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Cytoplasmic Acidification and Activation of Na^+/H^+ Exchange During Regulatory Volume Decrease in Ehrlich Ascites Tumor Cells

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Summary. Ehrlich ascites tumor cells undergoing regulatory volume decrease (RVD) exhibit cytoplasmic acidification as measured by an intracellular fluorescent pH indicator. The acidification results in an activation of the Na^+/H^+ exchanger. The intracellular pH 'set point' for the activation is estimated to be around 7.0. The activation of the Na^+/H^+ exchanger leads to an incomplete RVD. In support of this conclusion, amiloride and Na^+-free medium, known to limit the Na^+/H^+ exchange, indeed enhance the RVD response. Intracellular acidification and activation of Na^+/H^+ exchange may be a general response of cells undergoing RVD.

Key Words Ehrlich ascites tumor cells · volume regulation · cytoplasmic acidification · pH regulation · Na^+/H^+ exchange

Introduction

When Ehrlich cells are suspended in hypotonic medium, a regulatory volume decrease (RVD) response occurs; conductive Na^+ permeability decreases while conductive K^+ and Cl^- permeability greatly increases, leading to a net efflux of K^+ and Cl^- with concomitant loss of cell water to restore the cell volume (Hendil & Hoffmann, 1974; Hoffmann, 1978). The consequences of a loss of K^+ and Cl^- via separate, conductive transport pathways have been analyzed in detail by Lew and Bookchin (1986) in terms of an integrated mathematical cell model. For a cell with a Cl^-/HCO_3^- exchanger operating in its membrane, the K^+ loss can be predicted to be partially balanced by a proton uptake via the Jacobs-Stewart cycle with resulting intracellular acidification, and hence the K^+ loss will exceed the Cl^- loss to an extent which depends upon the cellular pH buffering capacity (*see* Freeman et al., 1987).

In the case of the Ehrlich ascites tumor cell, the K^+ loss during RVD exceeds the Cl^- loss by a factor of about 1.6 (Hoffmann, Simonsen & Lambert, 1984). The purpose of this study is to examine whether acidification which is to be expected on theoretical bases is actually taken during RVD in Ehrlich cells. This issue is of special physiological interest since cytoplasmic acidification may activate the Na^+/H^+ exchanger. Using an intracellular pH-sensitive fluorescent probe, intracellular pH (pH_i) measurements were carried out concomitantly with analyses of volume changes during RVD. The results demonstrate that both the expected acidification and activation of the Na^+/H^+ exchanger indeed take place. Similar results have previously been demonstrated for blood platelets (Livne et al., 1987*b*), thus possibly indicating a general pattern in cells undergoing RVD.

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 (in mM): NaCl: 150, KCl: 5, $MgSO_4$: 1, $CaCl_2$: 1, Na_2HPO_4: 1, MOPS: 4.4, 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 media with lower Cl^- concentration NaCl was replaced by NaGluconate in equimolar amounts.

Media used for measurements of cell volume in a Coulter counter (*see below*) were filtered through Millipore filters (0.45 μm) prior to experiments. Hyposmotic 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

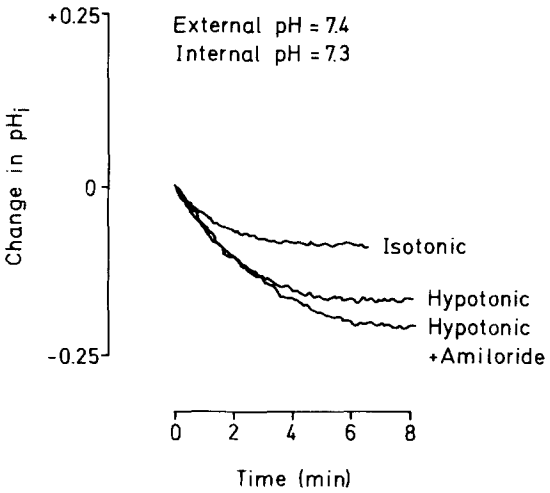

Fig. 1. Changes in intracellular pH (pH_i) of BCECF-loaded Ehrlich cells of an extracellular pH (pH_o) of 7.4 as a function of time after transfer to isotonic (300 mOsm) or hypotonic conditions (150 mOsm), with or without 100 μM amiloride. For amiloride treatment, the cells were preincubated for 1 min with the drug before they were added to the cuvette. The experiment is representative of four separate experiments

