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Shrinkage-induced Activation of the Na^+/H^+ Exchanger in Ehrlich Ascites Tumor Cells: Mechanisms Involved in the Activation and a Role for the Exchanger in Cell Volume Regulation

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Abstract. Amiloride-sensitive, Na^+-dependent, DIDS-insensitive cytoplasmic alkalinization is observed after hypertonic challenge in Ehrlich ascites tumor cells. This was assessed using the fluorescent pH-sensitive probe 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). A parallel increase in the amiloride-sensitive unidirectional Na^+ influx is also observed. This indicates that hypertonic challenge activates a Na^+/H^+ exchanger. Activation occurs after several types of hypertonic challenge, is a graded function of the osmotic challenge, and is temperature-dependent. Observations on single cells reveal a considerable variation in the shrinkage-induced changes in cellular pH_i, but the overall picture confirms the results from cell suspensions.

Shrinkage-induced alkalinization and recovery of cellular pH after an acid load, is strongly reduced in ATP-depleted cells. Furthermore, it is inhibited by chelerythrine and H-7, inhibitors of protein kinase C (PKC). In contrast, Calyculin A, an inhibitor of protein phosphatases PP1 and PP2A, stimulates shrinkage-induced alkalinization.

Osmotic activation of the exchanger is unaffected by removal of calcium from the experimental medium, and by buffering of intracellular free calcium with BAPTA.

At 25 mM HCO_3^-, but not in nominally HCO_3^--free medium, Na^+/H^+ exchange contributes significantly to regulatory volume increase in Ehrlich cells.

Under isotonic conditions, the Na^+/H^+ exchanger is activated by ionomycin, an effect which may be secondary to ionomycin-induced cell shrinkage.

Key words: Phosphorylation — Protein kinases — Protein phosphatases — Ca^{2+} — cAMP — PKC inhibitors

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Introduction

The existence of an amiloride-sensitive Na^+/H^+ exchanger in Ehrlich ascites tumor cells has previously been reported (Doppler, Maly & Grunicke, 1986; Wiener, Dubyak & Scarpa, 1986; Kramhøft, Lambert & Hoffmann, 1988; Levinson, 1991; Kramhøft, Hoffmann & Simonsen, 1994). The Na^+/H^+ exchanger is usually almost inactive at physiological pH values (Doppler et al., 1986; Kramhøft et al., 1988; Kramhøft et al., 1994) although chemical gradients favoring net inward transport of Na^+ and net outward transport of H^+ are present under these conditions.

In agreement with findings in other cell types, the Na^+/H^+ exchanger in Ehrlich cells is activated by various stimuli such as cytoplasmic acidification (Kramhøft et al., 1988), hypertonic stress (Levinson, 1991), addition of extracellular ATP (Wiener et al., 1986) and phorbol esters (Wiener et al., 1986; Kramhøft et al., 1988). Furthermore, the Na^+/H^+ exchanger in these cells is activated by addition of micromolar concentrations of $CuSO_4$ (Kramhøft et al., 1988).

In addition to the Na^+/H^+ exchanger, a DIDS-sensitive, Na^+-dependent Cl^-/HCO_3^- exchanger, and possibly a H^+ pump, have been suggested to be involved in the recovery of pH_i in Ehrlich cells after intracellular acidification, while a DIDS-sensitive, Na^+-independent Cl^-/HCO_3^- exchanger appears to be the main mechanism involved in the recovery of pH_i after intracellular alkalinization (Kramhøft et al., 1994). For recent reviews on the structure, function and regulation of the Na^+/H^+ exchanger, *see* Noël & Pousségur (1995), Tse et al. (1993), and Wakabayashi et al. (1992).

The present study was initiated to obtain further information about the activation of Na^+/H^+ exchange by hyperosmotic stress in Ehrlich cells, and about the cel-

lular signalling pathways involved in the activation process. Levinson (1991) suggested that protein phosphorylation events might be involved in the activation process. However, this was not followed up experimentally, and the question has not previously been addressed in this cell type. Phosphorylation and dephosphorylation processes appear to be involved in the regulation of the Na^+/H^+ exchanger in other cell systems (Bianchini et al., 1991; Sardet et al., 1990, 1991; Grinstein et al., 1992*b*).

In Ehrlich cells, PKC was suggested to be involved in the activation of the exchanger by extracellular ATP (Wiener et al., 1986). PKC was recently found to be involved in the regulation of another membrane transport system in Ehrlich cells, namely the $Na^+,K^+,2Cl^-$ cotransporter (Jensen, Jessen & Hoffmann, 1993). Consequently, the role of protein kinases and phosphatases in the hyperosmotic activation of Na^+/H^+ exchange in Ehrlich cells is addressed in the present study. The role of the intracellular free calcium concentration ($[Ca^{2+}]_i$) is also investigated, since contradictory observations regarding the role of Ca^{2+} in the activation of the Na^+/H^+ exchanger exist (*see* Mitsuhashi & Ives, 1988; Wøll et al., 1993).

In lymphocytes, *Amphiuma* red blood cells and other, mainly epithelial cell types, the main mechanism of Regulatory Volume Increase (RVI) is Na^+/H^+ exchange functionally coupled to Cl^-/HCO_3^- exchange (*see* Cala & Grinstein, 1988; Hoffmann & Simonsen, 1989). In other cell types, RVI is mediated by bumetanide-sensitive $Na^+,K^+,2Cl^-$ cotransport (*see* Hoffmann & Dunham, 1995). Since the Na^+/H^+ exchanger is activated by cell shrinkage in Ehrlich cells, and since Cl^-/HCO_3^- exchange systems are also present in these cells (Kramhøft et al., 1994), RVI by virtue of functional coupling of these transport systems should be possible. However, it was previously shown that RVI in Ehrlich cells is mediated by activation of a bumetanide-sensitive anion-cation transport system (Hoffmann, Sjøholm & Simonsen, 1983), which was later shown to be a $Na^+,K^+,2Cl^-$ cotransporter (Jensen et al., 1993). It was moreover found that in nominally HCO_3^--free medium, amiloride was without effect on RVI in these cells (Hoffmann & Simonsen, 1989).

The signal transduction pathways for activation of the RVI response in Ehrlich cells as well as in most other cell types, are still largely unknown (Hoffmann, Simonsen & Lambert, 1993; Hoffmann & Dunham, 1995). However, it has recently been shown that PKC is activated after shrinkage in Ehrlich cells (Larsen, Jensen & Hoffmann, 1994).

In the present study, the putative role of Na^+/H^+ exchange as a means of volume regulation in Ehrlich cells is addressed, and it is found that experimental conditions exist under which Na^+/H^+ exchange does become volume regulatory in Ehrlich cells.

A preliminary report of these results has previously been presented in abstract form (Pedersen et al., 1994).

Materials and Methods

Cell Suspensions and Incubation Media

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in white NMRI mice by weekly intraperitoneal transplantation. One week after transplantation, the mice were sacrificed by cervical dislocation and the cells contained in the ascites fluid were harvested in standard incubation medium containing heparin (2.5 IU/ml). The cells were washed 2–3 times by centrifugation (700 × *g*, 45 sec) in standard medium without heparin and subsequently suspended at a cytocrit of 4–8%. Usually, the cell suspensions were incubated in standard medium for 20–30 min before experiments.

Standard incubation medium was of the following composition (mM): 150 Na^+, 5 K^+, 150 Cl^-, 1 Mg^{2+}, 1 Ca^{2+}, 1 SO_4^{2-}, 1 PO_4^{3-}, 3.3 MOPS, 3.3 TES, 3.3 HEPES; pH was adjusted to 7.4 with NaOH unless otherwise indicated. In KCl- and NMDG-Cl media, KCl or NMDG-Cl, respectively, were substituted for NaCl in equimolar amounts. In these media, pH was adjusted with KOH. In Ca^{2+}-free medium, addition of Ca^{2+} was omitted and EGTA (2 mM) was added. In ''high $[K^+]$'' medium (53 mM K^+), K^+ was substituted for 48 mM of the Na^+-content. In bicarbonate media, 25 mM $NaHCO_3$ was substituted for 25 mM NaCl. In experiments performed at pH_o 8.3, MOPS, TES and HEPES were replaced by 5 mM BICINE and 5 mM TRICINE. In sucrose media, the desired osmolarity was obtained by addition of sucrose to the standard medium or NMDG-Cl medium. Medium osmolarity was (in mOsm): isotonic media: 310, hypotonic media: 160, hypertonic media: 420, 540 or 660. Medium osmolarity was measured by freezing point depression using a Knaur Osmometer. All media contained the same concentration of buffers as the standard medium. Unless otherwise indicated, experiments were carried out at 37°C.

RVD/RVI Protocol

Hypertonic challenge according to the ''RVD/RVI'' protocol was essentially as described by Hoffmann, Sjøholm & Simonsen (1983). Briefly: after harvest in standard medium, the cells were subjected to hypotonic conditions for 15–20 minutes. During this period, the initial osmotic swelling is succeeded by a period of Regulatory Volume Decrease (RVD). The cells were then transferred to isotonic medium. This results in immediate osmotic shrinkage, succeeded by Regulatory Volume Increase (RVI).

For measurements of intracellular pH, the cells were suspended in the hypotonic medium immediately after harvesting, during which time they were also loaded with BCECF-AM (see below). This procedure was found not to affect BCECF loading. For cell volume measurements, the cells were incubated for 20 min in standard medium prior to the hypotonic treatment.

Measurements of Intracellular pH in Cell Suspensions

Intracellular pH (pH_i) of cell suspensions was assessed fluorimetrically using the pH-sensitive fluorescent probe BCECF. Cell suspensions (4 ml, cytocrit 4%, or, in experiments using BAPTA-AM, 40 ml, cytocrit 0.4% were incubated at 37°C for 30–35 min with BCECF-AM (3.6 μM or 0.36 μM, respectively). The cells were then washed by

centrifugation (twice, 700 × *g*, 30 sec) in standard medium containing BSA (1 mg/ml) to remove extracellular BCECF, and subsequently resuspended at a cytocrit of 8% in standard medium containing BSA. BSA in the medium was found to be necessary to reduce extracellular fluorescence to acceptable levels (16% of the total fluorescence at the beginning of the experiments; *see also* Kramhøft et al., 1988).

Fluorescence measurements were performed after dilution of the cell suspension to a cytocrit of 0.16% in a thermostatically controlled (25°C) and magnetically stirred polystyrene cuvette in a Perkin Elmer LS-5 luminiscence spectrophotometer. The temperature 25°C was chosen to minimize leakage of BCECF from the cells (Kramhøft et al., 1988). At this temperature, total extracellular fluorescence was found to increase from 16 to 27% of the total fluorescence in 45 min, at which time experiments were terminated. Excitation wavelengths were 445 nm and 495 nm. Emission was measured at 525 nm. Excitation- and emission slit widths were 5 nm. Data were stored by a computer, the 445 nm/495 nm ratio was calculated on line by the computer software, and fluorescence traces were made using a computer spreadsheet. During the time course of an experiment, the contribution from extracellular fluorescence was calculated to change the 445/495 nm ratio by less than 2%, hence, this was not corrected for. Fluorescence ratios were converted to pH_i-values by *in situ* calibration after each experiment according to the nigericin method of Thomas et al. (1979), described for Ehrlich cells by Kramhøft et al. (1988). Amiloride was previously found to reduce BCECF fluorescence in single-wavelength measurements by about 10% (Kramhøft et al., 1988). In the present study, chelerythrine was found to increase the BCECF fluorescence ratio by about 10%. Therefore, in experiments with amiloride or chelerythrine, calibration was carried out in the presence of these compounds. In experiments measuring pH_i in BAPTA-loaded cells, loading of the cells with BAPTA was as previously described (N.K. Jørgensen, I.H. Lambert & E.K. Hoffmann, submitted), except that the cells were simultaneously loaded with BCECF, a procedure found not to affect BCECF fluorescence.

Measurements of Intracellular pH in Single Cells

Fluorescence was assessed using a Zeiss Axiovert 10 fluorescence microscope equipped with a 40X/1.3 NA oil immersion akrostigmat (UV) objective. The method was essentially as that described by N.K. Jørgensen et al. (submitted) for measurements of $[Ca^{2+}]_i$, but modified for measurement of pH_i.

