^+/H^+ exchanger seems to be implicated in a variety of cell functions, e.g. cell proliferation and pH regulation. This has been extensively reviewed in a collection of reviews edited by Aronson and Boron (1986), and in reviews by Frelin et al., (1986), and by Grinstein & Rothstein (1986). Recently, Na^+/H^+ exchange has also been reported in Ehrlich ascites tumor cells (Doppler, Maly & Grunicke, 1986; Wiener, Dubyak & Scarpa, 1986). The Na^+/H^+ exchange seems to be silent at normal physiological pH in Ehrlich cells, but it is activated after cytoplasmic acidification (Doppler et al., 1986; Kramhøft, Lambert & Hoffmann, 1987*a*) and following treatment of the cells with ATP or with the phorbol ester, TPA (Wiener et al., 1986). With respect to the effects of cytoplasmic acidification and TPA, Ehrlich cells thus respond like all other cells in which this has been investigated.

Many cells are capable of regaining their original volume after osmotic shrinkage. This regulatory volume increase (RVI) can be mediated by two different modes. In the majority of investigated cells it has been shown that osmotic shrinkage activates Na^+/H^+ exchange, which together with the simultaneous operation of Cl^-/HCO_3^- exchange causes primarily a net uptake of NaCl and osmotic-obliged water, by which the cells regain their original volume. This is the case in, e.g., lymphocytes (Grinstein, Clarke & Rothstein, 1983), *Amphiuma* red cells (Cala, 1985; 1986), dog red blood cells (Parker, 1983) and rabbit red blood cells (Jennings, Douglas & McAndrew, 1986). For a more complete list *see* Grinstein & Rothstein (1986). Other cells, among which is the Ehrlich cell, perform RVI by a net uptake of NaCl caused by the activation of a Na^+,Cl^- cotransport system (Kregenow, 1981; Hoffmann, Sjøholm & Simonsen, 1983; Geck & Pfeiffer, 1985) For a recent review *see* Hoffmann (1987). The present report shows that cytoplasmic acidification of Ehrlich cells results in amiloride-sensitive cell swelling, indicating the activation of Na^+/H^+ exchange following decreased cytoplasmic pH. Furthermore, it is confirmed that osmotic shrinkage activates Na^+,Cl^- cotransport rather than Na^+/H^+ exchange. This shows that regulatory swelling of Ehrlich cells can be brought about by two distinct mechanisms.

The existence of inducible Na^+/H^+ exchange in Ehrlich cells has thrown light upon previously reported inhibitory effects of the heavy metal salt $CuSO_4$ on regulatory volume decrease (RVD) in hypotonically swollen Ehrlich cells (Lambert, Kramhøft & Hoffmann, 1984). RVD by Ehrlich cells is due to the activation of separate, Ca^{2+}-sensitive, K^+ and Cl^--channels whereby the cells lose KCl and water (Hoffmann, Lambert & Simonsen, 1986). The Na^+ permeability was reported not to be affected (Hoffmann, 1978). Impairment of RVD must reflect either an inhibition of K^+ and/or Cl^- channels or an increased permeability of the cells to Na^+. A reduction of the Cl^- permeability by copper has been observed in warm-adapted frogs by Koefoed-Johnsen and Ussing (1973), whereas cold adapted frogs showed an increased Na^+ permeability in the presence of copper. In our previous work it was shown that inhibition of RVD by $CuSO_4$ required the presence of extracellular Na^+ (Lambert et al., 1984), and we suggested that an increased Na^+ permeability was involved in the inhibition of RVD. The present study provides evidence that the primary effect of $CuSO_4$ is an activation of the Na^+/H^+ exchange system in Ehrlich cells. When Na^+/H^+ exchange is operating, a net influx of Na^+ occurs, which is responsible for the impairment of RVD in the presence of $CuSO_4$. Preliminary results of this study have previously been published (Kramhøft, Lambert & Hoffmann, 1987*a,b*).

Materials and Methods

Cell Suspensions and Incubation Media

Ehrlich ascites tumor cells (hyperdiploid strain), maintained by serial intraperitoneal transplantation in white Theiller mice, were harvested 7–8 days after transplantation in standard incubation medium containing heparin (2.5 IU/ml). The cells were then washed twice (700 × *g*, 45 sec) with the same medium without heparin and incubated at a cytocrit of 4–8% for about 30 min prior to experimental treatment. The composition of standard incubation medium was (mM): NaCl, 150; KCl, 5; $MgSO_4$, 1; $CaCl_2$, 1; Na_2HPO_4, 1; MOPS, 3.3; TES, 3.3; and HEPES, 3.3. The pH was adjusted to 7.4 or 7.0 (*see below*). Hypotonic, half-strength medium, was prepared by dilution of standard medium with distilled water containing buffers alone. In propionate media 150 mM Na^+ or K^+-propionate was substituted for NaCl. The pH of the propionate media was adjusted to 6.6. When the effect of bumetanide was studied, $CaCl_2$ was omitted from the medium in order to avoid precipitation of bumetanide. KCl media were prepared by isosmotic substitution of KCl for NaCl. Nominally unbuffered medium was standard incubation medium without MOPS, TES and HEPES. Media used for measurements of cell volume in a Coulter counter (*see below*) were filtered through Millipore filters (0.45 μm) prior to experiments. Hypoosmotic conditions were established either by dilution with the appropriate hypotonic solution or by centrifugation (700 × *g*, 45 sec) of isotonic cell suspensions (cytocrit 4–8%) followed by resuspension in hypotonic medium. In all experiments with amiloride the cells were pretreated with amiloride for about 1 min prior to transfer to the experimental medium containing amiloride. Temperature was kept at 37°C throughout experiments except when otherwise indicated.

Cell Volume Measurements

Cell volumes were measured on cultures (cytocrit 4%), diluted 200–300 times with experimental incubation media, using a Coulter counter model ZB equipped with a Coulter channellyzer (C-1000). The mean cell volume (in fl, i.e., 10^{-15} liter) was calculated as the median from volume distribution curves after calibration with latex beads (12.9 μm, Coulter Electronics, England). Tube orifice was 100 μm.

Measurements of Ion Content

The cellular contents of Na^+ and K^+ were measured in cells from 1 ml cell suspension (cytocrit 5–8%), separated from the incubation medium by centrifugation (1 min, 20,000 × *g*), lysed in distilled water and deproteinized as previously described (Hoffmann et al., 1983). Na^+ and K^+ were measured by atomic absorption flame photometry using a Perkin Elmer atomic absorption spectrophotometer model 2380. Cellular ion content was corrected for trapped extracellular medium, defined as ^{3}H-inulin space, as described previously (Hoffmann et al., 1983).

Measurement of Intracellular pH

General Outline

Cell suspensions (3–4 ml, cytocrit 4%) were incubated with the acetoxymethylester of a fluorescent probe (BCECF-AM), final concentration 3.6 μM, for about 30 min at 37°C. One ml of this suspension was then washed twice in isotonic medium containing bovine serum albumin (BSA, 1 mg/ml) in order to remove excess probe, resuspended in 0.5 ml BSA medium, and subsequently incubated at 25°C. The fluorescence was recorded after 50-fold dilution of the cell suspension in a thermostated (25°C) and magnetically stirred cuvette (2 ml) using a Perkin Elmer LS-5 luminiscence spectrometer with excitation at 495 nm (slitwidth, 5 nm) and emission at 525 nm (slitwidth, 5 nm). The fluorimeter was connected to a recorder (Perkin Elmer R 100 A). A temperature of 25°C was chosen in order to minimize leakage of the intracellularily trapped BCECF from the cells. The degree of BCECF leakage was assessed in separate experiments, in which the BSA-washed cells were kept thermostated at 25, 30 and 37°C, respectively. At appropriate time points 40 μl of the cell suspension were transferred to 2 ml medium placed in the cuvette. The fluorescence was quickly read and the cells rapidly removed by centrifugation. The fluorescence remaining in the supernatant was then recorded. It was found that the amount of fluorescent probe in the supernatant increased in a linear fashion with time after wash with BSA medium. Furthermore, the degree of leakage was dependent on the incubation temperature as shown in Fig. 1, upper panel. Figure 1, lower panel, shows the rate of BCECF leakage as a function of the incubation temperature,