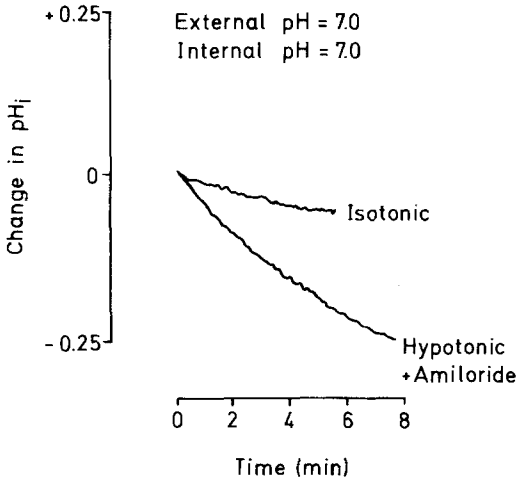

Fig. 2. Changes in intracellular pH (pH_i) of BCECF-loaded Ehrlich cells of an extracellular pH (pH_o) of 7.0 as a function of time after transfer to isotonic (300 mOsm) or hypotonic conditions (150 mOsm) with 100 μM amiloride. The experiment is representative of four independent experiments

amiloride for about 1 min prior to transfer to the experimental medium containing amiloride.

Cell Volume Measurements

Cell volume was measured in a Coulter counter model ZB equipped with a Coulter channellyzer (C-1000). The tube orifice was 100 μm. 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).

Measurement of Intracellular pH

Cell suspensions (cytocrit 4%) were incubated with the acetoxymethylester of a fluorescent probe (BCECF), final concentration 3.6 μM, for 30 min at 37°C. One ml of this suspension was then washed twice in isotonic medium containing bovine serum albumin (fraction V, 1 mg/ml) in order to remove excess probe, resuspended in this medium, and subsequently incubated at 25°C for 5–45 min. The fluorescence was recorded after 50-fold dilution of the cell suspension in the appropriate experimental medium (2 ml) in a thermostated (25°C) and magnetically stirred cuvette using a Perkin Elmer LS-5 luminescence spectrometer with excitation at 495 nm (slitwidth 5 nm) and emission at 525 nm (slitwidth 5 nm). A temperature of 25°C was chosen in order to minimize leakage of the intracellularly trapped BCECF from the cells (to 0.2%/min) as described by Kramhøft, Lambert and Hoffmann (1988).

Calibration was carried out after each pair of measurements 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 the K^+/H^+ ionophore nigericin (5 μM) was added, pH was manipulated by addition of increments of saturated solutions of Tris (increase in pH) or TES (decrease in pH) and a calibration curve was obtained by simultaneous recordings of extracellular pH in cuvette with a combined electrode (Ingold, Switzerland) and fluorescence. Calibration of amiloride experiments was always carried out in the presence of 100 μM amiloride, because amiloride was found to reduce the fluorescence by about 10%.

Reagents

All reagents were analytical grade. Amiloride and nigericin were from Sigma (St. Louis, MO). Nigericin was dissolved in ethanol (1 mg/ml) and kept at −20°C. Amiloride (10 mM) was dissolved in water and kept frozen and light-tight. BCECF-AM was purchased from Molecular Probes, Junction City, OR. It was dissolved in dry dimethylsulfoxide (1 mg/ml) and kept in 25-μl aliquots at −20°C.

Abbreviations

MOPS: 3-(N-morpholino)propanesulfonic acid.
TES: N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.
BCECF: 2′,7′-*bis*-(2-carboxyethyl)-5 (and-6)carboxyfluorescein.
Tris: tris(hydroxymethyl)aminomethane.
NMDG: N-methyl-D-glucamine.

Results

The effect of osmotic swelling on cytoplasmic pH was determined, utilizing the fluorescent probe