Cells were loaded with BCECF for 30 min as described above, after which time they were kept at 25°C to minimize leakage of BCECF (*see above*). For experiments, the cells were placed in a thermostatically controlled (25°C or 37°C) chamber (POC, Biophysica Technologies) on coverslips coated with poly-L-lysine (25 mg/ml). Excitation wavelengths were 436 nm and 490 nm (both 10 nm bandpass filters). Illumination was achieved using a 75 W Xenon lamp. Neutral density filters were inserted in the excitation light path to adjust the intensities of the excitation light. A K12 filter was used to ensure protection of the cells against infrared light. In addition, a BPB380/10 filter, used for measurements of $[Ca^{2+}]_i$, was present during measurements of pH_i, resulting in an additional 10% reduction of light intensity at both excitation wavelengths. The excitation- and neutral density filters were placed in an automated filter wheel (LUDL Electronic Products Ltd.) and selection of filters was computer-controlled by the digital image processing and quantitative fluorescence system (Image1/Fluor, Universal Imaging). In order to further minimize photobleaching and toxic effects of excessive illumination of the cells, a shutter was used to control illumination. Emitted light was passed through a BSP 510 dichroic mirror and filtered by a BP 520-560 filter. Fluorescence was viewed using an intensified CCD camera (CCD72 with a GenIIsys intensifier from Dage-MTI). To improve the signal-to-noise ratio, the images were collected as averages of 6 sequential video frames after 436 nm and 490 nm excitation, respectively. Fluorescence images were ratioed on a pixel by pixel basis. The images were stored as interlaced pair images on the hard disk of a 80386 33 Mhz computer (Victor) and displayed in pseudocolor on a high resolution RGB monitor (Sony Trinitron).

Calibration was performed after each experiment according to the nigericin method of Thomas et al. (1979). Three different values of pH_o were used in each calibration. The apparent pK_a of BCECF in Ehrlich cells under these conditions was estimated at 6.87, based on 3 separate sets of experiments using the nigericin calibration technique and 5 different values of pH_o. Autofluorescence as well as background contribution were found to be undetectable under the experimental conditions used.

Calculation of Rates of Change in pH_i, Intracellular H^+ Buffering Power (β_i), and H^+ Efflux Rates (J_{H^+})

Unless otherwise indicated, initial values of pH_i were virtually identical in all compared situations, hence, the rates of change in pH_i could be compared directly. In experiments assessing the effect of medium osmolarity, the rate of change in pH_i was calculated as the slope of the linear fit of the measurements obtained from time 2–4 min (at 25°C) or 1–2.5 min (at 37°C) after transfer of the cells to the experimental medium. During this period, the change in pH_i can be taken as linear ($R^2 > 0.98$). The activity of the Na^+/H^+ exchanger during recovery from an acid load was assessed using the NH_4^+ prepulse technique (*see* Boron, 1992). Briefly, cells were exposed to 15 mM NH_4Cl for 10 min, followed by resuspension of a small aliquot of the cells in standard isotonic medium in the cuvette. This causes an immediate intracellular acidification (due to NH_3 efflux) and subsequent recovery of pH_i, the amiloride-sensitive component of which was taken as the activity of the Na^+/H^+ exchanger. The rate of change in pH_i was calculated as the slope of the linear fit of the measurements obtained from time 0.5–1.5 min after transfer to the NH_4^+ free medium. The cellular H^+ buffering capacity (β_i) was determined by addition of 15 mM NH_4Cl to cell suspensions in Na^+-free media (NMDG-Cl substituted for NaCl; osmolarity adjusted to the desired value by addition of sucrose). The β_i was calculated from the initial change in pH_i resulting from addition of NH_4Cl (ΔpH_i) and from the intracellular concentration of NH_4^+ ($[NH_4^+]_i$) using the following relation: $\beta_i = [NH_4^+]_i/\Delta\ pH_i$ (*see* Boron, 1992). Solving the Henderson-Hasselbalch equation for $[NH_4^+]_i$ yields: $[NH_4^+]_i = [NH_3]_i \cdot 10^{pKa-pHi}$. The $[NH_3]_i$ is calculated from the Henderson-Hasselbalch equation for $[NH_4^+]_o$ and $[NH_3]_o$, and assuming that $[NH_3]_i = [NH_3]_o$ and $[NH_3]_o + [NH_4^+]_o = [NH_4Cl]$ added. The value of pKa of NH_4^+ used was 9.25 at 25°C. For the calculated β_i values, see legend to Table 3. Rates of H^+ efflux (J_{H^+}) were calculated as $dpH/dt \cdot \beta_i$, using the appropriate values of β_i.

Sodium Influx Measurements

The unidirectional Na^+ influx was determined using $^{22}Na^+$ (specific activity 40,000 Bq/ml) as a tracer. After harvesting, the cells were preincubated for 15–40 min in the relevant incubation medium (standard incubation medium for control experiments or hypotonic medium (160 mOsm) for RVD/RVI experiments). In experiments with chelerythrine, the drug (10 μM) was added 14 min prior to initiation of the flux. In all cases ouabain (1 mM) was added 3 min prior to initiation of the flux measurements. When used, amiloride (200 μM) was added 2.5 min prior to initiation of the flux measurements. Two min before ini-

tiation of the flux measurements, the cell suspension was mixed with 1/3 volume of either standard incubation medium (isotonic controls) or medium of double ionic strength (in order to restore tonicity to 310 mOsm in RVD/RVI experiments). The flux measurements were then initiated (zero time) by addition of $^{22}Na^+$ (40,000 Bq/ml). The cell suspensions were incubated at 37°C, and samples were subsequently collected at 0.25-min intervals. The cells were separated from the medium using cation exchange chromatography. This procedure was carried out essentially as described by Jessen et al. (1989, *see also* Jensen et al., 1993). All extracellular $^{22}Na^+$ is retained on the columns. The cells pass through the columns and are subsequently collected in plastic vials for measurement of radioactivity by liquid scintillation counting (Packard TriCarb 1900 TR Liquid Scintillation Analyzer). After the experiment samples (1 ml) of the cell suspensions were removed for determination of the dry weight of the cell suspensions. Unidirectional Na^+ influx is presented as $\mu mol \cdot g\ cell\ dry\ weight^{-1} \cdot min^{-1}$, calculated from the specific activity of the medium, the radioactivity of the cell lysates and the dry weight of the cell suspensions.

The flux measurements were performed in the absence and presence of amiloride (200 μM) in parallel, and the amiloride-sensitive flux was calculated as the difference between these fluxes.

Measurement of ATP, ADP and AMP

Aliquots of the cell suspension (1 ml) were centrifuged (17,000 × *g*, 5–10 min). The pellet was subsequently resuspended in 700 μl ice-cold PCA (0.4 N), whereby the nucleoside content was extracted, keeping the samples on ice for at least 5 min. The samples were centrifuged again, the supernatant saved and neutralised with KOH (5 N), and 40 μl samples of this extract were analysed for adenosine nucleotides by HPLC using a strong anion exchange column (Partisil-10 Sax, 2 mm ø × 200 mm, Whatman, Clifton, NJ). Samples of ATP, ADP and AMP (1 nmole, Sigma) were used as standards. The column was eluted as follows: 3 min with 5 mM KH_2PO_4, pH 3.5, followed by a linear gradient for 14 min to 400 mM KH_2PO_4 plus 800 mM NaCl, pH 3.5. Subsequently, an isocratic period of 20 min was allowed. The column was reequilibrated with the starting buffer prior to each new sample injection. The rate of flow as 0.7 ml/min. The absorbance at 254 nm was recorded, and the resulting peaks were automatically integrated.

Cell Volume Measurements

Cell volume was measured by electronic cell sizing in a Coulter Counter model ZB equipped with a Coulter channelyzer (C-1000). The tube orifice was 100 μM. Mean cell volume was calculated as the median of the volume distribution curves after calibration with latex beads (diameter 13.5 μm, Coulter Electronics LTD, Luton Beds, England). Media used for cell volume measurements were filtered (Millipore filters, 0.45 μm) prior to experiments.

Reagents

Unless otherwise indicated, all reagents were analytical grade and obtained from Sigma (Saint Louis, MO). BCECF-AM, fura-2-AM and BAPTA-AM were purchased from Molecular Probes (Eugene, OR). Chelerythrine and Calyculin A (CL-A) were from Alomone Labs (Jerusalem, Israel) and $^{22}Na^+$ was from Amersham (Buckinghamshire, UK). Heparin was from Løvens Kemiske (Ballerup, Denmark).

Chelerythrine (0.5 mM), amiloride (10 mM), DIDS (10 mM), bradykinin (1 mM), thrombin (1000 U/ml) and poly-L-lysine (250 mg/ml) were prepared as aqueous stock solutions. Ouabain was dissolved at a concentration of 10 mM in standard incubation medium. Nigericin (1 mg/ml), CL-A (20 μM), ionomycin (1 mM), H-7 (5 mM), pimozide (10 mM) and bumetanide (10 mM) were dissolved in 96% ethanol. All these reagents were kept at −20°C until use. Sodium azide (2 M) was dissolved in standard incubation medium, glucose (2 M), deoxyglucose (2 M), sucrose (2.5 M) and NH_4Cl (1 M) were prepared as aqueous stock solutions. These stock solutions were kept refrigerated. BCECF-AM (1.2 mM), fura-2-AM (1 mM) and BAPTA-AM (10 mM) were dissolved in dessicated DMSO, and kept at −20°C until use. The final concentration of DMSO was 0.3% for BCECF-AM, 0.2% for fura-2-AM and 0.5% for BAPTA-AM solutions. At these concentrations, the experiments were unaffected by the DMSO vehicle (*data not shown*). Where 96% ethanol was used as a solvent, the final concentration was about 0.5%. At this concentration, ethanol alone had no effect on pH_i (*data not shown*).

Statistical Evaluation

Results are presented either as means ± SEM, with the number of independent experiments in parenthesis or as single experiments representative of at least three individual experiments. Significance was tested using either a Student's *t*-test (level of significance $P < 0.05$), or analysis of variance (ANOVA).

Abbreviations

BAPTA-AM:	1,2-bis-(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester
BCECF-AM:	2′,7′-bis-(2-carboxyethyl)-5,6 carboxyfluorescein, tetraacetoxymethyl ester
BICINE:	N,N-bis (2-hydroxyethyl) glycine
BSA:	Bovine serum albumin, fraction V
DIDS:	4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid
DMSO:	dimethylsulfoxide
EGTA:	ethylene glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid
HEPES:	N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid
MOPS:	3-(N-morpholino) propanesulfonic acid
NMDG:	N-methyl-D-glucamine
PCA:	perchloric acid
TES:	N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid
TRICINE:	N-tris (hydroxymethyl) methylglycine
TRIS:	tris (hydroxymethyl) aminomethane

Results

Effect of Osmotic Shrinkage on pH_i

Amiloride-sensitive, Na^+-dependent, DIDS-insensitive Cytoplasmic Alkalinization

Figure 1 shows the results of measurements of pH_i in cells in suspension at 25°C as a function of time after exposure to hypertonicity using the RVD/RVI protocol (*see* Materials and Methods) and under various conditions. As seen (Control), a significant intracellular alkalinization is observed after a lag period of 1½–2 min.

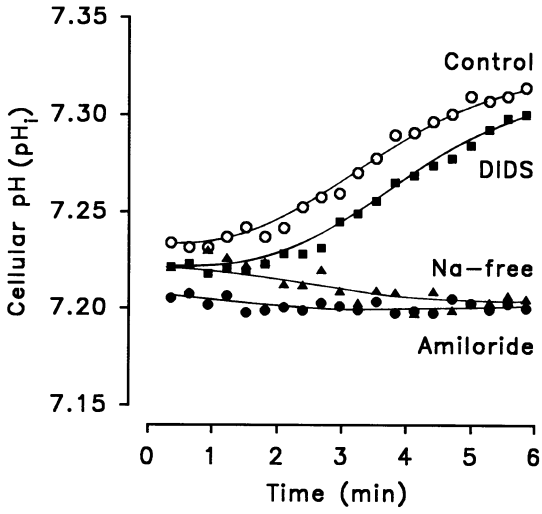

Fig. 1. Typical experiment showing cellular pH (pH_i) as a function of time after exposure to hypertonic challenge using the RVD/RVI protocol. Each curve was produced from the data points collected during the experiment, using a computer spreadsheet (*see* Materials and Methods). At zero time a sample of the hypotonic cell suspension was diluted in the experimental media: Standard medium (open circles), standard medium with amiloride (200 μM, filled circles), DIDS (300 μM, filled squares), or in Na^+-free NMDG medium (filled triangles). Cellular pH was assessed spectrophotometrically at 25°C. The figure is representative of 3–12 independent experiments.

