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Biochimica et Biophysica Acta 1328 (1997) 166–176

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Hypertonicity-induced alkalinization of rat hepatocytes is not involved in activation of Na^+ conductance or Na^+,K^+-ATPase

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Received 23 January 1997; revised 1 April 1997; accepted 8 April 1997

Abstract

We investigated whether cell alkalinization via activation of Na^+/H^+ exchange is involved in the stimulation of Na^+ conductance and Na^+,K^+-ATPase in rat hepatocytes under hypertonic stress. Osmolarity was increased from 300 to 400 mOsm/l at constant extracellular pH (7.4), whereas osmotically induced cell alkalinization (0.3 pH units in HCO_3^--free solutions) was mimicked by increasing extracellular pH from 7.4 to 7.8 in normosmotic solutions. In intracellular recordings with conventional and ion-sensitive microelectrodes, hypertonic stress led to a transient shift in the voltage response to low Na^+ solutions (95% in exchange for choline) by -4.3 ± 0.8 mV and a continuous increase in cell Na^+ from 13.7 ± 1.8 to 18.6 ± 3.0 mmol/l within 8 min. In the presence of 10^{-5} mol/l amiloride, these effects were reduced by 80 and 90%, respectively. In contrast, increasing pH did not change the voltage responses to low Na^+ or cell Na^+ concentrations significantly. In addition, application of 2 mmol/l Ba^{2+} pulses revealed that a sustained membrane hyperpolarization of 15.6 ± 1.4 mV following intracellular alkalinization exclusively reflects an increase in K^+ conductance. Increasing osmolarity at pH 7.4 augmented ouabain-sensitive $^{86}Rb^+$ uptake from 5.5 ± 1.1 to 8.5 ± 1.6 nmol mg protein^{-1} min^{-1}. In normosmotic solution at pH 7.8, $^{86}Rb^+$ uptake equalled 4.9 ± 1.6 nmol mg protein^{-1} min^{-1}, which is not significantly different from control. We conclude that, in rat hepatocytes, cell alkalinization under hypertonic stress is not responsible for the activation of Na^+ conductance and probably does not participate in the stimulation of Na^+,K^+-ATPase. © 1997 Elsevier Science B.V.

Keywords: Liver; Volume regulation; Electrophysiology; Fluorometry; $^{86}Rb^+$ uptake; Cell pH; Cell Na^+; Na^+ conductance; Na^+,K^+-ATPase

1. Introduction

Following an initial period of passive osmotic behavior, most animal cells are capable of actively readjusting their volumes despite continuous hypotonic and hypertonic challenges (see [1] for review). In rat hepatocytes under hypertonic stress, the key role in regulatory volume increase (RVI) was supposed to be activation of Na^+/H^+ exchange leading to a gain in cell Na^+ as the initial osmolyte and a coinciding alkalinization of the cytosol [2–4]; cell Na^+ is then exchanged for K^+ via stimulation of Na^+,K^+-ATPase [3,5,6]. In a recent report from this laboratory, however, evidence was provided that, in primary cultures of rat hepatocytes, hypertonic stress, in addition, leads to a prominent increase in Na^+ conductance [7]. Moreover, a quantitative analysis

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PII S0005-2736(97)00085-0

revealed that the net gain of cell Na^+ via Na^+ conductance by far (i.e., possibly by a factor of 10) exceeds that via Na^+/H^+ exchange [7]. Together with the fact that in most instances stable intracellular pH values are mandatory to maintain cellular functions, this raises the question if activation of Na^+/H^+ exchange may fulfil assignments in addition to an increase in cell Na^+, namely, if cell alkalinization may serve as a second messenger involved in the activation of Na^+ conductance and/or Na^+,K^+-ATPase.