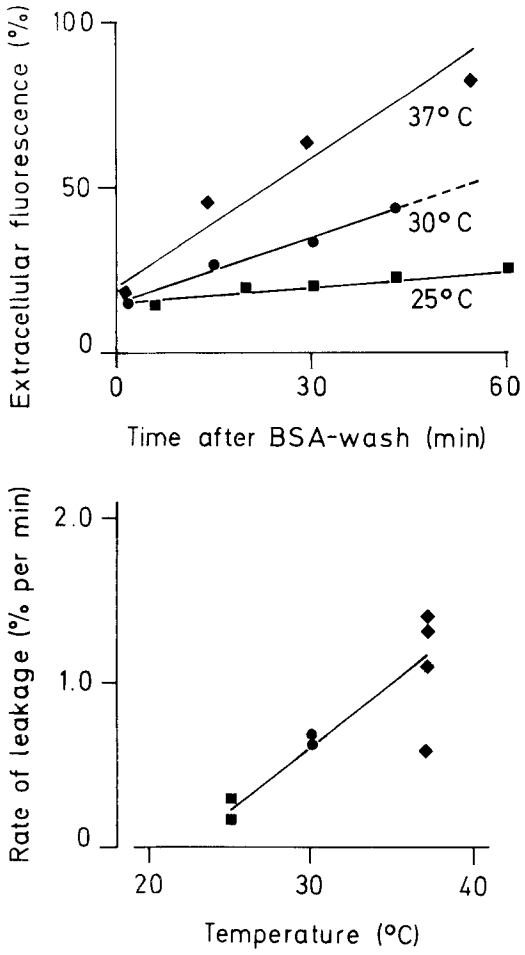

Fig. 1. (*Upper panel*) Leakage of BCECF from the cells as a function of time. Cells were loaded with BCECF-AM for 30 min. Excess probe was removed by centrifugation and the cells incubated at the temperatures indicated (*see* Materials and Methods). At appropriate time points after removal of the probe, cells were transferred to a thermostated cuvette. Total fluorescence and fluorescence remaining in the cuvette after removal of the cells (extracellular fluorescence) were measured. The figure shows the amount of extracellular BCECF as a function of time after the BSA wash at 25°C (■), 30°C (●) and 37° (◆), respectively. (*Lower panel*) Rate of BCECF-leakage as a function of the incubation temperature. The rate of leakage was calculated as the slopes of plots like the ones shown in the upper panel. The figure represents two experiments at 25°C and 30°C, respectively, and four experiments at 37°C. Symbols are as above

calculated from curves like the ones shown in Fig. 1, upper panel. The rate of leakage is given as per cent increase per min of extracellular fluorescence as a function of incubation temperature. It was concluded that the rate of leakage was acceptable (0.2%/min) at an incubation temperature of 25°C. At this temperature total extracellular fluorescence increased from about 18% at time zero (*see below*) to about 27% of the total fluorescence during 45 min. Changes in ionic gradients due to the temperature shift are negligible (Hoffmann, Simonsen & Sjøholm, 1979). At all three temperatures it was found that immediately after the BSA wash, 17.7 ± 2.6% (±SEM, $n = 8$), of 2, 2 and 4 experiments at 25, 30 and 37°C, respectively, of the total fluorescence was extracellular. Extracellular fluorescence was not corrected for in the present study. It should be noted that acetoxymethylesters release formaldehyde, which decrease the cellular ATP level (Tiffert, Garcia-Sancho & Lew, 1984). Thus, formaldehyde poisoning of the cells might present a serious problem. On the other hand, according to Tiffert and coworkers (Tiffert et al., 1984) red blood cells may be used for 3–6 hr after having been loaded with the acetoxymethyl ester of a Ca^{2+} indicator without meaningful ATP depletion. Under the present experimental conditions the cells were only incubated with acetoxymethylester for about 30 min. Significant adverse effects arising from ester hydrolysis are, therefore, not likely.

Calibration

Calibration was carried out after each pair of experiments according to the method of Thomas et al. (1979) in KCl media (KCl substituted for NaCl) of the same osmolarity and pH as that of the experimental solutions. After addition of the K^+/H^+ ionophore nigericin (5 μM), which renders intracellular pH equal to extracellular pH, pH was manipulated by addition of small amounts of saturated solutions of Tris (increase in pH) or TES (decrease in pH). A calibration curve was obtained by simultaneous recordings of pH in the cuvette with a combined electrode (Ingold, Switzerland) and fluorescence. $CuSO_4$ had no significant influence on the calibration curves (Students' t test, four independent paired experiments). Calibration of amiloride experiments was always carried out in the presence of amiloride, because amiloride was found to reduce the fluorescence by about 10%.

Comparison with Other Methods

A comparison of intracellular pH values (pH_i) measured in the present study with previous measurements on Ehrlich cells using different techniques gave the following results: At an extracellular pH (pH_o) of 7.0 we found a pH_i of 7.12 ± 0.02 (±SEM, $n = 14$), compared to 7.1 found by Gillies et al. (1982) and Bowen and Levinson (1984). At pH_o 7.4 the present study gave $pH_i = 7.34 \pm 0.02$ (±SEM, $n = 9$) compared to 7.39 and 7.36 reported by Gillies et al. (1982) and Bowen and Levinson (1984), respectively. Thus, excellent agreement exists between the pH_i values obtained with the present technique and the techniques applied in previous studies of pH_i in Ehrlich cells.

Measurements of Extracellular pH

Changes in extracellular pH was followed using a standard pH meter (PHM 82, Radiometer, Denmark) equipped with a combined electrode (Radiometer, Denmark). Immediately prior to the pH measurements 10 ml cell suspension (cytocrit 8%) was centrifuged (45 sec, 700 × g) and subsequently resuspended in nominally buffer free standard incubation medium. The pH was kept around 7.0 by manual addition of known amounts of KOH. The amount of H^+ expelled by the cells was calculated from the added amount of KOH and corrected for buffering capacity of the medium using a titration curve made in cell-free medium. After each experimental run, samples were removed for the determination of cell dry weight.

$CuSO_4$—Experiments

At pH 7.4 cupric salts precipitate as $Cu(OH)_2$. Therefore, a pH of 7.0 was chosen for the experiments with $CuSO_4$ as previously described (Lambert et al., 1984). Cells to be treated with $CuSO_4$ were equilibrated at pH 7.0 for 30 min prior to experimental treatment. The concentrations of $CuSO_4$ given in this paper are the concentration of $CuSO_4$ added to the cell suspension. From standard curves in standard medium and in unbuffered $NaNO_3$ solutions (pH 6.0) with the same ionic strength it was previously found (Lambert et al., 1984) that only about half the amount of the added $CuSO_4$ could be found in the medium after removal of the cells and that only 1–3% of the total amount of measured copper existed as free cupric ions. Copper is taken up by mammalian cells (Schmitt et al., 1983). It may, however, be calculated from their data that this uptake will not cause a measurable reduction in the extracellular copper concentration. The disappearance of $CuSO_4$ from the medium, therefore, probably reflects unspecific adsorption of $CuSO_4$ to the cell coat (glycocalyx), a labile structure covering the surface of Ehrlich cells (Rittenhouse, Rittenhouse & Takemoto, 1978).

Reagents

All reagents were analytical grade. Quinine, amiloride, monensin, nigericin and TPA were from Sigma (St. Louis, Mo.). Quinine (1 M), monensin (1 mg/ml), nigericin (1 mg/ml) and TPA (0.2 mM) were dissolved in ethanol and kept as stock solutions at -20°C. Amiloride (10 mM) was dissolved in water and kept frozen. Bumetanide, kindly donated by P.W. Feit, Leo Pharmaceuticals, Ballerup, Denmark, and pimozide, a gift from Lundbeck and Co., Copenhagen, Denmark, were dissolved in ethanol at a concentration of 5 and 1 mM, respectively, and kept at -20°C. ^{3}H-inulin was from Amersham or New England Nuclear, both England. BCECF-AM was purchased at Molecular Probes, Junction City, Or., and dissolved in dry DMSO at a concentration of 1 mg/ml. Stocks were kept in 25–30 μl portions at -20°C.

Miscellaneous

Except when otherwise indicated all figures are representative of at least three separately conducted experiments.

List of Abbreviations

MOPS:	3-(N-morpholino)propanesulfonic acid
TES:	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
HEPES:	N - 2 - hydroxyethylpiperazine - N' - 2 - ethane - sulfonic acid
BCECF-AM:	2',7' - bis - (2 - carboxyethyl) - 5 (and - 6)carboxy - fluorescein, tetraacetoxymethyl ester
BCECF:	free acid of BCECF-AM
DIDS:	4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid
DMSO:	dimethylsulfoxide
BSA:	bovine serum albumine, fraction V
Tris:	tris(hydroxymethyl)aminomethane
TPA:	12-O-tetradecanoylphorbol 13-acetate

Results

Effect of Cytoplasmic Acidification

Amiloride-Sensitive Cell Swelling

The operation of the Na^+/H^+ exchanger can be explored by monitoring the pH_i recovery following an acute acid load. This is often done by monitoring changes in cell volume after exposure of the cells to a weak organic acid—a technique introduced and described in detail by Grinstein and coworkers (Grinstein et al., 1984*b*).