Table 1. Rate of change in pH_i after hypertonic challenge using the RVD/RVI protocol

	Rate of change in pH_i (pH units/min)	
	Isotonic	Hypertonic
Control	-0.003 ± 0.001 ($n = 7$)	0.022 ± 0.002 ($n = 12$)*
Amiloride (200 μM)	-0.008 ± 0.002 ($n = 5$)	-0.002 ± 0.002 ($n = 3$)*
DIDS (300 μM)	-0.011 ± 0.002 ($n = 4$)	0.023 ± 0.004 ($n = 5$)*
Na^+-free	-0.007 ± 0.005 ($n = 3$)	-0.005 ± 0.002 ($n = 3$)

The cells were exposed to hypertonic challenge using the RVD/RVI protocol or osmotically undisturbed (isotonic). Changes in pH_i were measured spectrophotometrically at 25°C. The experimental procedure was as described in the legend to Fig. 1. The rates of change in pH_i were calculated as the slope of the linear fit of the measurements obtained in the interval from time 2 to time 4 after transfer to the experimental medium. During this period the pH_i change can be taken as linear ($R^2 > 98\%$, *see* Fig. 1). Positive values represent intracellular alkalinization, negative values intracellular acidification. Results are expressed as means ± SEM, with the number of independent experiments in parenthesis. *The values are significantly different from the corresponding isotonic values ($P < 0.001$). The value obtained under hypertonic conditions in the presence of DIDS is not significantly different from the hypertonic control value ($P > 0.1$).

The rate of alkalinization at 25°C, calculated as the slope of the linear fit of the measurements obtained in the interval from 2 to 4 min after transfer to the experimental medium, is 0.022 ± 0.002 pH units/min ($n = 12$). This alkalinization is completely abolished in the absence of extracellular Na^+ (NMDG medium) as well as in the presence of amiloride (200 μM), an inhibitor of the Na^+/H^+ exchanger. As seen, DIDS (300 μM), an inhibitor of Cl^-/HCO_3^- exchange, has no significant effect on the observed alkalinisation, indicating that activity of a Cl^-/HCO_3^- exchanger is negligible under the present conditions.

Table 1 summarizes the rates of change in pH_i after hypertonic challenge compared to isotonic cell suspensions treated in parallel. As seen, the rate of alkalinization observed under hypertonic conditions in the presence of DIDS is not different from that observed in hypertonic controls. Taken together, the above results demonstrate an amiloride-sensitive, Na^+-dependent, DIDS-insensitive alkalinization during RVI, indicating that a Na^+/H^+ exchanger is activated. This is in agreement with observations on Ehrlich cells by Levinson (1991). Table 1 also shows that under isotonic conditions, a slight cytoplasmic acidification occurs in the presence of amiloride or DIDS, in agreement with results previously presented by Kramhøft et al. (1988).

Shrinkage-induced cytoplasmic alkalinization is also seen with other protocols of hypertonic challenge. The results are summarized in Table 2. As seen, the rates of cytoplasmic alkalinization seen after other protocols of hypertonic challenge are not significantly different from that seen using the RVD/RVI protocol. Similar levels of activity of the exchanger after various protocols of osmotic shrinkage appears to be a general phenomenon, reported in several cell types, including Ehrlich cells (*see* Discussion). However, for reasons which are not fully understood, in Ehrlich cells and most other cell types, complete RVI is only seen after the RVD/RVI protocol of osmotic cell shrinkage (*see* Materials and Methods; for a discussion: *see* Cala & Maldonado, 1994). The RVD/RVI protocol is employed in this study, except in ATP-depletion- and single cell experiments, in which a simple hypertonic challenge had to be used for technical reasons.

Next, measurements of pH_i were performed at the single-cell level employing fluorescence microscopy with digital image processing. A representative example of the pH_i response of single cells to a hypertonic treatment of 660 mOsm at 37°C is shown in Fig. 2. Consistent with the observations on cell suspensions presented above, shrinkage-induced intracellular alkalinization was observed in the great majority of cells. However, some apparently healthy cells (as judged by the appearance of the cells using phase contrast microscopy) exhibiting either no change in pH_i or even a slight intracellular acidification were also observed, constituting about 10% of the population. Furthermore, the lag-time of activation

Table 2. Rate of cytoplasmic alkalinization in Ehrlich ascites tumor cells after exposure to various protocols of hypertonic stress

Protocol	Rate of change in pH_i (pH units/min)
RVD/RVI	0.022 ± 0.002 ($n = 12$)
Hypertonic sucrose (450 mOsm)	0.017 ± 0.001 ($n = 4$)
Hypertonic NaCl (420 mOsm)	0.022 ± 0.003 ($n = 5$)
Hypertonic NaCl (540 mOsm)	0.028 ± 0.002 ($n = 7$)

The cells were subjected to hypertonic conditions by exposure to: (i) the RVD/RVI protocol (*see* Materials and Methods); (ii) hypertonic sucrose medium (osmolarity 450 mOsm); (iii) hypertonic medium of increased electrolyte concentration (osmolarity 420 mOsm); (iv) hypertonic medium of increased electrolyte concentration (osmolarity 540 mOsm). Changes in pH_i were measured spectrophotometrically at 25°C. The rate of cytoplasmic alkalinization was calculated as described in the legend to Table 1. All values are given as means ± SEM with the number of independent experiments in parenthesis. None of the results shown differ significantly at the 5% level (Significance test: ANOVA analysis of variance).

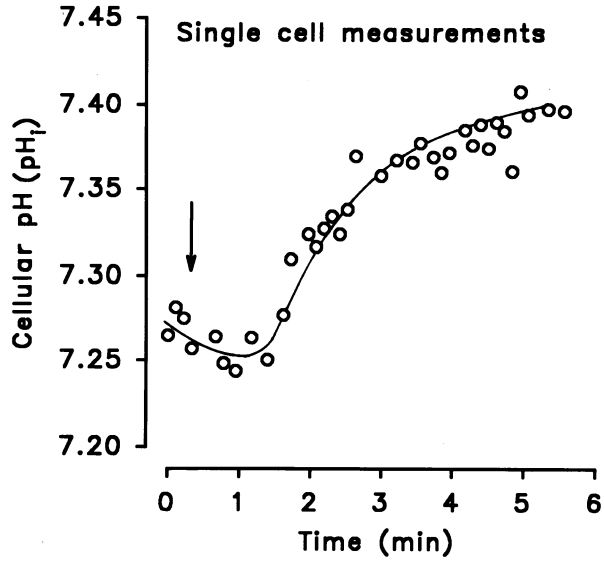

Fig. 2. Typical experiment showing cellular pH in a single cell measured as a function of time after exposure to hypertonic challenge. The graph was produced from the data points collected during the experiment, using a computer spreadsheet (*see* Materials and Methods). Cells were placed in a thermostatically controlled (37°C) microscope chamber and diluted to a cytocrit of 0.15% (*see* Materials and Methods). At the time indicated by the arrow, concentrated saline solution ($[Ca^{2+}]$ was 1 mM, as in the standard medium) was added to a final osmolarity of 660 mOsm. The trace is a representative measurement on a single cell out of 60 cells from 6 independent experiments.

varied (*not shown; see*, however, below). Shrinkage-induced alkalinization was found to be abolished in virtually all cells in the presence of amiloride (200 μM). Under these conditions, the average change in pH_i was -0.0027 ± 0.002 pH units/min (15 cells in 3 independent experiments), compared to 0.057 ± 0.002 pH units/min (60 cells in 6 independent experiments) in controls. Under isotonic conditions, a slight intracellular acidification was seen in virtually all cells (*data not shown*).

At the single cell level, measurements were performed at 25°C and 37°C, respectively. The lag time of the activation is temperature-dependent, decreasing from 1½–2 min at 25°C to ½–1 min at 37°C (*see* Fig. 2). Hence, rates of change in pH_i in single-cell experiments are calculated as the slopes of the linear fits of the measurements obtained in the interval from time 2 to 4 min (25°C) and 1–2.5 min (37°C), respectively, during which time the change in pH_i is linear. Intracellular H^+ buffering capacity (β_i) may vary with temperature and tonicity (*see* Boron, 1992). Consequently, β_i was determined for each experimental condition, and H^+ efflux rates (J_{H^+}) were compared rather than rates of intracellular alkalinization (*see* Materials and Methods). The values of β_i obtained in the present study (*see* legend to Table 3) range from about 19 to about 22 mmol $H^+ \cdot$ kg cell water$^{-1} \cdot$ pH unit^{-1}, depending on the temperature and on the tonicity of the experimental medium. In comparison, Bowen & Levinson (1984) found a value of β_i in Ehrlich cells of 27.7 ± 1.6 mmol $H^+ \cdot$ kg cell water$^{-1} \cdot$ pH unit^{-1}, or 100.3 mmol $H^+ \cdot$ kg dry wt$^{-1} \cdot$ pH unit^{-1}, obtained under isotonic conditions at 37°C. The shrinkage-induced J_{H^+} calculated using the appropriate values of β_i are shown in Table 3. As seen, the rate of shrinkage-induced J_{H^+} was found to be temperature-dependent. After exposure of the cells to a hypertonic challenge of 540 mOsm, a temperature coefficient (Q_{10}) of about 2 can be observed (2.79 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 25°C (29 cells in 3 experiments) compared to 4.60 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 37°C (44 cells in 4 experiments)). Furthermore, at 25°C as well as at 37°C, the shrinkage-induced J_{H^+} was found to increase with the magnitude of the hypertonic challenge. At 25°C: 2.37 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 420 mOsm, compared to 3.41 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 660 mOsm. At 37°C: 2.41 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 420 mOsm compared to 4.77 μmol $H^+ \cdot$ g dry wt$^{-1} \cdot$ min^{-1} at 660 mOsm (SEM values not provided, since J_{H^+} is the calculated product of dpH/dt and β_i. The corresponding rates of intracellular alkalinization are significantly different (25°C: $P < 0.02$, 37°C: $P < 0.001$, Student's *t*-test; 21–60 cells in 3–6 independent experiments)). Thus, the shrinkage-induced J_{H^+} appears to reflect the degree of hypertonic challenge, at 25°C as well as at 37°C (*see* Discussion).

If the shrinkage-induced cytoplasmic alkalinization demonstrated above is due to activation of a Na^+/H^+ exchanger, it should be possible to demonstrate an amiloride-sensitive increase in the unidirectional Na^+ influx in parallel with the cytoplasmic alkalinization. Table 4 shows the unidirectional Na^+ influx after hyper-

Table 3. Shrinkage-induced alkalinization and proton efflux. Dependence on temperature and magnitude of the hypertonic challenge

External osmolarity	25°C		37°C	
	ΔpH_i (pH units/min)	J_{H+} (μmol $H^+ \cdot$ g dry $wt^{-1} \cdot min^{-1}$)	ΔpH_i (pH units/min)	J_{H+} (μmol $H^+ \cdot$ g dry $wt^{-1} \cdot min^{-1}$)
420 mOsm	0.028 ± 0.003	2.37	0.030 ± 0.003[a]	2.41
540 mOsm	0.032 ± 0.002	2.79	0.056 ± 0.002	4.60
660 mOsm	0.037 ± 0.001	3.41	0.057 ± 0.002	4.77

Measurements were performed on single cells exposed to varying degrees of hypertonic challenge using the experimental procedure described in the legend to Fig. 2. The rates of change in pH_i (ΔpH_i, pH units/min) are calculated as the slope of the linear fit of the measurements obtained in the interval from time 1–2.5 min (37°C) or 2–4 min (25°C) after hypertonic challenge. During this period, the change in pH_i can be taken as linear ($R^2 > 98\%$). $J_{H^+} = dpH/dt \cdot \beta_i$ (in μmol $H^+ \cdot$ g dry $wt^{-1} \cdot min^{-1}$) was calculated in each case from the mean rate of change in pH_i and β_i determined at the appropriate temperature and tonicity (*see* Materials and Methods). The values of β_i were (in mmol $H^+ \cdot 1$ kg cell $water^{-1} \cdot$ pH $unit^{-1}$): At 25°C: 420 mOsm, 20.3 ± 1.5; 540 mOsm, 20.9 ± 1.6; 660 mOsm, 22.1 ± 2.2. At 37°C: 420 mOsm, 19.3 ± 0.27; 540 mOsm, 19.7 ± 0.38; 660 mOsm, 20.1 ± 0.55 (n = 3 in each case). The unit of β_i was converted to μmol $H^+ \cdot$ g dry $wt^{-1} \cdot$ pH $unit^{-1}$ using the dry weight of Ehrlich cells of 0.24 g dry wt per 1.00 g wet wt). The rates of change in pH_i are expressed as means ± SEM, and represent measurements on 21–60 cells in 3–6 independent experiments. The calculated values of J_{H+} are given without SEM. The values of ΔpH_i obtained at the osmolarities 420 mOsm and 660 mOsm are significantly different at 25°C ($P < 0.02$) as well as at 37°C ($P < 0.001$) (Significance test: Student's *t*-test). [a]Represents 3 experiments in 2 batches of cells.