pH effects on epithelial Na^+ conductances are amply documented. As determined in voltage-clamp experiments on intact tissues, intracellular acidification decreases the (amiloride-sensitive) apical Na^+ permeability of the toad urinary bladder [8]. Palmer and Frindt [9] examined the effects of cell pH on single Na^+ channels from rat cortical collecting duct by use of the patch-clamp technique and found that the open probability of these channels decreases from 0.41 to 0.19 and 0.05 when the pH is reduced from 7.4 to 6.9 and 6.4. In frog skin epithelium, cell acidification decreases and cell alkalinization increases apical Na^+ conductance (as well as basolateral K^+ conductance) and cell pH was discussed as a possible 'intrinsic regulator of epithelial ion transport' [10,11]. In A6 cells, the open probability of single Na^+ channels increases in a sigmoidal mode from virtually zero to one when the pH on the cytosolic side of excised patches is increased from 6.5 to 8.0; the pH dependence of P_o is steepest at pH 7.2 which is the intracellular pH under quasi-physiological conditions [12].

In their study on rabbit urinary bladder, Eaton et al. [13] analyzed in detail the effects of cell pH on the activity of Na^+,K^+-ATPase and reported a strong positive correlation between both parameters in the physiologically relevant range, i.e., between pH 6.5 and 7.5. In contrast, from their measurements on frog skin epithelium, Ehrenfeld et al. [10] concluded that protons have no direct effect on the Na^+,K^+-pump rate and that the activity of the transporter appears to be mainly regulated via cell Na^+. In their model, pH-dependent apical Na^+ conductance (see above) plays the decisive role in transepithelial Na^+ transport. Comparable conclusions are drawn by Rick [14] from his study on the same preparation, although a pH dependence of Na^+,K^+-ATPase is not excluded.

In the present paper, possible effects of cell alkalinization on rat hepatocyte Na^+ conductance and Na^+,K^+-ATPase were investigated and compared to those elicited under hypertonic stress. To this end, osmolarity was increased from 300 to 400 mOsm/l at constant extracellular pH (7.4), whereas osmotically induced cell alkalinization was mimicked by increasing extracellular pH from 7.4 to 7.8 under normosmotic conditions. Our results indicate that a cell alkalinization comparable in size to that following stimulation of Na^+/H^+ exchange in hypertonic solutions is not responsible for activation of Na^+ conductance or Na^+,K^+-ATPase.

2. Materials and methods

2.1. Primary culture of hepatocytes

Isolation of hepatocytes was the same as described previously [15,16]. After isolation, cells were plated on collagen-coated gas-permeable Petriperm® dishes and cultured in Dulbecco's Modified Eagles Medium (DMEM) fortified with 10% fetal bovine serum, 2 mmol/l glutamine, penicillin–streptomycin (100 U/ml, 100 μg/ml), 10^{-6} mol/l dexamethasone, 10^{-8} mol/l triiodothyronine/thyroxine (T_3/T_4), and 5 μg/ml bovine insulin at 37°C in 5% CO_2/air. Cells form confluent monolayers within 24 h and were used from days 1–3 after preparation.

Petriperm® dishes were purchased from Bachofer (Reutlingen, Germany), DMEM, penicillin–streptomycin, and glutamine from Flow (Bonn, Germany), collagenase and fetal bovine serum from Boehringer (Mannheim, Germany). All other compounds were obtained from Serva Chemical Company (Heidelberg, Germany).

2.2. Electrophysiology

Experimental set-up and recording techniques are described in detail in previous reports from this laboratory [15,17]. Briefly, circular sheets of gas-permeable membranes of approximately 1 cm^2 with confluent cell monolayers were cut from the bottom of the culture dishes and transferred to the superfusion chamber which is mounted on the stage of an inverted microscope (IM 35; Zeiss, Oberkochen, Ger-

many). The fluid volume above the tissue was 0.1 ml and cells were continuously superfused at a rate of 4 ml/min by means of a multi-channel peristaltic pump (PLG; Desaga, Heidelberg, Germany). Changes of the superfusate were performed by means of a four-way valve (ms-131 D; Whitey, Highland Heights, OH, USA) close to the experimental chamber. All storage vessels, superfusion lines, and the chamber were water-jacketed to achieve a constant temperature of the preparation (36.0 ± 0.5°C).