Cytoplasmic acidification by propionic acid induces Na^+-dependent and amiloride-sensitive cell swelling in Ehrlich ascites tumor cells as shown in Fig. 2, left panel. It is seen that in Na^+-propionate medium (control) the cells swell rapidly during the first 5 min, whereafter the swelling ceases. Amiloride, which is a specific blocker of Na^+/H^+ exchange (Benos, 1982), completely blocks cell swelling in Na^+-propionate medium at a concentration of 0.1 mM. Figure 2, right panel (lower part) shows that the inhibition of Na^+-propionate induced cell swelling by amiloride is bypassed upon addition of monensin. Monensin is an ionophore which promotes electroneutral exchange of H^+ for Na^+. Only a slight increase in cell volume is seen in cells tranferred to isotonic K^+-propionate medium (Fig. 2, left panel). Figure 2, right panel (upper part) shows that in K^+-propionate medium considerable cell swelling is provoked after addition of the K^+/H^+ ionophore nigericin. Thus, cells transferred to propionate media swell when the appropriate electroneutral cation/H^+ exchange system is introduced. By analogy with the results reported for lymphocytes and platelets (Grinstein et al., 1984*b*; Grinstein & Furuya, 1986), the results shown in Fig. 2 suggest that a Na^+ dependent and amiloride-sensitive mechanism, i.e., a Na^+/H^+ exchanger, is responsible for the acid-induced swelling of Ehrlich cells.

It has been reported for thymocytes that the activity of the Na^+/H^+ exchange system increases with decreasing intracellular pH (pH_i) but is, at the same time, inhibited as extracellular pH (pH_o) decreases because H^+ competes with Na^+ at the external site of the Na^+/H^+ exchanger (Grinstein et al., 1984*b*). In experiments like the ones shown in Fig. 2, pH_i is dependent on the amount of free propionic acid in the medium, which according to the Hasselbalch-Henderson relationship for a weak acid in turn is dependent on the pH_o. Consequently, an optimal pH_o for acid loading is expected. The

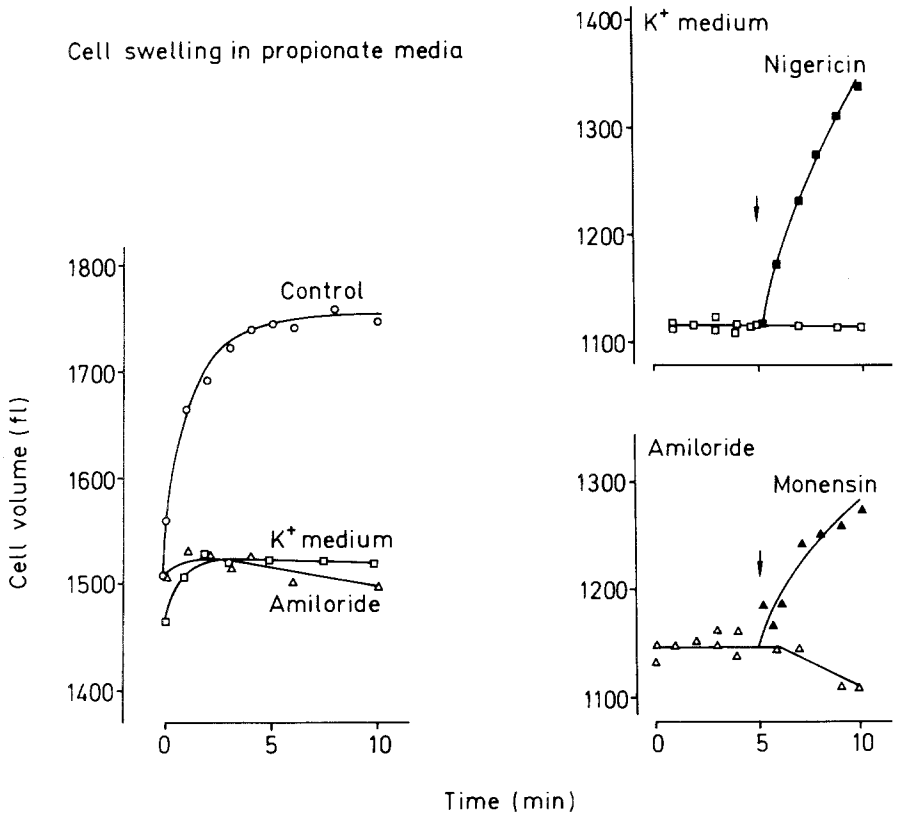

Fig. 2. Swelling of Ehrlich ascites tumor cells as a function of time after cytoplasmic acidification in propionate media. Cells suspended in isotonic standard incubation medium were diluted 200- to 300-fold in isotonic propionate medium (pH 6.6) at zero time. *Left panel* shows the cell swelling in Na^+-medium without (○) or with (△) 0.1 mM amiloride and in K^+-medium (□). *Right panel, upper part* shows that the inhibition of cell swelling in K^+-medium (□) can be overcome by addition of the K^+/H^+-ionophore nigericin (1 μM, ■). *Right panel, lower part* shows that the inhibition of cell swelling by amiloride (△) in Na^+-medium can be bypassed by addition of the Na^+/H^+-ionophore monensin (5 μM, ▲)

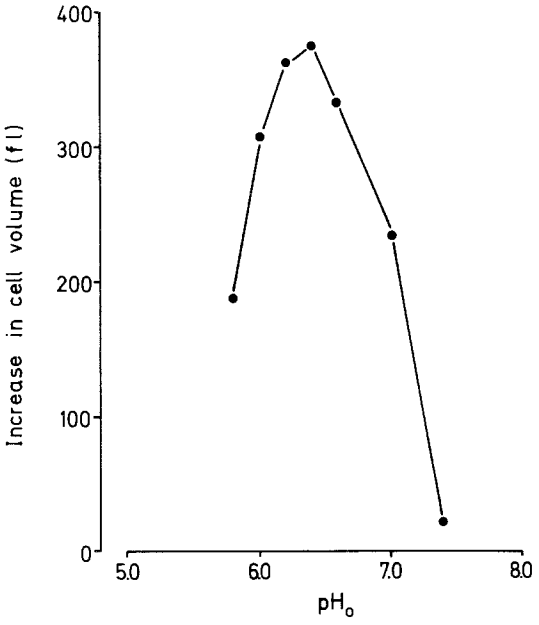

Fig. 3. Cell swelling in Na^+-propionate medium as a function of external pH. At time zero cells in standard isotonic medium were transferred by dilution (200- to 300-fold) to isotonic Na^+-propionate media at the pH values indicated, and the cell volumes were measured at time 5 min. The Na^+-dependent cell swelling was calculated by subtracting the cell volumes in K^+-propionate media from cell volumes in Na^+ media measured at the same pH values

dependence of Na^+-dependent cell swelling on pH_o is seen in Fig. 3, which shows the increase in cell volume measured 5 min after transfer of cells to Na^+-propionate medium. Cell volumes are corrected for the passive swelling, measured in parallel in isotonic K^+-propionate media at the same pH_o values. The passive swelling in K^+-propionate never exceeded 10% of the original cell volume. It appears from Fig. 3 that a maximum of acid-induced cell swelling occurs at pH_o 6.6. This is in good agreement with the optimal pH value for acid loading of platelets and leucocytes reported by Grinstein and coworkers (Grinstein et al., 1984*b*). Hence, this pH was chosen throughout the experiments described in this section.