Table 4. Unidirectional Na^+ influx in Ehrlich ascites tumor cells after hypertonic challenge and in osmotically undisturbed controls

Unidirectional Na^+ influx (μmol $\cdot$ g dry $wt^{-1} \cdot min^{-1}$)			
	Isotonic	Hypertonic	Shrinkage-induced increase
Control	17.7 ± 2.5 (n = 3)	42.4 ± 0.4 (n = 3)*	24.7 ± 2.5
Amiloride	17.4 ± 2.1 (n = 3)	32.5 ± 2.1 (n = 3)*,**	15.1 ± 3.0
Amiloride-sensitive, shrinkage-induced increase			9.6 ± 3.9

The unidirectional Na^+ influx was measured using $^{22}Na^+$ (specific activity 40,000 Bq/ml) as a tracer. For the RVD/RVI-experiment cells were preincubated in hypotonic medium (160 mOsm) for 15–40 min. Tonicity was restored to isotonicity (310 mOsm) by addition of the appropriate volume of double strength saline solution. Two minutes after restoration of tonicity, $^{22}Na^+$ was added (zero time). Ouabain (final concentration 1 mM) was added to all cell suspensions 3 min prior to initiation of the flux measurements. In experiments with amiloride, amiloride was added to the cell suspension (final concentration 200 μM) 2.5 min prior to initiation of the flux measurements. The cell suspensions were incubated at 37°C, and samples were removed at 0.25 min intervals from time 0.50 min to 1.5 min after addition of $^{22}Na^+$ for separation of cells from the medium (*see* Materials and Methods). The initial rate of flux was calculated by linear regression of measurements obtained within the first 1.5 min. In each case, $R^2 > 98\%$. All values are given as means ± SEM with the number of independent experiments given in parenthesis. *The values are significantly different from the unidirectional Na^+ influx in the corresponding osmotically undisturbed controls ($P < 0.01$). **The value is significantly different from the hypertonic control value ($P < 0.01$) (Significance test: Student's *t*-test).

tonic challenge, compared to the flux measured in osmotically undisturbed controls. The unidirectional Na^+ influx was calculated as the linear fit of measurements obtained in the interval from time 2 to 3.5 min after the hypertonic challenge, i.e., during the linear part of the cytoplasmic alkalinization (*see* Fig. 2). It should be noted that the bumetanide-*in*sensitive unidirectional Na^+ influx (primarily reflecting Na^+/H^+ exchange) during RVI in Ehrlich cells has previously been shown to be linear in the interval from 0 to 3 min after hypertonic challenge (Jensen et al., 1993). As seen, a significant, amiloride-sensitive increase in the unidirectional uptake of Na^+ of 9.6 ± 3.9 μmol $\cdot$ g dry $wt^{-1} \cdot min^{-1}$ is observed after the hypertonic treatment. This supports the notion that cell shrinkage activates the Na^+/H^+ exchange system in Ehrlich cells. Since measurements of pH_i were performed in the absence of bumetanide, this drug was absent in the flux studies for reason of comparison. However, the amiloride-*in*sensitive increase in the unidirectional Na^+ influx under hypertonic conditions of 15.1 ± 2.9 μmol $\cdot$ g dry $wt^{-1} \cdot min^{-1}$ is likely to primarily reflect the shrinkage-induced activation of the bumetanide-sensitive $Na^+,K^+,2Cl^-$ cotransporter (*see* Discussion).

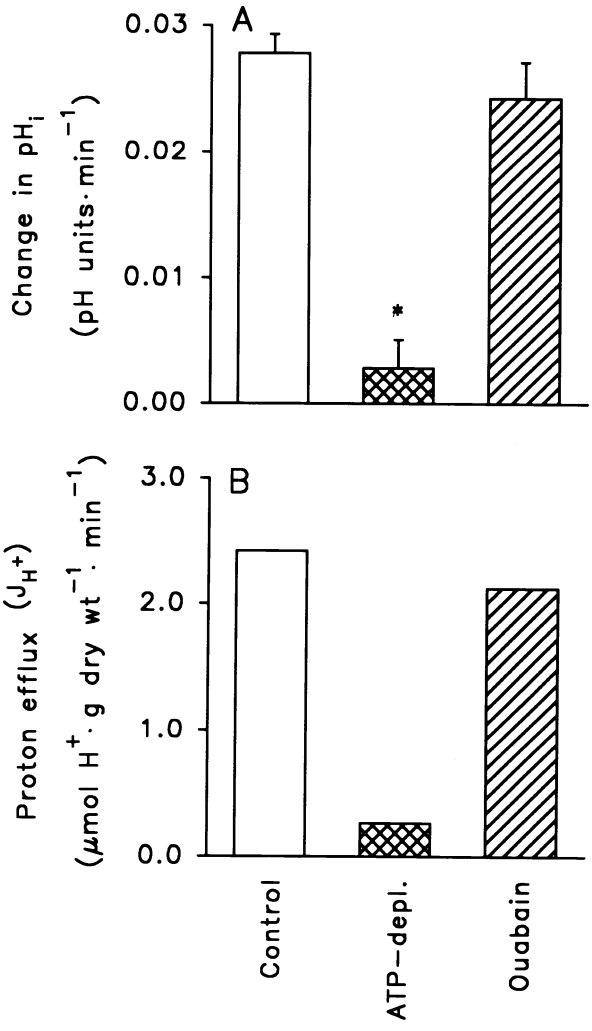

Fig. 3. Effect of ATP depletion on the rate of cytoplasmic alkalinization (*A*) and J_{H^+} (*B*) after hypertonic challenge. (*A*) The cellular ATP level was reduced by preincubation of the cells in standard incubation medium containing 10 mM sodium azide and 10 mM deoxyglucose for 30 min (during this time the cells were also incubated with BCECF-AM. This procedure was found not to affect fluorescence intensity). At zero time, the cells were exposed to hypertonic challenge by dilution of a sample of the cell suspension in concentrated saline solution (540 mOsm). Cellular pH (pH_i) was assessed spectrophotometrically at 25°C. The number of independent experiments were: ATP-depleted cells (n = 3), control cells in the absence ($n = 7$) or presence ($n = 3$) of ouabain (1 mM). ATP-depletion causes a decline in pH_i during the 30-min incubation period. In media of pH 7.4, the resulting pH_i is 7.00 ± 0.01 ($n = 3$) in depleted cells compared to 7.25 ± 0.03 ($n = 7$) in control cells. In order for rates of change in pH_i to be comparable in ATP-depleted cells and control cells, ATP depletion was carried out at pH_o 7.8, resulting in a pH_i of 7.10 ± 0.05 ($n = 5$) in ATP-depleted cells. The rates of cytoplasmic alkalinisation were calculated as described (*see* Materials and Methods; and also legend to Table 1). (*B*) The β_i was determined at the relevant pH_i and osmolarity (540 mOsm) in ATP-depleted cells and controls (*see* Materials and Methods). J_{H^+} was calculated as $dpH/dt \cdot \beta_i$. The values of β_i were (in mmol $H^+ \cdot 1$ cell $water^{-1} \cdot pH\ unit^{-1}$): control cells, 20.9 ± 1.6 ($n = 3$); ATP depleted cells 22.3 ± 1.5 ($n = 4$). The unit of the β_i values was converted to μmol $H^+ \cdot g$ dry $wt^{-1} \cdot pH\ unit^{-1}$ using the dry weight of Ehrlich cells of 0.24 g dry wt per 1.00 g wet wt. Results are expressed as means with SEM error bars, except in the case of J_{H^+} (*B*), which is the calculated product of dpH/dt and β_i, and for which no SEM is given. *The value is significantly different from the hypertonic control ($P < 0.01$). The values obtained in the presence of ouabain are not significantly different from those of the controls ($P > 0.1$) (Significance test: Student's *t*-test).

Mechanisms Involved in the Shrinkage-induced Activation of the Na^+/H^+ Exchanger in Ehrlich Ascites Tumor Cells

The aim of the following experiments was to investigate mechanisms involved in the shrinkage-induced activation of the Na^+/H^+ exchanger in Ehrlich cells. The following questions were asked: (i) Is the shrinkage-induced activation ATP-dependent, and does the activation of the Na^+/H^+ exchanger involve (ii) activation of protein kinases, (iii) inactivation of phosphatases, or (iv) changes in $[Ca^{2+}]_i$?

Dependence on ATP

According to Skovsgaard (1978), energy metabolism in Ehrlich cells should be blocked completely by incubation with sodium azide, an inhibitor of oxidative phosphorylation. However, in order to ensure maximal reduction of the cellular ATP level, cells were incubated in a combination of sodium azide (10 mM) and deoxyglucose (10 mM) for 30 min (during which time the cells were also loaded with BCECF, a procedure which was found not to affect BCECF loading). In control experiments it was shown that this treatment reduces the cellular ATP level to 0.115 ± 0.016 nmoles/mg dry wt (n = 4), compared to 12.4 ± 0.60 nmoles/mg dry wt (n = 4) in control cells. Upon addition of glucose (10 mM), the ATP content increases quickly and attains the normal level within 10 min. This indicates that viability of the cells is maintained even after ATP-depletion. Furthermore, leakage of BCECF from the cells is not increased after ATP-depletion, indicating that membrane integrity is maintained.

Figure 3 shows the rate of shrinkage-induced intracellular alkalinization (*A*) and J_{H^+} (*B*) obtained from measurements in cell suspensions at 25°C in ATP-depleted cells and in controls. As seen (*A*), the rate of shrinkage-induced cytoplasmic alkalinization is reduced by 90% in ATP-depleted cells. It should be noted that ATP-depletion causes a decline in pH_i of 0.25 pH units during the 30 min of incubation with the ATP depletion mixture, resulting in a difference between initial pH_i in ATP-depleted cells and controls. For rates of change in pH_i to be comparable in ATP-depleted cells and control cells, ATP-depletion was carried out at pH_o 7.8, resulting in a pH_i close to that of control cells at the time of initiation of the measurement of Na^+/H^+ exchanger activity (Fig. 3, legend).

Since β_i may be affected by the metabolic state of

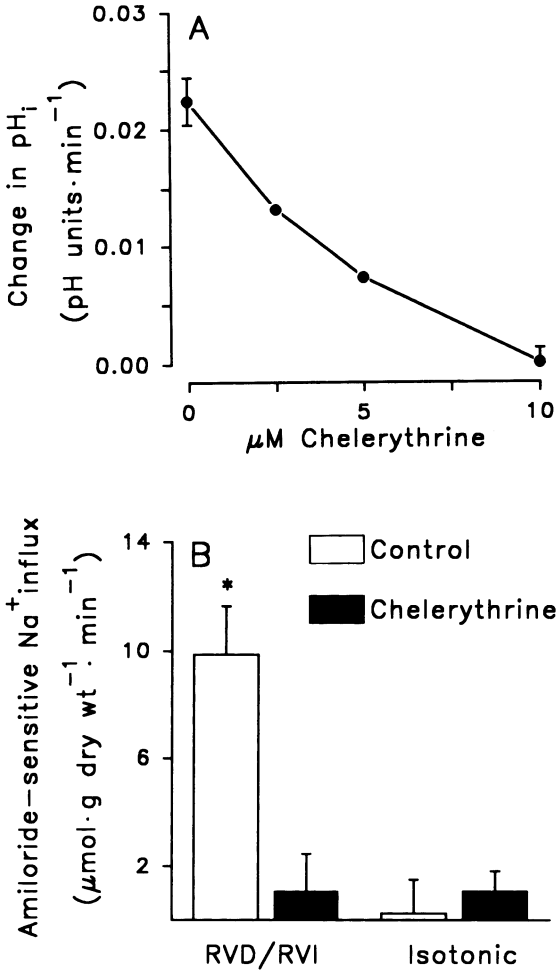

Fig. 4. Effect of chelerythrine on intracellular alkalinisation (*A*), and amiloride-sensitive unidirectional Na^+ influx (*B*), after a hypertonic challenge. (*A*) The cells were incubated with 2.5 μM (n = 3), 5.0 μM (n = 3) or 10 μM (n = 10) chelerythrine for 14 min prior to the measurements of intracellular pH. The number of experiments without chelerythrine was 12. The experimental procedure was as described in the legend to Fig. 1, except that chelerythrine was present in the experimental media at the concentrations indicated. The rates of cytoplasmic alkalinization were calculated as described (*see* Materials and Methods). Results are expressed as means with SEM error bars (some SEM values are too small to be seen on the graph). (*B*) The unidirectional Na^+ influx was measured using $^{22}Na^+$ as a tracer. The cells were preincubated for 15–40 min in either standard incubation medium (isotonic controls) or hypotonic medium (RVD/RVI protocol; *see* Materials and Methods). In experiments with chelerythrine, the drug (10 μM) was added 14 min before the flux was initiated by addition of $^{22}Na^+$. Chelerythrine (10 μM) was also present in the flux medium. Two min before addition of $^{22}Na^+$, the cell suspensions were mixed with 1/3 volume of either standard incubation medium (isotonic controls) or medium of double ionic strength (RVD/RVI experiments). In all cases, ouabain (1 mM) was added 3 min prior to addition of $^{22}Na^+$. Amiloride (200 μM) was added just prior to addition of $^{22}Na^+$ in part of the flux measurements. The initial rate of Na^+ influx was calculated as described in the legend to Table 4. The amiloride-sensitive Na^+ influx was calculated as the differences between parallel fluxes obtained in the absence and presence of amiloride (*see also* Table 4). The results are expressed as means of 3 independent experiments, with SEM error bars. *The value is significantly different at the 1% level from the other values shown in the figure (Significance test: ANOVA analysis of variance).

the cells (*see* Boron, 1992), Na^+/H^+ activity is also expressed as J_{H^+} (Fig. 3, *B*), using the values of β_i determined in ATP-depleted cells and control cells (*see* Materials and Methods). As seen, the calculated rate of shrinkage-induced decrease in J_{H^+} in ATP-depleted cells is reduced by 89% compared to control cells.