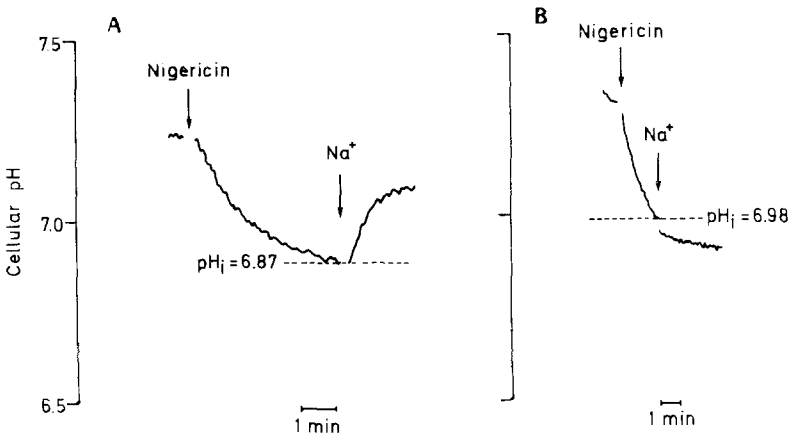

Fig. 3. Effect of external Na^+ on cytoplasmic alkalinization of acid-loaded Ehrlich cells. BCECF-loaded Ehrlich cells were suspended in N-methyl-D-glucamine medium. Cytoplasmic acid-loading was initiated by addition of nigericin (arrow, final concentration 0.67 μM) which exchanges cellular K^+ for external H^+. Forty sec before addition of 40 mM NaCl, BSA (3 mg/ml) was added. The interrupted line indicates the pH_i at the moment of the addition of Na^+

BCECF. As shown in Fig. 1, Ehrlich cells loaded with the fluorescent dye and diluted with isotonic media showed only limited changes in pH_i. In various other experiments no change in pH_i was observed under these conditions. In contrast, a gradual cytoplasmic acidification always took place following resuspension in hypotonic medium. The rates and extent of the acidification were increased in the presence of amiloride. A similar pattern was found at external and internal pH 7.0 where the cells in hypotonic medium in the presence of amiloride reached a pH_i as low as 6.75 (Fig. 2).

The greater extent of acidification in the presence of amiloride may indicate that the swelling-induced acidification is compensated in part by operation of the Na^+/H^+ exchanger. In many cell types the Na^+/H^+ exchanger is nearly quiescent at physiological pH, but is activated upon internal acidification (Aronson 1985, Grinstein & Rothstein, 1986). Therefore, it became of interest to examine the relationship between the internal acidification and the activation of the Na^+/H^+ exchanger. Elucidation of this relationship is necessary to evaluate the potential impact of the internal acidification during RVD on Na^+/H^+ activation.

The cells were acidified by exposure to nigericin by inducing an exchange of cellular K^+ for external H^+. When pH_i reached the value of 6.87 or lower, the subsequent addition of external NaCl (40 mM) caused a rapid alkalinization (Fig. 3*A*). This alkalinization was completely blocked by addition of 200 μM amiloride (*data not shown*). Thus, two of the characteristic properties of the Na^+/H^+ exchanger were demonstrated, namely, (*i*) dependence on external Na^+ and (*ii*) sensitivity to amiloride. If the internal acidification imposed by nigericin was limited to pH_i 6.98 (Fig. 3*B*) then the addition of external Na^+ did not elicit a net alkanization, but markedly reduced the rate of acidification. Thus, the pH 'set point' for the operation of Na^+/H^+ exchange in Ehrlich cells is slightly above pH_i 6.98. The value of the 'set point' can also be estimated from the maximal pH_i after addition of Na^+ as in Fig. 3*A*. Using this method the 'set point' can from two independent experiments be estimated at pH_i = 7.08. This value is within the range reported for several cell types (Frelin et al., 1986).

If the degree of internal acidification during RVD is large enough to activate the Na^+/H^+ exchange, the operation of the exchange will lead to the uptake of Na^+ and thus will counteract cell shrinkage and inhibit, at least partly, RVD. In this case, it should be possible to enhance RVD by treatments that limit the Na^+/H^+ exchange, such as amiloride. Furthermore, as acidification depends on cell swelling (Fig. 1), it is likely that the effect of amiloride will depend on the extent of swelling. To test this supposition cells were transferred to several different hypotonic solutions to impose different degrees of swelling. Figure 4 shows that upon more extensive swelling amiloride indeed stimulated the rate of RVD (Fig. 4*A*), whereas a progressively smaller swelling resulted in a corresponding smaller effect (Fig. 4*B*) or no effect (Fig. 4*C*) of amiloride. Another treatment, the replacement of external Na^+ with $NMDG^+$, is also expected to stimulate RVD, if Na^+/H^+ exchange is activated by cell swelling. As shown in Fig. 5, faster and more extensive RVD was observed in Na-free than in Na^+ medium.