In lymphocytes Na^+/H^+ exchange is activated both after cytoplasmic acidification and after cell shrinkage following hyperosmotic treatment (Grinstein et al., 1983, 1984*c*). Activation of the amiloride-sensitive Na^+/H^+ exchange in shrunken lymphocytes permits the re-uptake of ions and water usually denoted Regulatory Volume Increase (RVI). RVI in lymphocytes is therefore Na^+-dependent and inhibitable with amiloride. Ehrlich cells also perform RVI after hyperosmotically induced cell shrinkage. However, RVI in Ehrlich cells as well as in lymphocytes is only initiated if the cells

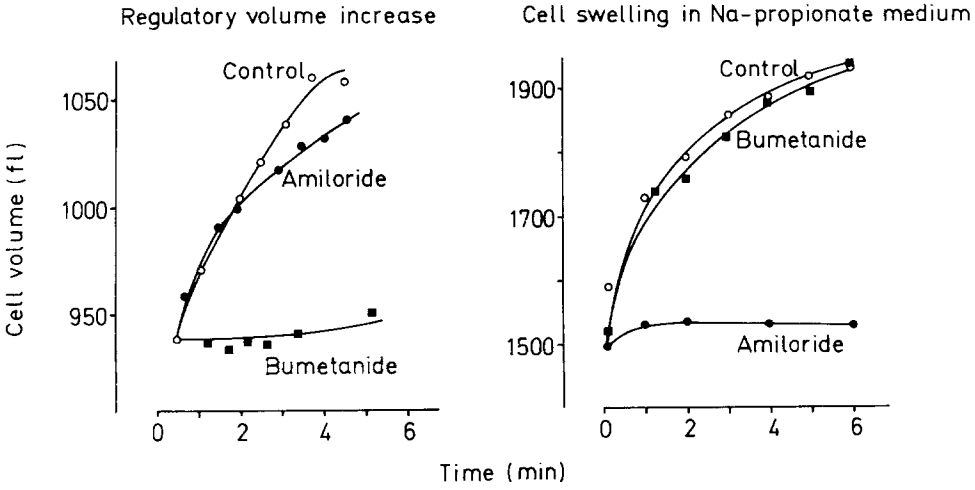

Fig. 4. Effect of bumetanide and amiloride upon regulatory volume increase, RVI, (*left panel*) and upon cell swelling in Na^+-propionate medium (*right panel*) in Ehrlich ascites tumor cells. (*Left panel*): The cells were pretreated by exposure to low external osmolarity (225 mOsm) for 15 min. At time zero a sample of the cell suspension was diluted 200- to 300-fold with isotonic (300 mOsm) medium, and the cell volume was followed with time. Control: ○. Cells in 20 μM bumetanide: ■. Cells in 0.1 mM amiloride: ●. (*Right panel*): At time zero a sample of a cell suspension in isotonic standard incubation medium was diluted 200- to 300-fold in isotonic Na^+-propionate medium (pH 6.6), and the cell volume was followed with time. Control: ○. Cells in 20 μM bumetanide: ■. Cells in 0.1 mM amiloride: ●

have been through a hypotonic pretreatment (*compare* Hendil & Hoffmann, 1974, with Hoffmann et al., 1983. *See also* Grinstein et al., 1984*c*). This is seen in Fig. 4, left panel, where cells after hypotonic challenge are returned to isotonic medium at time zero following the protocol described in Hoffmann et al. (1983). After an initial shrinkage (*not shown*) the cells swell again due to an activation of a cotransport of Na^+ with Cl^-. RVI in Ehrlich cells is therefore also Na^+ dependent (Hoffmann et al., 1983), but in contrast with lymphocytes, RVI in Ehrlich cells is only slightly affected by amiloride as shown in Fig. 4, left panel. Instead, RVI in Ehrlich cells is completely inhibitable with the loop diuretic bumetanide (Fig. 4, left panel). This confirms previous results showing that RVI in Ehrlich cells is mediated by cotransport of Na^+ and Cl^- (Hoffmann et al., 1983). Thus, in contrast to lymphocytes, Na^+/H^+ exchange does not seem to play any significant role for RVI in Ehrlich cells.

Figure 4, right panel, shows Na^+-propionate induced cell swelling in the presence of the same concentrations of bumetanide and amiloride as before. It is seen that bumetanide has only a weak effect on acid-induced cell swelling in Na^+-propionate medium, whereas amiloride, as also shown above (Fig. 2), completely blocks this cell swelling. Consequently, reswelling after osmotic-induced shrinkage and swelling following an acid-load of Ehrlich cells occurs by two distinct mechanisms, which are both Na^+-dependent but respond differently to inhibitors.

Volume Regulation After Acid-Induced Cell Swelling

Grinstein and coworkers (Grinstein et al., 1984*b*) reported that rat lymphocytes swell continuously for at least 15 min after transfer to Na^+-propionate-medium. As shown in Fig. 2 (left panel), Ehrlich cells behave differently because swelling ceases after about 5 min. In some experiments the period of swelling was followed by slow shrinkage. It is well known that Ehrlich cells respond to hypoosmotic swelling by activation of independent K^+ and Cl^- channels, thereby regaining their original volume by loss of KCl and cell water (Hoffmann, 1978; *see* Hoffmann et al., 1986). The possibility, therefore, exists that acid-induced cell swelling in Ehrlich cells could be partially offset by a swelling-induced activation of K^+ and Cl^- channels also under the present experimental conditions. It has previously been shown that the anti-calmodulin drug, pimozide, and the K^+-channel blocker, quinine, inhibit volume regulation by hypotonically swollen Ehrlich cells (Hoffmann, Simonsen & Lambert, 1984; Hoffmann et al., 1986). Figure 5 shows the effect of pimozide and quinine on the acid-induced volume response in Ehrlich cells. It is seen that while pimozide does not seem to affect the rate of cell swelling, the extent to which the cells swell is considerably increased. This result suggests that cells swollen in isotonic medium after cytoplasmic acidification possess a capacity for volume regulation via the pimozide-sensitive K^+ and Cl^- channels. In con-

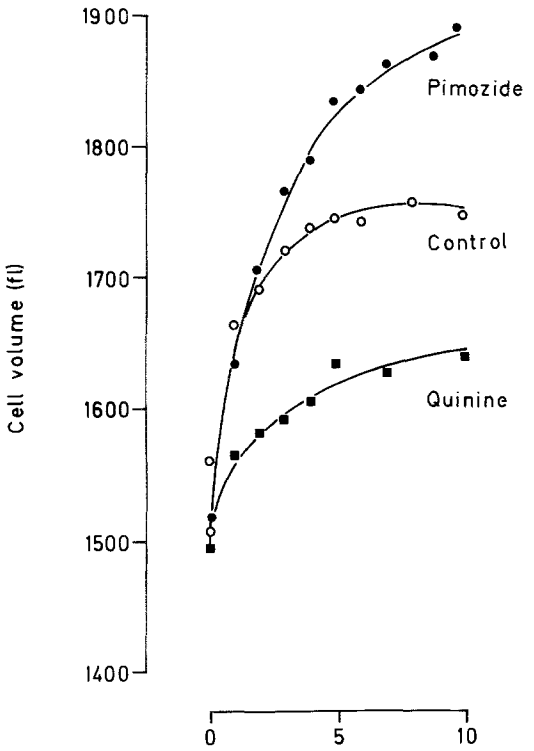

Fig. 5. The effect of pimozide and quinine on acid-induced cell swelling in Ehrlich cells. For experimental protocol *see* legend to Fig. 3. Pimozide (5 μM) and quinine (0.25 mM) were added at time zero. Control: ○. Cells in pimozide: ●. Cells in quinine: ■

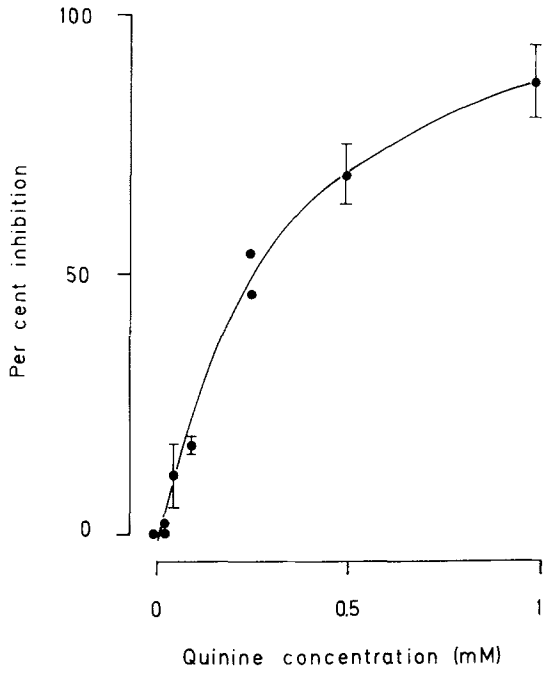

Fig. 6. Inhibition of acid-induced cell swelling by quinine as a function of the quinine concentration. For experimental protocol *see* legend to Fig. 3. The inhibitory effect of quinine was calculated in per cent from the cell volumes measured in the presence and in the absence of quinine at time 5 min after transfer of the cells to Na^+-propionate medium at pH 6.6. Cell swelling in cells without quinine at time 5 min was taken as 100%. The vertical bars represent SEM of three experiments. The other points show values from single experiments

trast, quinine reduces the rate of acid-induced cell swelling as shown in Fig. 5. A dose-response relation for the quinine inhibition was established and is shown in Fig. 6. It can be seen from Fig. 6 that half-maximal inhibition (IC_{50}) of acid-induced cell swelling occurs at a quinine concentration of about 0.25 mM. Since quinine inhibits amiloride-sensitive cell swelling, it is likely to inhibit Na^+/H^+ exchange. Quinidine—a close analogue to quinine—has previously been reported to inhibit Na^+/H^+ exchange in dog red blood cells (Parker, 1983). Quinine (and quinidine), therefore, seems to be a rather unspecific inhibitor of transport processes.