ATP-depletion could conceiveably result in inhibition of the plasma membrane Na^+/H^+-ATPase and, therefore, a dissipation of the transmembrane Na^+ gradient resulting, indirectly, in inhibition of the Na^+/H^+ exchanger. However, previous studies have shown that inhibition of the Na^+/H^+-ATPase in Ehrlich cells with ouabain results in a gradual increase in cellular Na^+ concentration from 19 to 22 mM during a period of 15 min (B. Kramhøft, *unpublished results*). Thus, during 30 min, an increase to only about 25 mM is to be expected. Since $[Na^+]$ of the standard medium is 150 mM, a considerable transmembrane Na^+ gradient should be present also in ATP-depleted cells. Moreover, as seen in Fig. 3, the shrinkage-induced decrease in $[H^+]_i$ is unaffected by ouabain (1 mM). The rate of recovery from cytoplasmic acidification following a NH_4Cl prepulse (*see* Materials and Methods) was also assessed in ATP-depleted cells and controls. The initial rate of recovery in the ATP-depleted cells is reduced to 0.0041 ± 0.0057 ($n = 3$), compared to 0.048 ± 0.0085 ($n = 3$) in control cells, a reduction of 91.5%. Thus, these results indicate that acidification-induced as well as shrinkage-induced activity of the Na^+/H^+ exchanger is dependent on intracellular ATP.

Involvement of Protein Kinase C

Figure 4 (*A*) shows the effect of chelerythrine, a highly specific inhibitor of PKC (Herbert et al., 1990), on the rate of shrinkage-induced intracellular alkalinization. Chelerythrine (2.5, 5 or 10 μM) was added 14 min prior to the hypertonic challenge. As seen, chelerythrine causes a concentration-dependent inhibition of the shrinkage-induced alkalinization. Based on these results, the IC_{50} of chelerythrine for shrinkage-induced alkalinization can be estimated at about 2.7 μM. At 10 μM, chelerythrine causes almost complete abolishment of the alkalinization response. In cells preincubated with chelerythine (10 μM), a slight intracellular acidification of 0.009 ± 0.001 pH units/min (n = 3) is observed under isotonic conditions.

As seen in Fig. 4 (*B*), chelerythine (10 μM) reduces the shrinkage-induced amiloride-sensitive unidirectional Na^+ influx to a level not significantly different from that of osmotically undisturbed controls. It should be noted

that in control experiments, chelerythrine (10 μM) was found to have no effect on cell volume (*data not shown*). Thus, taken together, the results shown in Fig. 4 strongly suggest that incubation of the cells with chelerythrine results in inhibition of shrinkage-induced Na^+/H^+ exchange.

The effect of H-7, another inhibitor of PKC was also assessed. After 20 min pretreatment with H-7 (100 μM) the rate of shrinkage-induced alkalinization at 25°C is 0.014 ± 0.003 pH units/min ($n = 3$), compared to 0.022 ± 0.002 pH units/min ($n = 12$) in hypertonic controls, i.e., a 38% inhibition. Under isotonic conditions, H-7 has virtually no effect on pH_i (*data not shown*). The observed effects of chelerythrine and H-7 indicate a role for a PKC isoform in the shrinkage-induced activation of the Na^+/H^+ exchanger (*see* Discussion).

Involvement of Protein Phosphatases

Figure 5 (*A*) shows the effect of Calyculin A (CL-A), a potent inhibitor of the serine/threonine protein phosphatases PP1 and PP2A (Hosoya et al., 1993), on pH_i of osmotically undisturbed Ehrlich cells. It has previously been shown that CL-A does not cause cell shrinkage in Ehrlich cells (Jakobsen, Jensen & Hoffmann, 1994). Therefore, the effects of CL-A on pH_i observed in the present study are not due to effects of CL-A on cell volume. Measurements of pH_i were performed on cell suspensions at 25°C. Under isotonic conditions addition of CL-A (100 nM) results in a slight initial cytoplasmic acidification followed by return to normal pH_i. Thus, inhibition of dephosphorylation does not seem to result in stimulation of the Na^+/H^+ exchanger under isotonic conditions. The slight acidification (0.03 pH units) seen in the presence of CL-A under isotonic conditions is unaffected by DIDS (*data not shown*), indicating that it is not caused by the activation of a DIDS-sensitive pH_i regulatory system, but rather reflects an increased metabolic production of acid equivalents in the presence of the phosphatase inhibitor.

Figure 5 (*B*) shows the effect of CL-A on cytoplasmic alkalinization after hypertonic challenge using the RVD/RVI protocol. CL-A (100 nM) causes an increase of 36% in the rate of shrinkage-induced alkalinization (measured as the linear fit of the measurements obtained from time 2–4 min after hypertonic challenge) from 0.022 ± 0.002 pH units/min ($n = 12$) in controls to 0.030 ± 0.002 pH units/min ($n = 5$), a rate significantly different ($P < 0.03$) from that of hypertonic control cells. Furthermore, the resulting final pH_i level is seen to be increased in cells treated with CL-A as compared to the pH_i level obtained in the controls. These data indicate that when dephosphorylation is inhibited, shrinkage-induced activation of the Na^+/H^+ exchanger is stimulated. In support of the above, similar effects of CL-A were observed in single cell experiments (*data not shown*). In preliminary experiments, okadaic acid (OA), another inhibitor of protein phosphatases PP1 and PP2A, had little effect on Na^+/H^+ exchange activity in Ehrlich cells (*data not shown*). This may be due to a lower plasma membrane permeability of OA compared to CL-A in these cells, as has recently been observed in rabbit red blood cells (M. Jennings, *personal communication*).

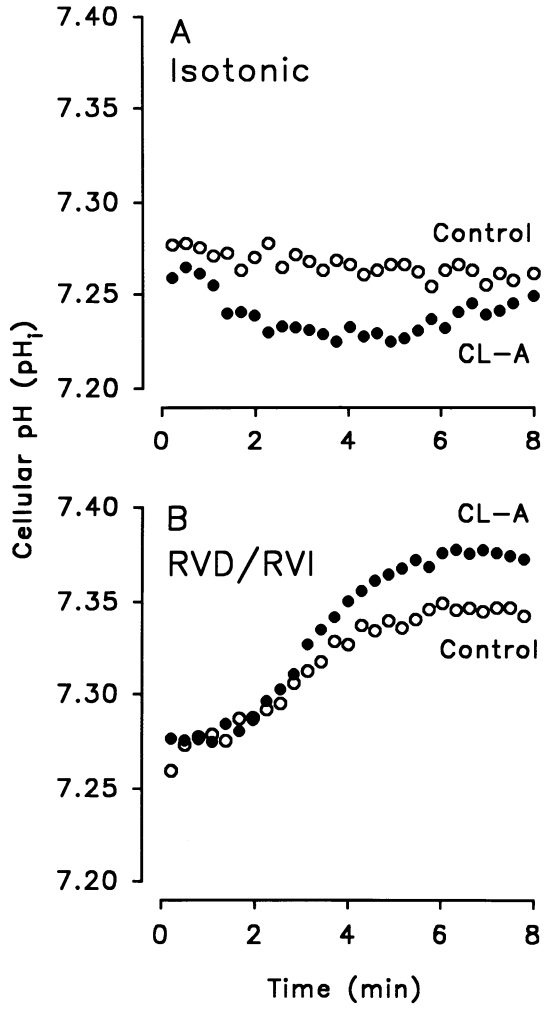

Fig. 5. Typical experiments showing the effect of calyculin A (CL-A) on intracellular pH. Each graph was produced from the data points collected during that experiment, using a computer spreadsheet (*see* Materials and Methods). (*A*) Osmotically undisturbed cells. (*B*) Cells exposed to hypertonic challenge using the RVD/RVI protocol. The experimental procedure was as described in the legend to Fig. 1 except that CL-A (100 nM) was present from time zero. Both panels are representative of at least 3 individual experiments. The rate of shrinkage-induced intracellular alkalinization (calculated as described in Materials and Methods) in the presence of CL-A is 0.030 ± 0.002 pH units/min ($n = 5$), and significantly different from the rate of 0.022 ± 0.002 pH units/min ($n = 12$) in controls ($P < 0.03$) (Significance test: Student's *t*-test).

The Role of $[Ca^{2+}]_i$ and the Ca^{2+}/Calmodulin System

The possible involvement of changes in $[Ca^{2+}]_i$ in shrinkage-induced activation of the Na^+/H^+ exchanger was investigated in a set of experiments in which (i) Ca^{2+} was removed from the experimental medium, or (ii) changes in $[Ca^{2+}]_i$ were prevented using BAPTA, an EGTA-derivative, which is a potent chelator of intracellular calcium (Tsien, 1980). The experiments were performed on cell suspensions at 25°C, using the RVD/RVI protocol. The rate of shrinkage-induced cytoplasmic alkalinization in standard incubation medium (1 mM Ca^{2+}) is 0.022 ± 0.002 pH units/min ($n = 12$), compared to 0.021 ± 0.002 pH units/min ($n = 3$) in Ca^{2+} free medium (2 mM EGTA), and 0.025 ± 0.005 pH units/min (n = 3) in cells which had been loaded with BAPTA (*see* Jørgensen et al., 1995; *see* also Materials and Methods). Hence, these data strongly suggest that neither influx of Ca^{2+} across the plasma membrane, nor other changes in $[Ca^{2+}]_i$, are required for the shrinkage-induced activation of the Na^+/H^+ exchanger in Ehrlich cells.

Finally, the possible role of the Ca^{2+}/calmodulin system in shrinkage-induced activation of the exchanger was investigated using the anti-calmodulin drug pimozide. Under isotonic conditions, pH_i is virtually unaffected by pimozide (*data not shown*). Addition of pimozide (10 μM) simultaneous to the hypertonic challenge results in a significant decrease in the shrinkage-induced cytoplasmic alkalinization from 0.022 ± 0.002 pH units/min ($n = 12$) in controls to 0.014 ± 0.001 pH units/min ($n = 4$), corresponding to a 37% inhibition of the alkalinization response ($P < 0.03$). Thus, although the shrinkage-induced activation of the Na^+/H^+ exchanger is partly inhibited by pimozide, it appears to primarily involve pathways which are independent of the Ca^{2+}/calmodulin-system.