Conventional two-channel microelectrodes were pulled from 1.5-mm o.d. 'Thick-Septum-Theta' borosilicate glass capillaries (WPI, New Haven, CT, USA) on a Kopf vertical puller (750; David Kopf Instruments, Tujunga, CA, USA) and had resistances of 80–130 MΩ when filled with 0.5 mol/l KCl and immersed in control Tyrode solution. One channel was used to measure voltage, the second to inject constant-current pulses for determination of input resistances. Criteria for successful impalements have previously been described [15].

Na^+-sensitive microelectrodes were constructed as reported in a previous publication [7]. Briefly, electrodes were pulled from single-channel borosilicate filament glass capillaries of 1.0 mm o.d. and 0.5 mm i.d. (Hilgenberg, Malsfeld, Germany) to give resistances of 80–120 MΩ when filled with 0.5 mol/l KCl. They were silanized by exposure to dimethyldichlorosilane vapor (Fluka, Neu-Ulm, Germany) at 200°C and baked for 1 h. The electrodes were then backfilled with Na-Ionophore 1-Cocktail A (71176; Fluka) and 0.5 mol/l NaCl. They were calibrated in mixed NaCl/KCl standard solutions at a constant sum of Na^+ plus K^+ of 150 mmol/l, the Na^+ concentrations being 150, 50, 15, 5, and 1.5 mmol/l; solutions were buffered to pH 7.4 with 5 mmol/l TRIZMA (tri-(hydroxymethyl)-aminomethane; Sigma, München, Germany). The activity coefficients of Na^+ and K^+ were assumed to be constant and equal to 0.77 [18]. The slope of the electrodes and the selectivity coefficients of Na^+ over K^+ were obtained by fitting the measured potential difference and Na^+ activities of the standard solutions by use of a non-linear least-square fit routine to the Nicolsky equation [7,18,19] and equalled 60.9 ± 3.2 mV/decade and 60 ± 21, respectively (mean ± S.D.). In the experiments, a single cell was first impaled with an internal reference electrode pulled from the same capillaries as the ion-sensitive one, but filled with 0.5 mol/l KCl. Once a stable registration was achieved, the same cell was impaled with the Na^+-sensitive electrode which was commonly accompanied by a short transient deflection in membrane voltage (cf. [7]). Only those experiments were accepted in which the membrane voltage was restored to within 2 mV of the original value. Na^+-sensitive electrodes were calibrated before and after use and the intracellular Na^+ activities were calculated according to Horisberger and Giebisch [18] (see also [7]).

In all measurements, a custom-made 0.5 mol/l KCl flowing junction in series with an Ag–AgCl wire was used as the extracellular references electrode to avoid liquid junction potentials. This electrode was placed in an additional 1-ml compartment which is connected to the chamber via a hole 1.5 mm in diameter and 15 mm in length and which also contains the suction cannula of the superfusion system. Because there is a continuous flow of experimental solutions from the chamber into this compartment, any contamination of the preparation via leakage from the reference electrode can be excluded.

2.3. Microfluorometry

Intracellular pH was monitored by use of the fluorescent dye BCECF (2′,7′-bis-2-carboxyethyl)-5-(and-6)carboxyfluorescein; Molecular Probes Inc., Eugene, OR, USA) as previously described [7,16]. Briefly, after dye loading, cell monolayers were transferred to a Perspex chamber of 0.1 ml volume and continuously superfused. Cell fluorescence was excited by use of the 488-nm band of an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) and the 442-nm band of a helium–cadmium laser (4310 N; Liconix, Santa Clara, CA, USA) on a confocal laser-scan unit (MRC-600; BioRad, Hemel Hempsted, UK) coupled to a standard microscope (Diaphot; Nikon, Düsseldorf, Germany). Cell pH was determined from the fluorescence ratio from both excitation wavelengths and a calibration was performed at the end of each experiment in 140 mmol/l KCl and 10 μmol/l nigericin at various pH values bracketing the pH range under consideration according to Thomas et al. [20].