To investigate the assumption that the source of the acidification is recycling of extracellular Cl^- for cellular HCO_3^- via the anion exchanger (*see* Freeman et al., 1987), we have performed the volume change experiment at varying external Cl^- and followed changes in cytoplasmic pH. Figure 6 shows that, when extracellular chloride is reduced to 19 mM, no acidification occurred. At an extracellular chloride concentration of 38 mM a slight acidification was still seen, and at zero extracellular chloride

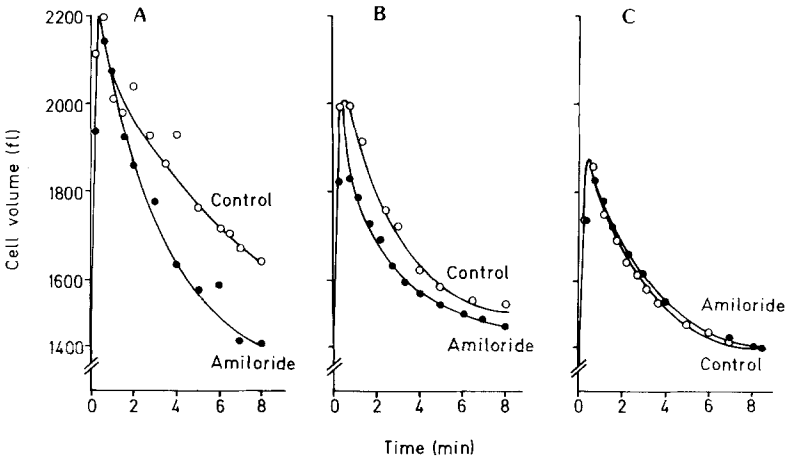

Fig. 4. Regulatory volume decrease in Ehrlich cells in the presence and absence of amiloride. After preincubation in 300 mOsm incubation medium for 15 min a sample of the cell suspension was diluted 1000- to 1500-fold with hypotonic medium of 145 mOsm (left frame), 160 mOsm (middle frame) and 175 mOsm (right frame).

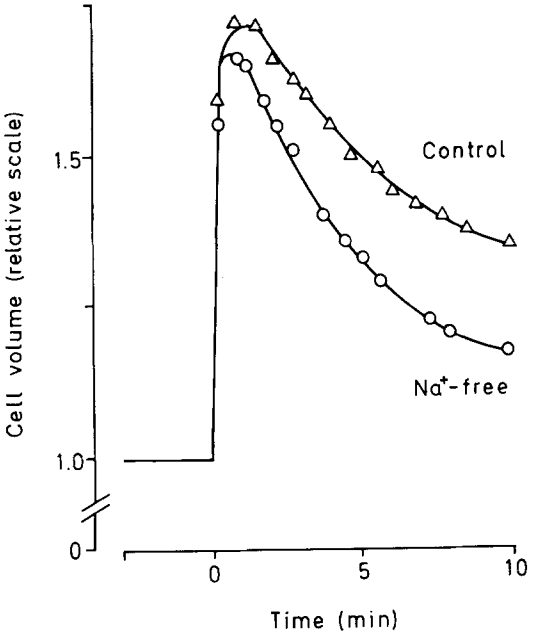

Fig. 5. Effect of Na^+ substitution on regulatory volume decrease in Ehrlich ascites tumor cells. At zero time a sample of the cell suspension was diluted in a hypotonic (150 mOsm) standard medium (control) or a hypotonic (150 mOsm) N-methyl-D-glucamine medium (Na^+-free), where all Na^+ was substituted by N-methyl-D-glucamine, and the cell volume was followed with time. The figure is representative of three independent experiments. The rate of cell shrinkage measured in the interval 1–3 min after dilution in hypotonic medium was 115 ± 15 and 75 ± 13 fl/min in the Na^+-free and in the Na^+-containing (control) medium, respectively. In paired experiments the rate was 1.54 ± 0.07 times higher in Na^+-free than in Na^+-containing medium

a slight alkalinization was observed (*data not shown*). The cytoplasmic chloride concentration is estimated at 29 mM after the initial swelling, assuming that the cells initially swell as perfect osmometers.

Discussion

Two lines of reasoning make it likely that Ehrlich tumor cells acidify internally during the process of RVD: (*i*) theoretical, in relation to the operation of bicarbonate chloride exchange (Lew & Bookchin, 1986); (*ii*) experimental, based on the precedence shown in blood platelets (Livne et al., 1987*b*). It may be concluded that the intracellular acidification of Ehrlich tumor cells during swelling leads to activation of Na^+/H^+ exchange which, in turn, affects RVD. This conclusion is supported by several observations. (*i*) Acidification accompanies RVD (Fig. 1). The extent of acidification roughly corresponds to the extent required to attain the pH_i 'set point' for Na^+/H^+ activation (Fig. 3). (*ii*) Amiloride accelerates RVD, at least following a moderate or an extensive swelling (Fig. 4*A,B*). (*iii*) RVD is enhanced when Na^+ is replaced by an impermeable cation (Fig. 5). (*iv*) Amiloride and Na^+-free medium facilitate a more complete RVD, so that the original volume is more closely reached.