Role of Na^+/H^+ Exchange in Regulatory Volume Increase

As demonstrated above, cell shrinkage causes activation of a Na^+/H^+ exchanger in Ehrlich cells. However, as previously reported, Regulatory Volume Increase (RVI) following osmotic shrinkage in these cells is not mediated by Na^+/H^+ exchange but by Na^+, K^+, $2Cl^-$-cotransport (Jensen et al., 1993). Previous experiments investigating volume regulation after shrinkage in Ehrlich cells were all conducted in the nominal absence of HCO_3^-. Therefore, in the present study, the possible role of the Na^+/H^+ exchanger in RVI in these cells in the presence of HCO_3^- was investigated. Representative experiments are shown in Fig. 6 (*see* legend for average values and statistics). Using the same protocol of hypertonic challenge, Hoffmann et al. (1983) reported a degree of cell shrinkage of about 25%. Figure 6 (*A*) shows the changes in cell volume, relative to isotonic cell volume, as a function of time after osmotic cell shrinkage using

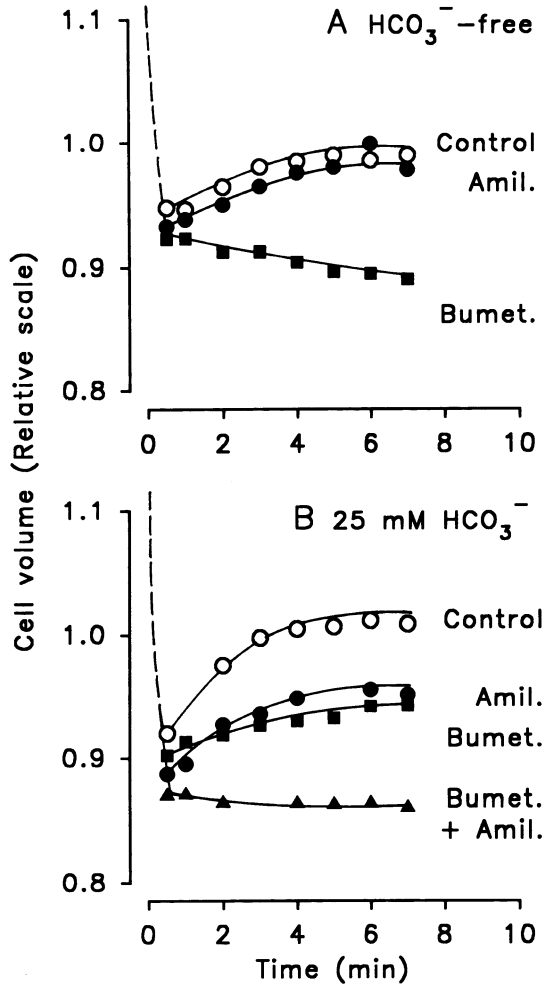

Fig. 6. Typical experiments showing cell volume recovery after osmotic shrinkage. The RVD/RVI protocol was used (*see* Materials and Methods). At zero time tonicity was restored by 500-fold dilution of the cells in standard medium (*A*) or medium containing 25 mM HCO_3^- (*B*), and in the absence (Controls, open circles) or presence of amiloride (100 μM, filled circles), bumetanide (20 μM, filled squares), or amiloride (100 μM) plus bumetanide (20 μM) (filled triangles). Cell volume was followed with time at 37°C by electronic cell sizing using a Coulter counter. Cell volume is shown relative to the volume of isotonic control cells. Using the same protocol of hypertonic challenge, Hoffmann et al. (1983) found a cell shrinkage of about 25% upon restoration of tonicity, as indicated by the broken line. The numbers of independent experiments were 4 (*A*), and 6 (*B*), respectively. The average, initial rates of volume recovery, calculated by linear regression of the measurements obtained in the interval from time 0.5 (the time of minimum volume) to time 2 min after the hypertonic challenge are as follows (positive values represent an increase, negative values a decrease in cell volume). (*A*) Standard medium: control 17.6 ± 2.7; amiloride 15.0 ± 3.2; bumetanide − 4.1 ± 2.4 B. 25 mM HCO_3^- control 30.4 ± 1.1*; amiloride 19.9 ± 1.9*; bumetanide 7.0 ± 1.2; bumetanide, amiloride −7.5 ± 4.0.[a] *The values are significantly different (Student's *t*-test, $P < 0.01$). The values obtained in nominally HCO_3^- free medium in the absence and presence of amiloride (100 μM), and the value obtained in 25 mM HCO_3^- medium in the presence of amiloride (100 μM) are not significantly different ($P > 0.1$). [a]The value represents 4 independent experiments.

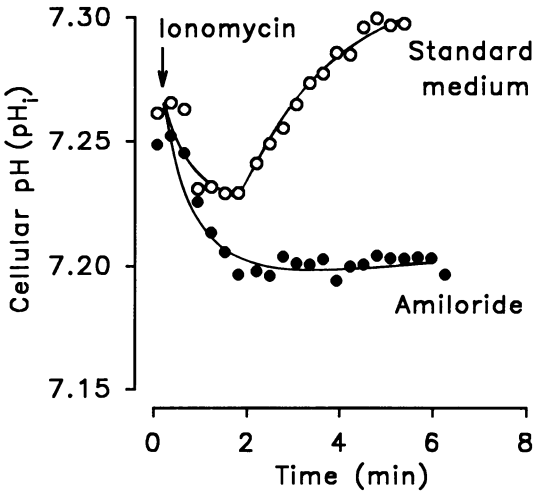

Fig. 7. Representative experiment showing the effect of ionomycin on pH_i as a function of time. Each curve was produced from the data points collected during the experiment, using a computer spreadsheet (*see* Materials and Methods). The cells were diluted at a cytocrit of 0.16% in the experiment medium, and ionomycin (0.5 μM) was added to the cell suspension at the time indicated by the arrow. Cellular pH was assessed spectrophotometrically at 25°C. The experimental media were: standard medium (open circles), and standard medium with amiloride (200 μM, filled circles). The experiment shown is representative of 3 independent experiments.

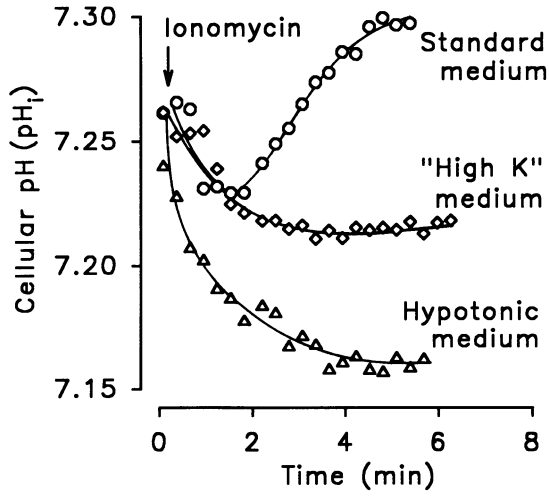

Fig. 8. Typical experiment showing the effect of ionomycin on pH_i as a function of time in various media. Each curve was produced from the data points collected during the experiment, using a computer spreadsheet (*see* Materials and Methods). The cells were diluted at a cytocrit of 0.16% in the experimental medium, and ionomycin (0.5 μM) was added to the cell suspension at the time indicated by the arrow (0.5 min). Cellular pH was assessed spectrophotmetrically at 25°C. The experimental media were: standard medium (open circles), ''high K^+'' medium (*see* Materials and Methods, open diamonds), and hypotonic medium (open triangles). The experiment shown is representative of 3 independent experiments. In control experiments, it was found that when ionomycin (0.5 μM) was added at time 0.5 min after suspension of the cells in hypotonic medium, this resulted in a cell volume which was, after 7 min, still substantially larger (by about 200 fl) than that of control cells (standard medium).

the RVD/RVI protocol and nominally HCO_3^--free medium. Under these conditions, volume recovery after shrinkage is largely unaffected by amiloride (100 μM) but is completely abolished in the presence of bumetanide (20 μM). In fact, in the presence of bumetanide, cell volume continues to decrease slightly.

Markedly different results were obtained in medium containing 25 mM HCO_3^-, in which volume recovery is partly inhibited (by 35%) by amiloride (100 μM), and considerable volume recovery is observed in the presence of bumetanide (20 μM). Furthermore, addition of amiloride and bumetanide in combination completely prevents volume recovery (Fig. 6, *B*). Thus, it appears that two mechanisms: $Na^+,K^+,2Cl^-$-cotransport, and Na^+/H^+ exchange functionally coupled to Cl^-/HCO_3^- exchange, contribute to volume recovery after cell shrinkage in the presence of HCO_3^-. The notion that the Cl^-/HCO_3^- exchanger plays a role in the presence of HCO_3^- is further supported by the fact that the rate as well as the magnitude of the shrinkage-induced alkalinization were observed to be reduced by about 60% under these conditions (*data not shown*).

Activation of the Na^+/H^+ Exchanger Under Isotonic Conditions

Ionomycin Activates the Na^+/H^+ Exchanger in Ehrlich Cells Under Isotonic Conditions

In isotonic medium, the Na^+/H^+ exchanger is activated by the Ca^{2+} ionophore ionomycin (Fig. 7). As seen, ionomycin (0.5 μM) alone (control, open circles) produces a biphasic change in pH_i: initially a slight acidification is seen, which is succeeded by a cytoplasmic alkalinization resulting in a considerable pH_i ''overshoot.'' In the presence of amiloride (200 μM, filled circles) the alkalinization, but not the initial acidification, is abolished, indicating that alkalinization is due to activation of a Na^+/H^+ exchanger.

Ca^{2+} ionophores have previously been shown to cause KCl loss and cell shrinkage in Ehrlich cells (*see* Hoffmann et al., 1993). Hence, the activation of the Na^+/H^+ exchanger by ionomycin could conceivably be a secondary effect of either the loss of KCl or the concomitant cell shrinkage. To investigate these possibilities, the effect of ionomycin on pH_i was assessed in buffers of various compositions. The results are shown in Fig. 8. It has previously been shown that when Ehrlich cells are suspended in ''high K^+'' medium, Ca^{2+}-induced KCl-loss and cell shrinkage are virtually prevented (Jensen, 1994; *see also* Materials and Methods). As seen, under these conditions, the ionomycin-induced intracellular alkalinization, but not the initial acidification, is eliminated (Fig. 8, open diamonds).

The absence of ionomycin-induced intracellular alkalinization in ''high K^+'' medium could in principle be due to either maintenance of cellular KCl-content or ab-

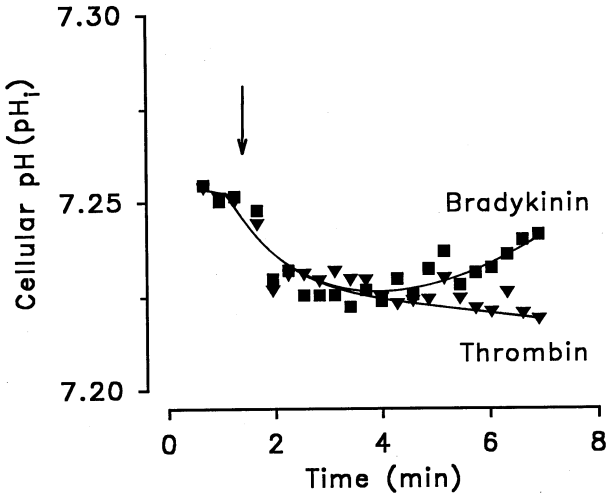

Fig. 9. Typical experiment showing the effect of thrombin and bradykinin on pH_i under isotonic conditions as a function of time. Each curve was produced from the data points collected during the experiment, using a computer spreadsheet (*see* Materials and Methods). The cells were diluted in standard medium at a cytocrit of 0.16%, and thrombin (10 U/ml, filled triangles) or bradykinin (10 μM, filled squares) were added at the time indicated by the arrow. Cellular pH was assessed spectrophotometrically at 25°C. The experiment shown is representative of 3 independent experiments.

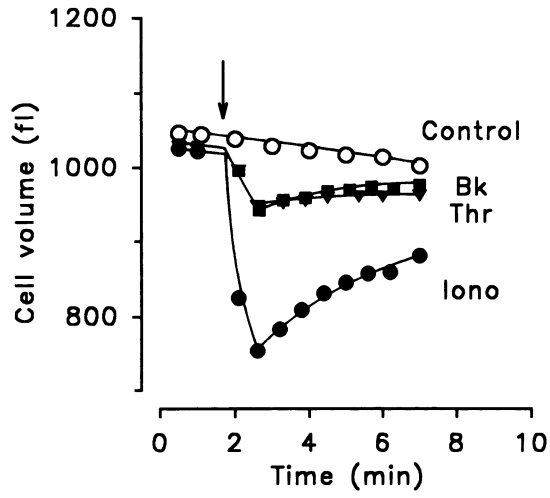

Fig. 10. Representative experiment showing the effects of thrombin, bradykinin and ionomycin on cell volume under isotonic conditions. A sample of the cell suspension was diluted 500-fold in isotonic standard medium and bradykinin (10 μM, filled squares), thrombin (10 U/ml, filled triangles), or ionomycin (0.5 μM, filled circles) were added at the time indicated by the arrow. The volume of control cells is shown as open circles. Cell volume was followed with time at 37°C by electronic cell sizing using a Coulter counter. The experiment shown is representative of 3 independent experiments.

sence of cell shrinkage under these conditions. Therefore, we repeated the experiment in hypotonic medium (160 mOsm), in which the cells were allowed to swell osmotically for 30 sec before addition of ionomycin. Under these conditions, the cells will swell osmotically and loose KCl without shrinking below the original volume (*data not shown*). As seen, in hypotonic medium (Fig. 8, open triangles) ionomycin fails to cause intracellular alkalinization. Instead, a continuous cytoplasmic acidification is observed. Thus, it appears likely that the ionomycin-induced activation of the Na^+/H^+ exchanger is secondary to the cell shrinkage caused by addition of ionomycin.