The intracellular pH of rat hepatocytes (pH_i) was determined at extracellular pH values (pH_e) between

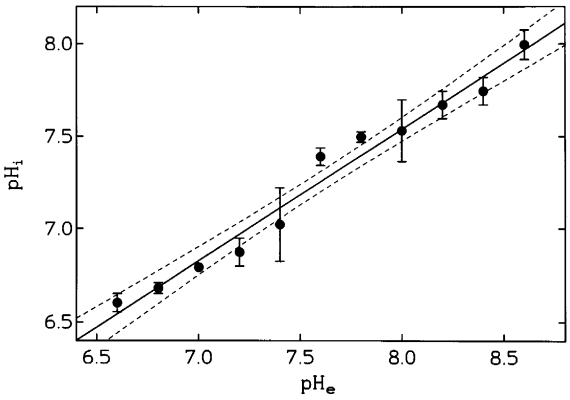

Fig. 1. Effects of extracellular pH (pH_e) on the intracellular pH (pH_i) of rat hepatocyte monolayers. $n = 4–9$ for each datapoint. The solid line was obtained by linear regression analysis of the mean values ($r = 0.987$), the dotted line represents the 95% confidence interval.

6.6 and 8.6. As Fig. 1 reveals, pH_i was a quasi-linear function of pH_e and followed the form

$$pH_i = 1.84 + 0.71 \times pH_e$$

within this range. According to these calibration measurements, increasing the pH of the superfusate from 7.4 to 7.8 will lead to an intracellular alkalinization by 0.3 pH units which is equivalent to the pH response of rat hepatocytes to a 400 mOsm/l solution under HCO_3^--free conditions [7].

2.4. Confocal laser-scanning microscopy

Cell volumes were determined as previously described [21]. Briefly, after loading with BCECF (see above), hepatocytes were optically scanned in the *z*-axis at the excitation wavelength of 442 nm, where the dye is pH insensitive (isosbestic point). The spatial resolution of this procedure is 0.9 μm and a set of optical sections was acquired within 5 s. Cell volumes were then computed from the measured surface areas of every cross-section.

2.5. Measurements of Rb^+ uptake

Circular sheets of 16-mm diameter with confluent monolayers were cut from the bottom of the culture dishes, washed, and transferred to standard scintillation vials of 20-ml volume filled with 5 ml of experimental solution; solutions were kept at 36°C and continuously gassed with humidified O_2. Rb^+ uptake was determined by transferring monolayers for 2, 4, 6, and 8 min to identical solutions labeled with 1–5 μCi/ml $^{86}Rb^+$ (cf. [7]). Uptake was measured in control tyrode (300 mOsm/l, pH 7.4) and 5 min after transfer to 400 mOsm/l (pH 7.4) or pH 7.8 (300 mOsm/l). Each experimental protocol was preceded by an isotonic preperiod of 10 min at pH 7.4. In half of the measurements, 2 mmol/l ouabain was present throughout the experiments. Influxes were terminated by removing the monolayers from the vials and washing them with ice-cold experimental solution of appropriate osmolarity and pH. The lower cell-free surface of the membranes was then carefully blotted on filter paper and the membranes were transferred to 0.5 ml of 2% sodium dodecyl sulfate in 2 mmol/l ethylenedinitrilotetraacetic acid. As pilot experiments with ^{14}C-labeled sucrose as a second marker revealed, there was no extracellular compartment that was not accessible to the washing and drying procedure used (cf. [7]). After 60 min of cell lysis, aliquots were sampled for liquid scintillation counting and determination of protein content.