The changes affected by amiloride and Na-free medium are in harmony with the proposition that Na^+ taken up by the cells through the activated Na^+/H^+ exchange hampers the rate and extent of RVD. The Na^+ taken up by the cells is osmotically active, whereas the cytoplasmic buffering power will replenish most of the extruded H^+. Cu^{2+}, which is found to activate Na^+/H^+ exchange in Ehrlich cells (Kramhøft et al., 1988), is also found to impair RVD by activating an amiloride-sensitive influx of Na^+. Activation of the exchange appears to be a secondary effect, initiated by cytoplasmic acidification. Activation by reduced pH_i appears to be a general property of Na^+/H^+ exchange, shared by many cell types. The reason for the acidification in osmotically swollen Ehrlich cells is likely to result from recycling of Cl^- for HCO_3^- (Freeman et al., 1987), yet metabolic acid production or activation

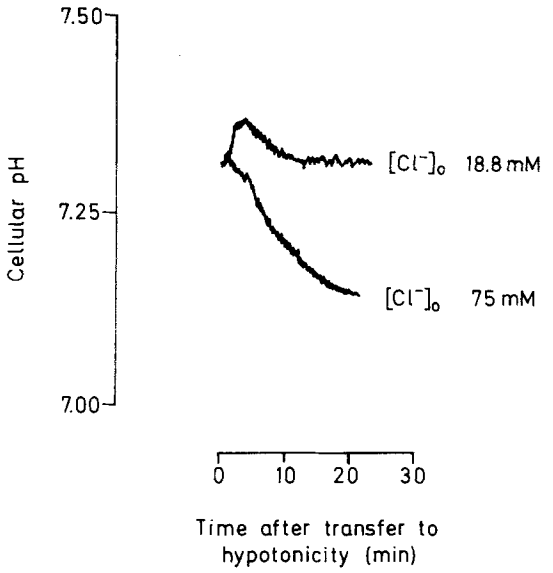

Fig. 6. Effect of Cl^- substitution on the shifts in intracellular pH of Ehrlich cells as a function of time after transfer to hypotonic conditions. At zero time BCECF-loaded cells were transferred to a hypotonic (150 mOsm) standard medium (75 mM Cl^-) or a hypotonic (150 mOsm) medium where ¾ of the NaCl was substituted by NaGluconate in equimolar amounts (18.75 mM Cl^-)

of K^+/H^+ exchange as described for *Amphiuma* red cells (Cala, 1985) should also be considered. The assumption that cellular bicarbonate is extruded in exchange for extracellular chloride is, however, supported by the experiments in which external chloride was reduced (*see* Fig. 6). At an extracellular Cl^- concentration in the hypotonic solution of 19 mM, which is well below the initial cytoplasmic Cl^- concentration immediately after the cell swelling, acidification was no longer seen, showing that an inward chemical gradient for chloride is required.

RVD has been reported to be incomplete in a number of different cell types (*see* review by Hoffmann & Simonsen, 1989). It is conceivable that in such cases an intracellular acidification activated Na^+/H^+ exchange, thereby partly neutralizing RVD, as previously demonstrated for platelets (Livne et al., 1987*b*) and shown for Ehrlich cells in this report.

The physiological significance of RVD has been documented in several studies (*see* review by Hoffmann & Simonsen, 1989). Cytoplasmic pH may play an important role as a second messenger in relation to several fundamental cellular functions (Busa, 1986). The present study and the previous study on platelets (Livne et al., 1987) illustrate the interplay between volume and pH regulatory mechanisms with the participation of the Na^+/H^+ exchange system. Since the properties of the Na^+/H^+ exchanger in Ehrlich cells (*see* Kramhøft et al., 1988) and in platelets (*see* Livne et al. 1987*a,c*) are in general similar to those of the exchanger in many other cells (*see* e.g., Frelin et al., 1986; Grinstein & Rothstein, 1986) and since activation of separate K^+ and Cl^- channels after cell swelling are now demonstrated in several cell types (*see* Hoffmann & Simonsen, 1989) it is suggested that such interplay between volume and pH regulatory mechanisms is a general phenomenon in animal cells.

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