Activation of Na^+/H^+ Exchange by $CuSO_4$

Inhibition of RVD by $CuSO_4$

The previous section confirms, although indirectly, that a Na^+/H^+ exchange system exists in Ehrlich cells as in numerous other cell systems and that this system is activated after cytoplasmic acidification of cells in isotonic medium (Doppler et al., 1986; Wiener et al., 1986). The present section presents direct evidence that a Na^+/H^+ exchange system is activated under hypotonic as well as under isotonic conditions after treatment of the cells with the heavy-metal salt $CuSO_4$.

When Ehrlich cells are transferred from isotonic to hypotonic conditions, they swell initially as nearly perfect osmometers. This is followed by a shrinkage phase (10–15 min) during which they gradually approach their original volume. This phase, designated Regulatory Volume Decrease (RVD), is due to a cellular loss of KCl through separate, conductive K^+ and Cl^- channels, activated by cell swelling (Hendil & Hoffmann, 1974; Hoffmann et al., 1986). Cellular permeability to Na^+ was reported to be practically unchanged under these conditions (Hoffmann, 1978).

We have previously shown that low concentrations of $CuSO_4$ (about 0.5 ppm) added to Ehrlich cells undergoing RVD inhibited volume regulation (Lambert et al., 1984). Full inhibition was observed at concentrations above 1 ppm of $CuSO_4$. This is equal to 16 μM $CuSO_4$ and corresponds to about 0.5 μM of free Cu^{2+} ions as measured with a Cu^{2+}-specific electrode (see Lambert et al., 1984). It was shown that $CuSO_4$ inhibited RVD only in the pres-

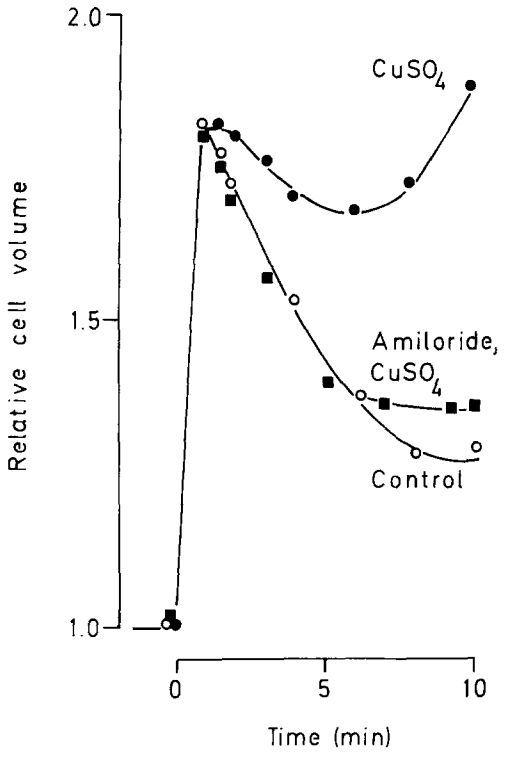

Fig. 7. The effect of $CuSO_4$ and/or amiloride on regulatory volume decrease (RVD) of Ehrlich cells after hypotonic challenge. Cells were diluted 200- to 300-fold with hypotonic medium (150 mOsm) at time zero, and the changes in cell volume were followed with time. $CuSO_4$ (40 μM) was added at time 0.80 min. Control with 0.1 mM amiloride: ○. $CuSO_4$: ●. Amiloride plus $CuSO_4$: ■

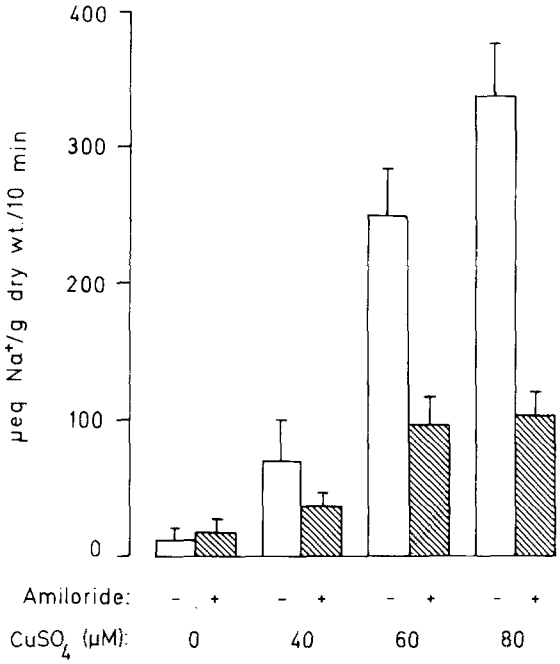

Fig. 8. Net Na^+-uptake by hypotonically treated Ehrlich cells without or with $CuSO_4$ and/or amiloride. Na^+-content (μeq/g dry wt) was measured 10 min after transfer to hypotonic medium (150 mOsm). The net Na^+ uptake was calculated as the difference between Na^+ content in the hypotonically treated cells and isotonic control cells. Amiloride was used at a concentration of 0.5 mM. The vertical bars indicate SEM of three independent experiments

ence of extracellular Na^+ and that the inhibition was anion-independent. It was argued that the $CuSO_4$-induced inhibition of volume regulation resulted from an increased permeability of the cell membrane to Na^+ as suggested by Riisgård and coworkers (Riisgård, 1979; Riisgård, Nørgård-Nielsen & Søgård-Jensen, 1980) for similar observations in the marine flagellate *Dunaliella marina*. Figure 7 confirms that $CuSO_4$ strongly inhibits RVD. Figure 7 also shows that amiloride, added 1 min prior to the osmotic shock, practically abolishes the effect of $CuSO_4$ on RVD. Control cells were also treated with amiloride. It is generally accepted that the free Cu^{2+} ion is the most toxic of the various copper species found in aqueous solutions (Florence & Batley, 1977). As a control, it was demonstrated that amiloride had no effect on the concentration of free cupric ions as measured with a $CuSO_4$-specific electrode. Assuming that $CuSO_4$ impairs RVD by increasing cellular permeability to Na^+, the result shown in Fig. 7 strongly suggests that $CuSO_4$ induces or activates an amiloride-sensitive influx of Na^+.

This suggestion is confirmed by measurements of net Na^+ transport during RVD in the absence and presence of $CuSO_4$ and with or without amiloride. Figure 8 shows the changes in cellular Na^+ content (μeq/g dry wt) measured 10 min after transfer of cells from isotonic to hypotonic conditions with three different concentrations of $CuSO_4$. In the control groups the net Na^+ uptake was small both with and without amiloride. Addition of $CuSO_4$ causes a considerable net uptake of Na^+, the extent of which is dependent on the concentration of $CuSO_4$ added. However, amiloride added prior to experimental treatment greatly reduces the $CuSO_4$-induced Na^+ uptake. At 80 μM $CuSO_4$ (i.e., about 1 μM free Cu^{2+}, *see* Materials and Methods) 67% of the $CuSO_4$-induced Na^+ uptake is amiloride sensitive. The present results, therefore, confirm the suggestion that $CuSO_4$ activates a Na^+ influx, of which the major part is amiloride inhibitable.

$CuSO_4$ caused an excess loss of K^+ compared to the K^+ loss seen during RVD in untreated cells (*data not shown*). The magnitude of the $CuSO_4$-induced K^+ loss was, however, less than the concommitant Na^+ uptake. The Cl^- loss during RVD in the presence of $CuSO_4$ was reduced to the same extent as the Na^+ uptake exceeded the additional K^+ loss.

Table 1. The effect of $CuSO_4$ and/or amiloride on the NPS content in Ehrlich cells after a hypoosmotic shock.