The effects of the Ca^{2+}-mobilizing agents thrombin and bradykinin, well-known activators of the inositol phosphate cycle, on cellular pH were also tested. Both agonists were previously shown to cause release of Ca^{2+} in Ehrlich cells (*see* Hoffmann & Ussing, 1992; *see* Hoffmann et al., 1993). Figure 9 shows the effect of these compounds on pH_i in cells suspended in isotonic standard medium. As seen, neither bradykinin (10 μM) nor thrombin (10 U/ml) have any significant effects on pH_i. Bradykinin causes a slight decrease in pH_i (about 0.02 pH units), followed by a return to prestimulation level, while thrombin only causes a slight decrease in pH_i (about 0.03 pH units during the 7-min time-course of the experiment).

To elucidate why ionomycin, but neither bradykinin nor thrombin causes substantial activation of the Na^+/H^+ exchanger, we assessed the effects of these compounds on cell volume, using electronic cell sizing. As seen in Fig. 10, addition of ionomycin (0.5 μM) results in a considerable cell shrinkage (about 25%), whereas bradykinin and thrombin only cause slight cell shrinkage (about 10%). A similar degree of cell shrinkage in response to thrombin has previously been reported in Ehrlich cells (*see* Hoffmann & Ussing, 1992). It may also be noted that Hoffmann et al. (1983) found a cell shrinkage of about 25% in response to a hypertonic challenge using the RVD/RVI protocol (final osmolarity 225 mOsm).

The above result further supports the notion that ionomycin-induced activation of the Na^+/H^+ exchanger is secondary to the cell shrinkage caused by addition of ionomycin. It also follows that the cell shrinkage induced by thrombin or bradykinin is insufficient to activate the Na^+/H^+ exchanger.

Discussion

Activation of Na^+/H^+ Exchange After Osmotic Shrinkage

When Ehrlich cells are exposed to hypertonic challenge, an amiloride-sensitive, Na^+-dependent, DIDS-insensitive intracellular alkalinization is observed (Fig. 1 and Table 1), in parallel with an amiloride-sensitive increase in unidirectional influx of Na^+ (Table 4). Thus, the present results elaborate the results previously found by Levinson (1991), who reported that activation of Na^+/H^+ ex-

change in Ehrlich cells can be demonstrated following hypertonic challenge. However, unidirectional Na^+ influx measurements during the period of cytoplasmic alkalinization were not included in the study by Levinson. Similar results have been obtained with other cell types such as rat thymic lymphocytes, fibroblasts (Grinstein et al., 1986; 1992*b;* 1994), human bladder carcinoma cells (Bianchini et al., 1991), and barnacle muscle fibres (Davis et al., 1992).

Our observation of a rate of shrinkage-induced alkalinization of 0.056 ± 0.002 pH units/min at 540 mOsm and 37°C (Table 3) is in agreement with that observed by Levinson (1991), who published an alkalinization rate of about 0.06 pH units/min. In the present study, the rate of shrinkage-induced alkalinization was found to be temperature-dependent with a temperature coefficient (Q_{10}) of about 2 at 540 mOsm, as was the lag time of the activation process. The J_{H^+} at 540 mOsm and 37°C is calculated at 4.60 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$ (Table 3). At 540 mOsm and 37°C, the rate of shrinkage-induced alkalinization is close to that obtained using the RVD/RVI protocol (Table 2). The amiloride-sensitive unidirectional Na^+ influx, measured using the RVD/RVI protocol at 37°C, was 9.6 ± 3.9 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$ (Table 4). A 1 (Na^+):1 (H^+) stochiometry is to be expected for a mammalian Na^+/H^+ exchanger. The observed deviation from a 1:1 stochiometry may be due to the fact that the J_{H^+} and the Na^+ influx are not directly comparable, due to the difference in the protocol of hypertonic challenge. Furthermore, metabolic processes in the cell, as well as H^+ influx across the plasma membrane result in an ongoing intracellular acidification (*see* Boron, 1992; *see* Grinstein & Cohen, 1987; also Table 1, this study) which can be expected to attenuate the measured intracellular alkalinization in response to an osmotic challenge.

The present observation that shrinkage-induced activation of the Na^+/H^+ exchanger occurs independent of the nature of the hypertonic challenge (Table 2) is in accordance with the studies of Levinson (1991) on Ehrlich cells as well as studies on other cell types such as Chinese hamster ovary cells (Rotin & Grinstein, 1989). It should be noted that intracellular ion concentrations, as well as ionic gradients across the plasma membrane vary considerably with the protocol of hypertonic challenge (Levinson, 1991). Furthermore, the shrinkage-induced activation of the Na^+/H^+ exchanger appears to increase with increasing magnitude of the hypertonic challenge, and, therefore, of the magnitude of the initial cell shrinkage, assuming that Ehrlich cells initially behave as perfect osmometers when exposed to anisotonic conditions (Table 3). Taken together, these results indicate that the trigger for hyperosmotic activation of the Na^+/H^+ exchanger in Ehrlich cells is the degree of change in cell volume, rather than the magnitudes of intra- and extracellular ion concentrations.

It is not yet established which NHE isoform(s) is present in Ehrlich cells. However, the Na^+/H^+ exchanger in these cells (i) is amiloride-sensitive (e.g., this study; Kramhøft et al., 1988; Levinson 1991), (ii) is activated by cell shrinkage (this study, Levinson, 1991), (iii) by cytoplasmic acidification (this study; Kramhøft et al., 1988), and (iii) by phorbol esters (*ibid.*). These pharmacological and functional characteristics of the Ehrlich cell Na^+/H^+ exchanger are characteristic of the NHE1 isoform (*see* Noël & Pousségur, 1995). Furthermore, the NHE1 isoform is ubiquitously distributed, while the NHE2, NHE3 and NHE4 isoforms appear to be restricted mainly to epithelial tissues (*ibid.*). Taken together, these observations indicate that the Ehrlich cell Na^+/H^+ exchanger is the NHE1 isoform.

Mechanisms Involved in the Shrinkage-induced Activation of the Na^+/H^+ Exchanger

Shrinkage-induced Activation of the Na^+/H^+ Exchanger is Dependent on ATP

ATP depletion causes a dramatic decrease, of about 90%, in shrinkage-induced intracellular alkalinization and J_{H^+} (Fig. 3). This effect is not due to reduced cell viability, nor to inhibition of the Na^+/H^+ ATPase, since ouabain is without effect on the intracellular alkalinization. Furthermore, the rate of recovery from an acid load was found to be drastically reduced (by 91.5%) in ATP-depleted cells compared to controls, in close agreement with Little et al. (1988), who found a 95% reduction in the rate of recovery from an acid load in cultured rat aortic smooth muscle cells after ATP-depletion. Thus, although ATP is not hydrolyzed during the transport cycle of the Na^+/H^+ exchanger, exchanger activity appears to be ATP-dependent in acidified as well as in shrunken cells.

The ATP dependence observed in the present study is in agreement with findings in many other cell types (Bianchini et al., 1991; Grinstein et al., 1992*b;* Little et al., 1988). Bianchini et al. (1991) observed that treatment of ATP-depleted cells with the phosphatase-inhibitor okadaic acid (OA) restores the alkalinization response to hypertonicity, indicating the involvement of phosphorylation events. However, other interpretations are possible. ATP-depletion may inhibit G-proteins, via reduction of cellular GTP-levels. G proteins have been suggested to be involved in the shrinkage-induced activation of the exchanger (Davis et al., 1992). Alternatively, ATP may bind directly to the exchanger or to an associated protein (Grinstein et al., 1994). Finally, it has been suggested that ATP-dependent interactions with the cytoskeleton or with plasma membrane phospholipids

may be responsible for the ATP dependence of the Na^+/H^+ exchanger (Grinstein et al., 1994).

PKC or a Similar Protein Kinase may be Involved in the Shrinkage-induced Activation of the Na^+/H^+ Exchanger

The shrinkage-induced activation of the Na^+/H^+ exchanger in Ehrlich cells is inhibited by chelerythrine (Fig. 4), which is reported to be a highly selective inhibitor of PKC (Herbert et al., 1990). A similar result was obtained using the less specific PKC-inhibitor H-7. These observations indicate the involvement of phosphorylation by PKC or a similar protein kinase in shrinkage-induced activation of the exchanger in Ehrlich cells.

In lymphocytes (*see* Grinstein et al., 1994), and in a rat bone cell line (Dascalu et al., 1992), shrinkage-induced activation of the Na^+/H^+ exchanger appears to be independent of PKC. Activation of PKC after cell shrinkage could not be demonstrated in lymphocytes (Grinstein et al., 1986). In contrast, PKC is known to be activated in Ehrlich cells within one min after exposure to hypertonic stress (Larsen et al., 1994), indicating that the signaling pathways in RVI may differ between these cell types. Thus, shrinkage-induced activation of Na^+/H^+ exchange appears to be PKC-*independent* in lymphocytes, but to involve PKC in Ehrlich cells. However, several observations in this and previous studies indicate that other, PKC-independent mechanisms must be involved as well: (i) Concentrations of chelerythrine (Fig. 4) or H-7 sufficient to completely inhibit the shrinkage-induced activation of PKC (Larsen et al., 1994), cause only about 40% inhibition of shrinkage-induced alkalinization. IC_{50} for inhibition of PKC by chelerythrine has been reported to be 0.7 μM (Herbert et al., 1990), compared to an IC_{50} of 2.7 μM for inhibition of shrinkage-induced alkalinization by chelerythine, observed in the present study. Regarding the effect of H-7, it is noteworthy that this drug inhibits PKA as well as PKC (*ibid.*). cAMP has been shown to inhibit shrinkage-induced intracellular alkalinization in Ehrlich cells (S.F. Pedersen, *unpublished data*). Assuming that this effect of cAMP is an effect on the Na^+/H^+ exchanger via PKA, inhibition of PKA by H-7 would thus be expected to counteract the effect on the exchanger of inhibition of PKC, resulting in incomplete inhibition of shrinkage-induced Na^+/H^+ exchange by H-7. (ii) Changes in $[Ca^{2+}]_i$ do not seem to be required for osmotic activation of the Na^+/H^+ exchanger. This does not exclude the involvement of calcium-independent isoforms of PKC (Nishizuka, 1989). In fact, regulation of Na^+/H^+ exchange by a Ca^{2+}-independent PKC-isoform has recently been suggested in MDCK-cells (Wojnowski et al., 1994). (iii) The inability of thrombin and bradykinin, well-known activators of the inositol phosphate cycle, to activate the Na^+/H^+ exchanger indicates that a diacylglycerol- and Ca^{2+}-dependent PKC isoform does not activate Na^+/H^+ exchange under the present experimental conditions.

Thus, whether the inhibitory effects of chelerythrine and H-7 on shrinkage-induced Na^+/H^+ exchange reflect the involvement of a PKC-isoform or their possible inhibition of other kinases remains unclear. It should also be noted that while the present findings suggest a role for protein phosphorylation events in the shrinkage-induced activation of the Na^+/H^+ exchanger in Ehrlich cells, it is not revealed whether phosphorylation affects the exchanger through direct phosphorylation, phosphorylation of accessory regulatory protein(s), or some other phosphorylation-dependent mechanism. Observations in lymphocytes indicate that osmotic activation of the exchanger is not associated with direct phosphorylation of the exchanger protein (Grinstein et al., 1992*b*).

Inhibition of Protein Phosphatases PP1 and PP2A Increases Shrinkage-induced Activation of the Na^+/H^+ Exchanger

In the present study, it is demonstrated that the shrinkage-induced activity of the Na^+/H^+ exchanger is increased in the presence of CL-A (Fig. 5, *B*). This is in agreement with the results of Parker, Colclasure and McManus (1991), who found that okadaic acid (OA), an inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), stimulates shrinkage-induced activation of Na^+/H^+ exchange in dog red blood cells.

It is also seen from Fig. 5 (*A*), that CL-A causes a slight cytoplasmic acidification under isotonic conditions. Thus, Na^+/H^+ exchange seems not to be activated by CL-A under isotonic conditions in Ehrlich cells. This is at variance with observations by Bianchini et al. (1991), who found that in rat thymic lymphocytes and human bladder carcinoma cells, OA caused parallel activation of the exchanger and accumulation of phosphoproteins under isotonic conditions. These authors observed that osmotic and OA-induced activation of the exchanger were not additive, leading them to suggest similar, albeit not identical mechanisms.