2.6. Solutions

The normosmotic control solution (300 mOsm/l, pH 7.4) contained (in mmol/l): NaCl, 144; KCl, 2.7; NaH_2PO_4, 0.4; Na^+-HEPES, 2.5; HEPES, 2.5; $CaCl_2$, 1.8; $MgCl_2$, 1.1; glucose, 5.6. In one series of experiments, the extracellular pH was varied in the range of 6.4–8.6. pH was adjusted by the addition of 1 M HCl and 4 M NaOH as appropriate and for pH values higher than 7.4, HEPES was replaced by TRIZMA. Increases in osmolarity were achieved by the addition of 100 mmol/l sucrose. In the ion substitution experiments, Na^+ was isosmotically reduced 20-fold in exchange with choline. Changes in K^+ conductance upon cell alkalinization were assessed by application of 2 mmol/l Ba^{2+} pulses. All experimental solutions were continuously gassed with humidified O_2 and kept at 36.0 ± 0.5°C.

Na^+-HEPES and HEPES were bought from Serva Chemical Co., NaCl and KCl from Baker (Deventer, NL), and amiloride from Sigma. All other substances were obtained from E. Merck (Darmstadt, Germany).

2.7. Statistics

Mean values ± S.E. are presented, unless otherwise indicated, with n denoting the number of cell cultures. t-Tests for paired and unpaired data were applied as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

In the present paper, the possible effects of cell alkalinization on hepatocyte Na^+ conductance and on the activity of Na^+,K^+-ATPase were investigated. Experiments were performed in HCO_3^--free solutions to avoid possible interferences of alkaline pH with Cl^-/HCO_3^- exchange [22] and Na^+/HCO_3^- symport [23,24]. In contrast, changes in the activity of Na^+/H^+ exchange upon intracellular alkalinization are not to be expected (see [25] for review). As confocal laser microscopy revealed, increasing extracellular osmolarity under these conditions from 300 to 400 mOsm/l decreased the volume of confluent rat hepatocytes in primary culture to 88.4 ± 0.7% within 2 min (Fig. 2A) [1]. Thereafter, cell volume gradually increased to 93.3 ± 0.8% of the control value within 8 min, i.e. hepatocytes exhibited an RVI of 42.7 ± 0.1% ($P < 0.001$). As shown in Fig. 2B, this RVI was completely blocked in the continuous presence of 10^{-5} mol/l amiloride. These changes in cell volume are very similar to the volume responses of rat hepatocytes in the presence of HCO_3^- [7] and, thus, validate the use of HCO_3^--free solutions for the analysis of RVI (see Section 4: Discussion).

The overall voltage response of rat hepatocytes to hypertonic stress was an initial membrane depolarization by 6.9 ± 0.6 mV ($n = 9$, $P < 0.001$) followed by a slowly developing membrane hyperpolarization