Medium	NPS content (μmol/g dry wt)
Isotonic	325 ± 14 (n = 12)
Hypotonic	
Control (no additions)	225 ± 8 (n = 6)
80 μM $CuSO_4$	243 ± 9 (n = 6)
0.1 mM amiloride	193 ± 6 (n = 6)
0.1 mM amiloride, 80 μM $CuSO_4$	211 ± 9 (n = 6)

Cells in isotonic standard medium (300 mOsm) were transferred to hypoosmotic conditions (150 mOsm) by centrifugation. The NPS content was measured using taurine as a standard on samples removed just prior to and 10 min after the reduction in osmolarity. Figures are average ± SEM of n independent experiments.

Consequently, the net effect was an inhibition of RVD.

It has previously been shown (Hoffmann & Hendil, 1976; Hoffmann & Lambert, 1983) that a net loss of amino acids, mainly taurine, takes place during RVD. Consequently, the possibility exists that $CuSO_4$, in addition to its effects on Na^+, might also inhibit amino acid loss during RVD. As shown in Table 1 this is, however, not the case. Table 1 shows measurements of the cellular content of Ninhydrin-Positive Substances (NPS) measured with taurine as a standard in isotonic cells and 10 min after a hypoosmotic treatment without and with $CuSO_4$ and/or amiloride. Confirming previous results (Hoffmann & Hendil, 1976; Hoffman & Lambert, 1983), it is seen that untreated cells lose about 30% of their NPS content during the first 10 min of RVD. Neither $CuSO_4$, nor amiloride or both in combination had any significant effect on the net loss of NPS (Student's t test, $P < 0.01$), showing that the effect of $CuSO_4$ on RVD does not involve NPS.

Effects of $CuSO_4$ on Intracellular pH

The results presented above show that the major cause of the $CuSO_4$-induced inhibition of RVD in Ehrlich cells is an amiloride-sensitive uptake of Na^+. Amiloride being a specific blocker of Na^+/H^+ exchange (Benos, 1982), this suggests that $CuSO_4$ possesses a capability of activation of a Na^+/H^+ exchange system. If this is true, then cells treated with $CuSO_4$ should expel H^+ from the cytoplasm with a resultant cytoplasmic alkalinization. Figure 9 shows changes with time of intracellular pH (pH_i) after a hypoosmotic shock imposed at time zero without and with 50 μM $CuSO_4$ (For absolute pH_i values, *see* legend to Fig. 9). Amiloride was present in the experiments shown in the right panel. pH_i was measured fluorometrically with the fluorescent pH_i indicator BCECF (*see* Materials and Methods). The traces are representative of six paired experiments without amiloride and of four paired experiments with 0.2 mM amiloride. As shown in Fig. 9, left panel, a gradual decrease of cellular pH with time is seen after a hypoosmotic shock in accordance with the results of Livne and Hoffmann (1988). Addition of $CuSO_4$ (at time 0.25 min) strongly reduces the rate of cytoplasmic acidification during the first 5 min after the osmotic shock. It may be estimated from the slopes of the traces in Fig. 9, left panel, that $CuSO_4$ reduces the rate of cytoplasmic acidification to about 25% of the rate seen in untreated cells. After this time period the rate of acidification again increases. In cells treated with amiloride, cytoplasmic acidification was observed both in the presence and absence of $CuSO_4$ after the osmotic shock and to the same extent as that seen in untreated controls (Fig. 9, right panel). That hypotonicity results in a decrease in cytoplasmic pH is in agreement with Livne and Hoffmann (1988). They also found an increased acidification in the presence of amiloride when the initial degree of swelling was 1.9. This was interpreted as a result of an activation of Na^+/H^+ exchange by the pH reduc-

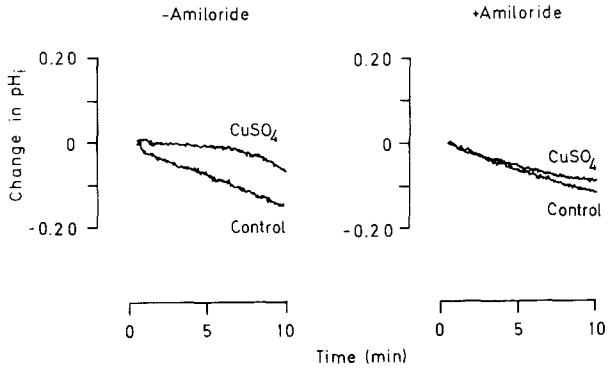

Fig. 9. Changes in intracellular pH (pH_i) of Ehrlich cells as a function of time after transfer to hypotonic conditions (150 mOsm). $CuSO_4$ (50 μM) was added at time 0.25 min and recording was initiated at time 0.50 min after the osmotic shock. Amiloride (right panel) was present at a concentration of 0.2 mM. The pH_i measured at time 0.50 min was in controls without and with amiloride 7.23 ± 0.12 (± SD, n = 6) and 7.24 ± 0.12 (± SD, n = 4), respectively. In the presence of $CuSO_4$ the equivalent pH_i values were 7.14 ± 0.06 (± SD, n = 6) and 7.19 ± 0.12 (± SD, n = 4), respectively. The rate of acidification of controls was estimated at 0.011 ± 0.001 pH units/min (± SD, n = 6). In the presence of $CuSO_4$ the reduced rate (measured between time 0.50 and time 5 min) was 0.004 ± 0.001 (± SD, n = 6). The rates of acidification of amiloride-treated cells were 0.014 ± 0.006 and 0.012 ± 0.003 pH units/min (± SD, n = 4) without and with $CuSO_4$, respectively

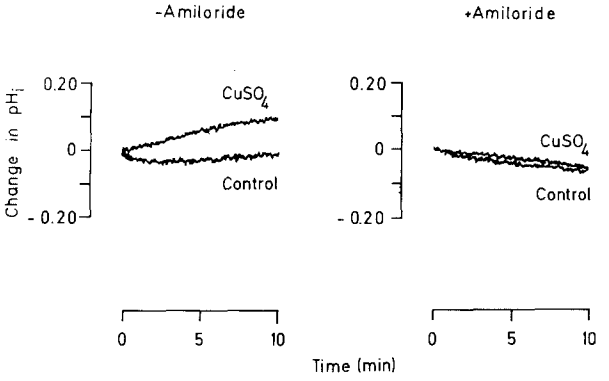

Fig. 10. Changes in intracellular pH of Ehrlich cells in isotonic standard incubation medium as a function of time after transfer of the cells to the cuvette. $CuSO_4$ (20 μM) was added immediately after transfer of the cells (time zero), and recording was initiated at time 0.50 min. Amiloride (right panel) was present at a concentration of 0.2 mM. The initial pH_i (time 0.50 min) value in isotonic cells was 7.12 ± 0.02 (± SEM, n = 14), both without and with amiloride. In the presence of $CuSO_4$ the initial pH_i value was 7.04 ± 0.02 (± SEM, n = 6), with and without amiloride

tion. In the present experiments the initial swelling was only 1.7, and the resulting acidification was also smaller. Hence, no activation of Na^+/H^+ exchange seems to occur under the present experimental conditions. It is concluded from Fig. 9 that addition of $CuSO_4$ to hypotonically treated cells causes, however transiently, a *relative* alkalinization of the cytoplasm which is sensitive to amiloride.

The results shown in Fig. 9 were obtained on a background of strong cytoplasmic acidification occuring during RVD. In order to avoid a possible interference with the results from events attributed to nonsteady-state cells undergoing RVD, e.g. changes in cell volume, similar experiments were carried out with steady-state cells under isotonic conditions. $CuSO_4$ has no effect on the cell volume of isotonic cells under the present experimental conditions (Lambert et al., 1984). The results are shown in Fig. 10, which depicts changes in pH_i with time after addition of $CuSO_4$ (For absolute pH_i values, *see* legend to Fig. 10). Amiloride was present in the experiments shown in the right panel. Figure 10, left panel, shows that control cells maintain a practically constant pH_i throughout the experimental period. In contrast, addition of $CuSO_4$ provokes an increase in cytoplasmic pH amounting to about 0.1 pH unit in the course of the first 10 min. As shown in Fig. 10 (right panel), amiloride completely abolishes the cytoplasmic alkalinization induced by $CuSO_4$. In contrast a slight acidification of the cytoplasm is seen both with and without added $CuSO_4$.

Table 2. The effect of $CuSO_4$ and/or amiloride on intracellular pH of Ehrlich cells under isotonic conditions

	Change in pH_i/10 min
Control	−0.010 ± 0.010 (n = 6)
20 μM $CuSO_4$	+0.087 ± 0.004 (n = 7)
0.2 mM amiloride	−0.054 ± 0.007 (n = 5)
Amiloride, $CuSO_4$	−0.058 ± 0.003 (n = 4)

The changes in pH_i within the first 10 min were estimated from the change in fluorescence occurring within the first 10 min after transfer of the cells to the fluorescence cuvette. Figures are average ± SEM of n independent experiments.