It has been proposed that volume regulation involves changes in the cellular kinase/phosphatase equilibrium, cell shrinkage being associated with increased phosphorylation of a regulatory protein (Parker et al., 1991). The observation that CL-A appears to have no effect on the Na^+/H^+ exchanger under isotonic conditions, but stimulates shrinkage-induced activation of the exchanger, might be explained if cell shrinkage leads to increased activity of protein kinase(s), and if protein phosphorylation is involved in the shrinkage-induced activation of the Na^+/H^+ exchanger. Hence, preventing phosphatase activity in shrunken cells will result in increased activity of the Na^+/H^+ exchanger, while having no effect in os-

motically undisturbed cells. In support of this interpretation, it has recently been suggested that cell shrinkage activates a kinase, rather than inhibiting a phosphatase (Grinstein, Furuya & Bianchini, 1992*a*).

Several mechanisms can account for the observed effects of CL-A. Since substrates of PKC are primarily dephosphorylated by the protein phosphatases PP1 and PP2A (Bianchini et al., 1991), the observed effect of CL-A on the Na^+/H^+ exchanger could reflect a potentiation of an effect of PKC (or a similar kinase). Furthermore, CL-A is a potent inhibitor of type 1 protein phosphatases (PP1), which is suggested to play an important role in regulation of cell shape and cytoskeletal structure (Hosoya et al., 1993). Grinstein et al. (1994) have recently suggested, that direct physical interaction with cytoskeletal elements may underlie the osmotic activation of the Na^+/H^+ exchanger. Hence, if changes in cytoskeletal structure, e.g., an increased level of phosphorylation of cytoskeletal proteins, activate the Na^+/H^+ exchanger during cell shrinkage, the observed effect of CL-A could be a potentiation of shrinkage-induced cytoskeletal changes. It has been shown in Ehrlich cells that the cytoskeleton is involved in the regulation of the $Na^+,K^+,2Cl^-$ cotransporter (Jessen & Hoffmann, 1992).

Changes in $[Ca^{2+}]_i$ are not Essential for the Shrinkage-induced Activation of the Na^+/H^+ Exchanger

Shrinkage-induced alkalinization is observed in EGTA-buffered, Ca^{2+}-free media as well as in cells in which changes in $[Ca^{2+}]_i$ have been buffered by BAPTA. Hence, it is concluded that changes in intracellular Ca^{2+} are not necessary for the shrinkage-induced activation of the Na^+/H^+ exchanger in Ehrlich cells. Similarly, in other cell types, changes in $[Ca^{2+}]_i$ were found not to be involved in the shrinkage-induced activation of the Na^+/H^+ exchanger (Mitsuhashi & Ives, 1988; Dascalu et al., 1992). In Ehrlich cells no changes in $[Ca^{2+}]_i$ could be detected in response to a hypertonic challenge (Pedersen et al., *unpublished data*). However, highly localized changes in $[Ca^{2+}]_i$ may have occurred, which could not be detected in our experimental setup.

The anti-calmodulin drug pimozide causes 40% inhibition of shrinkage-induced alkalinization in Ehrlich cells. This is in accordance with the results of Dascalu et al. (1992), who observed inhibition of shrinkage-induced activation of the Na^+/H^+ exchanger by the anti-calmodulin drugs W-7 and chlorpromazine. The observed calmodulin-dependence might indicate the involvement of CaM kinase II in the shrinkage-induced activation of the Na^+/H^+ exchanger. However, since shrinkage-induced activation of the Na^+/H^+ exchanger seems to be $[Ca^{2+}]_i$-independent, calmodulin might be involved in the regulation of the exchanger in a manner not requiring Ca^{2+}-binding, a phenomenon previously observed in a rat bone cell line (Dascalu et al., 1992). Recently, it was shown that the Na^+/H^+ exchanger (NHE1 isoform) is a calmodulin-binding protein, and, furthermore, deletion of a high-affinity calmodulin binding site was shown to result in 80% reduction of shrinkage-induced activation of the exchanger (Bertrand et al., 1994). Alternatively, it cannot be excluded, that either the low resting level of $[Ca^{2+}]_i$ in BAPTA-loaded cells is sufficient for CaM kinase II activity, or that the effect of pimozide is via other pathways, given the lack of specificity of anticalmodulin drugs (*see* Veigl, Klevit & Sedwick, 1989).

Role of the Na^+/H^+ Exchanger in RVI

The results of cell volume measurements, which were conducted in the nominal absence of HCO_3^-, confirm the previous notion that RVI in Ehrlich cells is mediated by the $Na^+,K^+,2Cl^-$ cotransporter, with the Na^+/H^+ exchanger giving only an insignificant contribution (Fig. 6, *A*). Thus, under these standard conditions, the Na^+/H^+ exchanger appears to be volume-activated, but not volume regulatory. In striking contrast, when HCO_3^- is available, volume recovery is partially inhibited by bumetanide as well as by amiloride and completely abolished in the presence of both inhibitors in combination (*B*). This suggests that both the $Na^+,K^+,2Cl^-$ cotransporter and the Na^+/H^+ exchanger contribute to RVI in the presence of HCO_3^-. Furthermore, volume recovery, expressed as the rate of recovery observed during the first 1.5 min, is considerably enhanced in the presence of HCO_3^- (Fig. 6, legend). This effect is due to the additional activity of the Na^+/H^+ exchanger, since the rate of volume recovery in the presence of amiloride is similar in the absence and presence of HCO_3^-.

Measurements of Na^+ influx in the nominal absence of HCO_3^- (Table 4) show, as discussed above, a rate of amiloride-sensitive Na^+ influx after hypertonic challenge at 37°C of 9.6 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$. As discussed below, the Cl^-/HCO_3^- exchanger is unlikely to be active under these circumstances. Jensen et al. (1993, Table 5) observed a bumetanide-sensitive K^+-influx during RVI of 24.1 $\mu mol \cdot dry\ wt^{-1} \cdot min^{-1}$ under similar conditions. Thus, activity of the $Na^+,K^+,2Cl^-$ cotransporter may account for a total uptake of ions of about 96 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$ (24 Na^+ + 24 K^+ + 48 Cl^-). This is 10-fold the observed amiloride-sensitive Na^+ influx seen after cell shrinkage. Hence, it is conceiveable that the fact that the Na^+/H^+ exchanger is volume activated but not volume regulatory in the nominal absence of HCO_3^- is due to a limited ability of the exchanger to contribute to net salt uptake under these conditions.

In nominally HCO_3^--free medium, at normal resting pH_i (7.25), the Na^+-dependent as well as the Na^+-independent Cl^-/HCO_3^- exchanger have previously been

found to be virtually quiescent in Ehrlich cells (Kramhøft et al., 1994). The present study indicates that this is also the case in shrunken Ehrlich cells in the nominal absence of HCO_3^-, since DIDS has no effect on the rate of change in pH_i after cell shrinkage under these conditions (Fig. 1). It should be noted, however, that theoretically, the finding that DIDS is without effect on shrinkage-induced alkalinization might result from the simultaneous operation of the Na^+-dependent and the Na^+-independent Cl^-/HCO_3^- exchangers, since they have opposing effects on pH_i and are both DIDS-sensitive (Kramhøft et al., 1994).

The activation of the Na^+/H^+ exchanger by cell shrinkage will cause a reduction in $[H^+]_i$ and a subsequent increase in $[HCO_3^-]_i$. This will result in secondary activation of Cl^-/HCO_3^- exchange (Hoffmann & Simonsen, 1989). In media containing 25 mM HCO_3^-, pH_i was found, in the present study, to be 7.31 ± 0.01 (n = 3), slightly more alkaline than in nominally bicarbonate-free media. The secondary activation of Cl^-/HCO_3^- exchange following shrinkage-induced activation of the Na^+/H^+ exchanger is likely to be supported by the increased HCO_3^--concentration as well as by the increased pH_i, as discussed by Mason et al. (1989). Furthermore, in an open system, CO_2/HCO_3^- has been reported to increase the total cellular β_i (contribution: $\beta_{CO2} = 2.3\ [HCO_3^-]_i$, *see* Boron, 1992). Thus, it is likely that an increase in β_i due to increased CO_2/HCO_3^- buffering will also facilitate volume regulation via the Na^+/H^+ exchanger, by reducing the shrinkage-induced alkalinization, and thus retarding inactivation of the exchanger by pH_i (Cala & Maldonado, 1994). In support of this interpretation, a reduced rate of shrinkage-induced intracellular alkalinization in the presence of HCO_3^- was observed in the present study, as reported above.

Consequently, it is suggested that in the presence of HCO_3^-, two mechanisms: (i) $Na^+,K^+,2Cl^-$ cotransport, and (ii) the parallel activity of the Na^+/H^+ exchanger and the Na^+-independent Cl^-/HCO_3^- exchanger, are involved in RVI.

Activation of the Na^+/H^+ Exchanger by Ionomycin

The present study demonstrates that the Ca^{2+} ionophore ionomycin (0.5 μM) causes intracellular alkalinization in Ehrlich cells under isotonic conditions. This effect is completely inhibited by amiloride (Fig. 7). This strongly indicates that the observed alkalinization is a result of activation of the Na^+/H^+ exchanger and is not due to $Ca^{2+}/2H^+$ exchange caused by the ionophore itself, which cannot be inhibited by amiloride. In agreement with the present results, calcium ionophores have been reported to activate Na^+/H^+ exchange in several other cell types (Grinstein & Cohen, 1987; Dascalu et al., 1992; Wøll et al., 1993).

As seen in Fig. 7, ionomycin initially causes a slight acidification of about 0.04 pH units. This is likely to be a result of (i) membrane hyperpolarization, known to be caused by Ca^{2+} ionophores in Ehrlich cells (Lambert, Hoffmann & Jørgensen, 1989), which increases the driving force for uptake of H^+ across the plasma membrane, and (ii) an increase in the rate of metabolic production of H^+, as suggested by Grinstein and Cohen (1987) in lymphocytes. In hypotonic medium, a continuous acidification can be seen after addition of ionomycin (Figure 8). Most likely, the dramatic loss of KCl, which can be expected to result from the combination of hypotonicity and ionomycin, will partly be compensated for by Cl^-/HCO_3^- exchange, thus resulting in intracellular acidification (Livne & Hoffmann, 1992).

The subsequent activation of Na^+/H^+ exchange is likely to result from the ionomycin-induced cell shrinkage, and not from the initial intracellular acidification. This is suggested by the fact that ionomycin-induced intracellular alkalinization is prevented under conditions where shrinkage is prevented, i.e., in ''high K^+''-medium and hypotonic medium, although the initial acidification is still seen under these circumstances (Fig. 8). It should be noted that a substantial inward Na^+ gradient is present in both ''high K^+''-medium and hypotonic medium.

The present suggestion that activation of Na^+/H^+ exchange by ionomycin is secondary to ionomycin-induced cell shrinkage is in agreement with observations on lymphocytes (Grinstein & Cohen, 1987) and Ha-ras oncogene expressing fibroblasts (Wøll et al., 1993). In further support of this notion, ionomycin (0.5 μM) was found to cause considerable cell shrinkage in Ehrlich cells, to about 75% of the normal volume (Fig. 10), in agreement with N.K. Jørgensen et al. (submitted).

In contrast to observations in other cell types (Sardet et al., 1990; Wøll et al., 1993), the Ca^{2+} mobilizing agents bradykinin and thrombin do not cause cytoplasmic alkalinization, and, thus, do not appear to activate the Na^+/H^+ exchanger in Ehrlich cells (Fig. 9). The present study shows that cell shrinkage induced by ionomycin is substantially greater than that induced by either bradykinin or thrombin (Fig. 10). Therefore, the fact that Na^+/H^+ exchange in Ehrlich cells is activated by ionomycin, but not by thrombin or bradykinin, is suggested to reflect the different degree of cell shrinkage induced by these compounds. The activation of the Na^+/H^+ exchanger by bradykinin and thrombin observed in other cell types may either involve a mechanism other than cell shrinkage, or the degree of shrinkage induced by these agents in other cell types may suffice to activate Na^+/H^+ exchange in these cells.

In conclusion, cell shrinkage activates Na^+/H^+ exchange in Ehrlich ascites tumor cells. Cellular phosphorylation events are involved in the activation, whereas changes in $[Ca^{2+}]_i$ do not seem to be directly implicated in the activation process. Finally, whereas the Na^+/H^+

exchanger is not involved in volume regulation in these cells under nominally bicarbonate-free conditions, the exchanger contributes substantially to volume regulation after hypertonic treatment in the presence of bicarbonate.

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