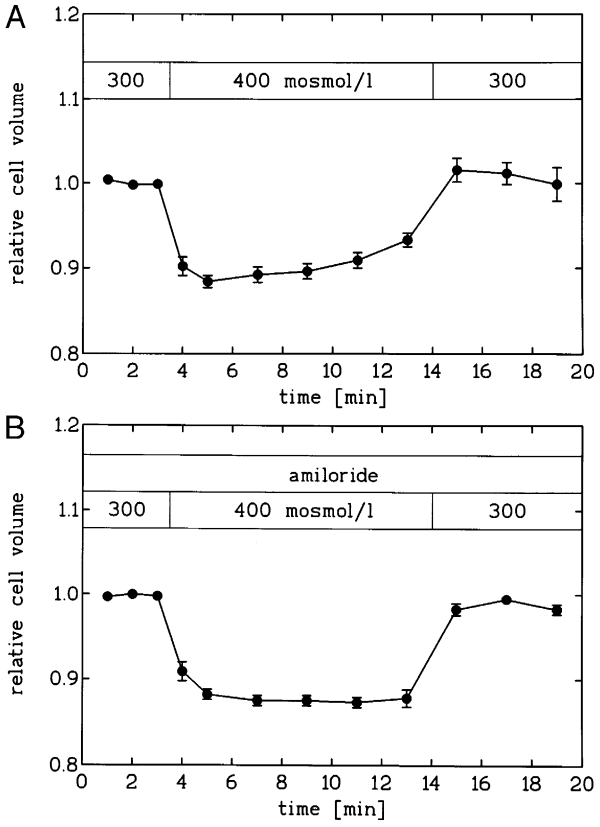

Fig. 2. A: effects of increased extracellular osmolarity on the cell volume of rat hepatocytes in confluent monolayers. For the time indicated, 100 mmol/l sucrose was added to the normosmotic superfusate of 300 mOsm/l. B: same experiments as in A, but in the continuous presence of 10^{-5} mol/l amiloride. $n = 19$ and 14 for A and B, respectively.

which equalled −7.5 ± 0.8 mV ($P < 0.001$) at 13 min in 400 mOsm/l (Fig. 3A). These effects most likely reflect activation of Na^+ conductance and an increasing electromotive force for K^+ upon activation of Na^+,K^+-ATPase, respectively (cf. [7]). The changes in hepatocyte Na^+ conductance were monitored by means of low Na^+ pulses in which Na^+ was 20-fold reduced in exchange with choline. Although a quantitative analysis of Na^+ conductance by this approach is complicated by the presence of Na^+/H^+ exchange and the pH dependence of K^+ conductance [27,28] which in concert lead to significant membrane depolarizations [15,29], changes in Na^+ conductance become detectable as shifts in the voltage responses to low Na^+ solutions [7,29]. Moreover, no direct evidence for a change in K^+ conductance under hypertonic conditions could be obtained so far and

[1] This apparently low degree of cell shrinkage is, at least in part, due to the rapid activation of volume regulatory mechanisms. In the continuous presence of 1 mmol/l amiloride (to block Na^+ conductance as well as Na^+/H^+ exchange) plus 100 μmol/l furosemide (to block Na^+–K^+–$2Cl^-$ symport) initial cell shrinkage equals approximately 13%, from which an osmotically inactive space of 46% can be calculated (F. Wehner and H. Tinel, unpublished). This is in good agreement with data reported by Corasanti et al. [26] for isolated rat hepatocytes, namely 38%.