Table 3. The effect of $CuSO_4$ on H^+ excretion by Ehrlich cells

	μeq H^+/g dry wt/10 min
Control	19.5 ± 1.6 (n = 4)
0.2 mM amiloride	22.7 ± 0.3 (n = 3)
60 μM $CuSO_4$	40.7 ± 4.9 (n = 3)
0.2 mM amiloride, 60 μM $CuSO_4$	21.0 ± 3.1 (n = 3)

H^+ production was followed during the first 10 min after transfer of the cells to weakly buffered medium. In two of the experiments 100 μM DIDS was added in order to prevent possible interference on pH shifts from Cl^-/HCO_3^- exchange. There was no difference between values of H^+ excretion with or without DIDS present. Figures are average ± SEM of n experiments.

The rates of pH_i changes in a number of experiments like the ones shown in Fig. 10 have been summarized in Table 2. In conclusion: the results shown in Figs. 9 and 10 and Table 2 show that treatment of cells with $CuSO_4$ causes an amiloride-sensitive cytoplasmic alkalinization.

Parallel to alkalinization of the cytoplasm the operation of a Na^+/H^+ exchange system promotes excretion of H^+ from the cells, which will cause acidification of the extracellular medium if this is only weakly buffered. It has been found by Heinz, Sachs and Schafer (1981) that Ehrlich cells, even in the absence of glucose, excrete protons, causing acidification of the extracellular medium. This is confirmed in Table 3, from which it is seen that untreated cells excrete acid equivalents at a rate equal to 18 μeq/g dry wt/10 min. Addition of $CuSO_4$ (60 μM) causes a doubling in the rate of H^+ excretion, and this increase is completely abolished by amiloride. Accordingly, $CuSO_4$ causes a transmembrane flux of H^+ which is amiloride-sensitive. We, therefore, conclude that addition of $CuSO_4$ to Ehrlich cells promotes the activation of a Na^+/H^+ exchange system, which is the cause of inhibition of volume regulation after a hypoosmotic shock.

Effect of a Phorbol Ester

In many cells tumor-promoting phorbol esters such as TPA are shown to activate Na^+/H^+ exchange (Moolenaar, Tertoolen & deLaat, 1984; Moolenaar, 1986; Grinstein & Rothstein, 1986). A weak effect of TPA on the pH_i of Ehrlich cells was recently demonstrated by Wiener and coworkers (Wiener et al., 1986). In the present study the effect of TPA on pH_i of Ehrlich cells was assessed at pH_o 7.4 and 7.0, respectively. It was found that no measurable change in pH_i could be demonstrated at pH_o 7.4, whereas at pH_o 7.0 cytoplasmic alkalinization at a rate of 0.17 ± 0.03 pH units/10 min (± SEM, $n = 4$) was found. Since pH_i is 7.34 and 7.12 at pH_o, 7.4 and 7.0, respectively, the present results suggest a pH dependence of the effect of TPA on Ehrlich cells similar to that found in lymphocytes (Grinstein & Rothstein, 1986).

Discussion

Demonstration of Na^+/H^+ Exchange Activity by Electronic Cell Sizing

As seen from Figs. 3, 4 and 5, an amiloride-sensitive cell swelling occurs after transfer of Ehrlich cells to a medium in which the usual NaCl has been replaced by the isosmotic amount of the Na salt of a weak, organic acid. A similar phenomenon has previously been reported for platelets and lymphocytes (Grinstein et al., 1984*b*). In the case of lymphocytes and platelets, extensive evidence was provided that transfer of cells to Na^+-propionate medium imposed an acid load on the cells leading to the activation of a Na^+/H^+ exchange system. Swelling was a consequence of net accumulation of Na^+-propionate. The present study strongly suggests that a similar mechanism operates in Ehrlich cells after transfer of the cells to Na^+-propionate medium. Evidences for this hypothesis were: (i) cells swell in Na^+-propionate but not in K^+-propionate (Fig. 2, left panel), i.e., cell swelling is Na^+-dependent; (ii) cell swelling in Na^+-propionate medium is completely abolished by amiloride, which is known to be a specific blocker of Na^+/H^+ exchange in numerous cell systems (Benos, 1982) (Fig. 2, left panel); (iii) the Na^+/H^+ ionophore monensin can bypass the amiloride blockage of cell swelling (Fig. 2, right panel). This confirms that cell swelling in Na^+-propionate is by a specific, amiloride-sensitive and Na^+-dependent pathway and shows that absence of cell swelling in the presence of amiloride is not due to a collapsed Na^+ gradient; (iv) addition of the K^+/H^+ ionophore nigericin to cells in K^+-propionate medium causes considerable cell swelling, demonstrating that an outward-going H^+-gradient also exists in cells in K^+-propionate medium (Fig. 2, right panel). This observation confirms that cell swelling in Na^+-propionate is mediated by a Na^+-specific mechanism. Thus, by the simple cell sizing technique, evidence, however indirect, for the activation of a Na^+/H^+ exchange system in Ehrlich cells after acid loading was obtained. A Na^+/H^+ exchange system in these cells has recently been demonstrated by Doppler and coworkers (Doppler et al., 1986), who demonstrated an amiloride-sensitive Na^+ influx which was strongly dependent on pH_i. They also found that pH_i became more alkaline as the extracellular concentration of Na^+ increased, suggesting a Na^+-dependent efflux of H^+ from the cells. Wiener and his group (Wiener et al., 1986) showed by measurements of pH_i that addition of ATP to suspensions of Ehrlich cells caused Na^+-dependent and amiloride-sensitive cytoplasmic alkalinization. Furthermore, they showed that the phorbol ester TPA, which is known to activate Na^+/H^+ exchange in several cell systems (Moolenaar et al., 1984; Moolenaar, 1986), also increased pH_i of Ehrlich cells, although the effect was rather small. These results taken together with the present study has made it reasonable to conclude that an inducible Na^+/H^+ exchange system exists in Ehrlich ascites tumor cells.

Activation of Na^+/H^+ Exchange by $CuSO_4$

The effects of $CuSO_4$ on Na^+ influx and pH_i of Ehrlich cells provide direct evidence for the existence of a Na^+/H^+ exchanger in these cells which is inducible by treatment of the cells with the heavy-metal salt $CuSO_4$. The evidence is the following observations: (i) the inhibitory effect of $CuSO_4$ on RVD is completely antagonized by amiloride (Fig. 7); (ii) in cells undergoing RVD, $CuSO_4$ causes a large net influx of Na^+, of which the major part is amiloride-sensitive (Fig. 8); (iii) $CuSO_4$ causes amiloride-sensitive cytoplasmic alkalinization (Figs. 10 and 11); and (iv) treatment of Ehrlich cells with $CuSO_4$ causes a net H^+ efflux (Table 3).

In addition to this, $CuSO_4$ also causes an amiloride-insensitive influx of Na^+ as shown in Fig. 8. This is also reflected in Fig. 7, where it is seen that only during the first 5 min after the hypoosmotic shock amiloride can completely abolish the effect of $CuSO_4$. This suggests that, whereas the primary effect of $CuSO_4$ seems to be the activation of Na^+/H^+ exchange, secondary effects of $CuSO_4$ on Na^+ transport are also seen. Amiloride-insensitive net

Na^+ influx may reflect inhibition of the Na^+/K^+ pump or an increase in the diffusional Na^+ leak permeability of the cytoplasmic membrane by the presence of $CuSO_4$.

Stoichiometric Considerations

Isotonic Cells

The operation of a Na^+/H^+ exchange system with the expected 1:1 stoichiometry should result in a net proton efflux of the same order of magnitude as the simultaneously measured net Na^+ influx. Under isotonic conditions the amiloride-sensitive, $CuSO_4$-induced contribution to cellular H^+ excretion was 20 μeq H^+/g dry wt/10 min at a $CuSO_4$ concentration of 60 μM (cf. Table 3). In an additional experiment (*data not shown*) an amiloride-sensitive, $CuSO_4$-induced net uptake of Na^+ of 19 and 28 μeq/g dry wt/10 min in 60 and 80 μM $CuSO_4$, respectively, was found. Thus, there is good agreement between net Na^+ and net H^+ fluxes in the presence of $CuSO_4$ in isotonic cells. $CuSO_4$-induced net H^+ efflux (*see* Table 3) should correspond to the measured change in pH_i provided cellular buffering capacity is taken into account. From Table 2 it is seen that the $CuSO_4$-induced, amiloride-sensitive change in pH_i is about 0.1 pH units/10 min in 20 μM $CuSO_4$. In three additional experiments with 40 μM $CuSO_4$, a $CuSO_4$-induced change in pH_i of 0.17 pH units/10 min was found. Thus, a concentration dependence of the $CuSO_4$ effect on pH_i seems to exist. The net H^+ flux corresponding to a given pH_i change may be calculated as the product of cellular buffering capacity and the pH_i change (Grinstein, Cohen & Rothstein, 1984*a*). The buffering capacity of Ehrlich cells was previously found to be 100 μeq/g dry wt/pH (Bowen & Levinson, 1984). Using this value for cellular buffering capacity, a net efflux of 10 and 17 μeq H^+/g dry wt/10 min in 20 and 40 μM $CuSO_4$, respectively, can be calculated from the measured changes in pH_i. This is in reasonably good agreement with the measured efflux of 20 μeq H^+/g dry wt/10 min found in 60 μM $CuSO_4$, taking the differences in $CuSO_4$ concentration and experimental conditions into account. Consequently, the present estimates strongly support the hypothesis that $1Na^+:1H^+$ exchange can be induced by $CuSO_4$ in Ehrlich cells under isotonic conditions.

Hypotonic Cells

In hypotonically treated cells the amiloride-sensitive Na^+ uptake induced by $CuSO_4$ (Fig. 8) amounts to 34, 152, and 233 μeq/g dry wt/10 min in 40, 60, and 80 μM $CuSO_4$, respectively. Assuming that $CuSO_4$ only activates 1:1 Na^+/H^+ exchange, this should lead to an equivalent H^+ extrusion. An extrusion of protons of this magnitude should result in an increase in pH_i of 0.3, 1.5, and 2.3 pH units, respectively, again using a cellular buffering capacity of 100 μeq/g dry wt/pH. Even taking into account that pH_i experiments and measurements of ion movements can hardly be directly compared because of the large differences in the cytocrit between these two types of experiments, the calculated theoretical pH_i changes are not in agreement with the observed change (about 0.1 pH units/10 min in 50 μM $CuSO_4$, *see* Fig. 9). Consequently, it may be hypothesized that the observed change in pH_i is likely to reflect the net result of oppositely directed H^+ movements. The operation of a K^+/H^+ exchange system simultaneously with the Na^+/H^+ exchange would cause H^+ movement in the opposite direction of that caused by the operation of the Na^+/H^+ exchange. Following a hypoosmotic shock in the presence of $CuSO_4$, a K^+ loss in excess of that seen in untreated controls during RVD was observed (data not shown). In 80 μM $CuSO_4$ this K^+ loss was partly inhibited by amiloride. Simultaneous activation of both Na^+/H^+ exchange and K^+/H^+ exchange by an exogenous toxic substance (the tumor-promoting phorbol ester TPA) has previously been reported for *Amphiuma* red blood cells (Cala, 1986). In this case both alkalimetal/H^+ exchange systems were inhibitable with amiloride, and it was suggested that K^+/H^+ exchange and Na^+/H^+ exchange are mediated by the same membrane protein (Cala, 1986). Thus, the possibility exists that $CuSO_4$ in addition to the activation of a Na^+/H^+ exchanger also activates a K^+/H^+ exchange system in Ehrlich cells under hypotonic conditions. As argued above, such a phenomenon, however, does not seem to play a significant role in $CuSO_4$-treated isotonic cells. Another possibility is that $CusO_4$ under hypotonic conditions in addition to the activation of Na^+/H^+ exchange also induces amiloride-sensitive, conductive Na^+ channels.

Mechanism of the Activation of the Na^+/H^+ Exchanger

Activation of Na^+/H^+ exchange has previously been reported to occur in numerous cell systems when cells are exposed to a variety of stresses. These include decrease of cellular pH, exposure to increased medium osmolarity and treatment of cells with the tumor-promoting phorbol ester TPA (*see* Grinstein & Rothstein, 1986). Induction of Na^+/H^+ exchange by $CuSO_4$ has, to our knowledge, not previously been reported.

Both osmotic shrinkage and treatment with TPA shift the pH_i dependence of the Na^+/H^+ exchanger in a more alkaline direction in lymphocytes (Grinstein et al., 1985*b*; Grinstein & Rothstein, 1986), i.e., Na^+/H^+ exchange becomes active at a higher pH value in shrunken and TPA-treated lymphocytes than in untreated controls. The effect of TPA in Ehrlich cells suggests that a similar phenomenon exists in these cells. As shown in Fig. 10, little activity of Na^+/H^+ exchange is seen at pH_i 7.12 as estimated from the weak cytoplasmic acidification seen in the presence of amiloride (Fig. 10, right panel). This is consistent with the pH_i dependence of the Na^+/H^+ exchanger in lymphocytes, where the "set-point" is around pH_i 7.0–7.2 (*see* Grinstein & Rothstein, 1986). The "set-point" is the pH_i below which Na^+/H^+ exchange can be measured. Livne and Hoffmann (1988) have recently determined the "set-point" for the Na^+/H^+ exchanger in Ehrlich cells to be in the range 7.0–7.1. Treatment of Ehrlich cells with TPA at pH_o 7.0 (pH_i 7.1) results, as mentioned above, in significant cytoplasmic alkalinization, suggesting that Na^+/H^+ exchange is operating. In lymphocytes, TPA is reported to shift the "set-point" of the exchanger about 0.2 pH units in alkaline direction and considerable activity of Na^+/H^+ exchange is found in TPA-treated cells at pH_i 7.1 (Grinstein et al., 1985*b*). At pH_o = 7.4 pH_i in Ehrlich cells is 7.3. A "set-point" shift by TPA in Ehrlich cells of similar magnitude as that seen in lymphocytes may be expected to move the "set-point" to pH_i 7.2–7.3, and this will not suffice to activate the exchanger. This is consistent with the observation that no cytoplasmic alkalinization was observed in the present study after treatment of Ehrlich cells with TPA at pH_o 7.4. This probably also explains the rather small effect of TPA seen by Wiener and coworkers, who performed their experiments at pH 7.4 (Wiener et al., 1986).

The effect of TPA and $CuSO_4$ on intracellular pH is additive. Addition of 40 μM $CuSO_4$ to cells already treated with TPA at pH_o 7.0 resulted in a new rate of cytoplasmic alkalinization of about 0.3 pH units/10 min (*data not shown*). Thus, addition of $CuSO_4$ after TPA resulted in a doubling of the rate of alkalinization. The possibility that $CuSO_4$ in analogy with TPA shifts the "set-point" of the exchanger, and in the same direction, is not possible to test because of precipitation of $Cu(OH)_2$ when pH_o is increased above 7.0. As appears from the legends to Figs. 9 and 10, $CuSO_4$ apparently causes a quick acidification of the cytoplasm of 0.05 to 0.09 pH units. The possibility that this acidification in itself is sufficient to trigger the Na^+/H^+ exchanger can, therefore, not be excluded. It is well known that TPA activates Na^+/H^+ exchange via the protein-kinase C system (Berridge, 1984). The mechanism by which $CuSO_4$ can activate Na^+/H^+ exchange is at present unknown. Recently, a model for the functioning of the renal Na^+/H^+ exchange system has been presented which underlines the importance of a carboxylic group and a histidine group on the external site of the exchanger (Igarashi & Aronson, 1987). Since copper is known to have high affinity to $-COOH$ and $-NH_2$ groups (Passow, Rothstein & Clarkson, 1961), it is tempting to suggest that $CuSO_4$ modifies the exchanger by binding to the external site. If this is true, then TPA and $CuSO_4$ activate Na^+/H^+ exchange by two distinct mechanisms, and this is in agreement with the fact that the effects of TPA and $CuSO_4$ are additive.

In conclusion, the present work has confirmed the existence of a Na^+/H^+ exchange system in Ehrlich ascites tumor cells which is activated following a decrease in intracellular pH. Furthermore, the exchanger is activated after treatment of the cells with the heavy-metal salt $CuSO_4$. Na^+/H^+ exchange is, therefore, responsible for the inhibitory effect of $CuSO_4$ on RVD previously reported.

The work was supported by the Carlsberg Foundation (B. Kramhøft) and the Danish Natural Science Research Council (I.H. Lambert, grant no. 11-4614 and grant nos. 11-5608 and 11-4420 [equipment]).

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Received 10 August 1987; revised 14 December 1987