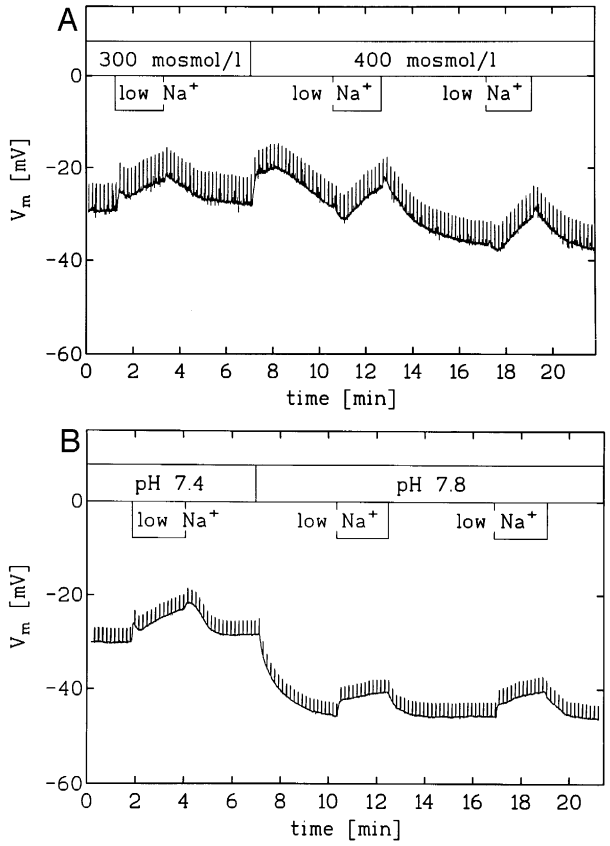

Fig. 3. A: effects of increased osmolarity on membrane voltage (V_m) and on the voltage response to low Na^+ (20-fold reduced with choline as the substitute for the times indicated). Vertical deflections result from injected constant current pulses of 1 nA. B: effects of increased extracellular pH on membrane voltage and on the voltage response to low Na^+. Current pulses of 1 nA.

ion substitution experiments (in HCO_3^--free solutions) clearly show that Cl^- conductance is not changing [7] [2]. Accordingly, in normosmotic solutions, a 20-fold reduction of extracellular Na^+ in exchange for choline led to slowly developing membrane depolarizations (Fig. 3A) which equalled 2.1 ± 0.5 mV at 30 s in low Na^+ solution, which is the most sensitive time point for this kind of analysis [7]. Increasing osmolarity from 300 to 400 mOsm/l clearly shifted this membrane response to more negative values: taking the above time point in low Na^+ solution as reference, membrane voltages changed by -2.1 ± 0.6 and -0.8 ± 0.5 mV after 200 and 500 s in 400 mOsm/l, respectively ($n = 9$; Fig. 3A and Fig. 4A), equivalent to negative shifts in the voltage responses to low Na^+ by -4.3 ± 0.8 mV ($P < 0.001$) and by -2.9 ± 0.6 mV ($P < 0.01$). In the continuous presence of 10^{-5} mol/l amiloride, which selectively blocks the volume-activated Na^+ conductance in rat hepatocytes without exhibiting significant effects on Na^+/H^+ exchange [7] these negative shifts were reduced to -0.8 ± 0.3 mV ($P < 0.005$) and -0.4 ± 0.3 mV ($P < 0.005$, $n = 8$).

A possible role of cell alkalinization in the activation of Na^+ conductance was then examined in Na^+ substitution experiments, in which Na^+ was reduced in normosmotic solutions at pH 7.4 and after change to pH 7.8. A typical measurement from this series is depicted in Fig. 3B and data are summarized in Fig. 4B. These experiments clearly show that under isotonic conditions, a cell alkalinization by 0.3 pH units does not change the voltage response of rat hepatocytes to low Na^+ solutions. The pronounced hyperpolarization of membrane voltages after change to pH 7.8 which equalled -15.6 ± 1.4 mV ($n = 9$) and which coincided with a decrease in cell input resistance to $80 \pm 5\%$ of the control values most probably reflects an increase in cell membrane K^+ conductance [27,28].

It may be argued that the above-mentioned increase in K^+ conductance at pH 7.8 could mask a parallel activation of Na^+ conductance. To elucidate this point, we applied (2 mmol/l) Ba^{2+} pulses in normosmotic solutions at pH 7.4 and after change to pH 7.8. Ba^{2+} is an effective blocker of K^+ conductance in hepatocytes [17,30] and if cell alkalinization exclusively results in a change of K^+ conductance, then membrane voltages in the presence of Ba^{2+}, i.e., at the azimuth of the Ba^{2+} pulses, are expected to be virtually identical and independent of pH. As Fig. 5 exemplifies, this, in fact, is the case: membrane

[2] An additional validation for the use of Na^+ substitution experiments in the analysis of hypertonicity-induced changes of Na^+ conductance is provided on base of cable analysis of specific cell membrane resistances. In an earlier report from this laboratory, it became obvious that, in HCO_3^--containing solutions, hypertonic stress led to a significant decrease of this parameter which coincided with a pronounced negative shift in the voltage response to low Na^+ [7]. Preliminary cable analysis experiments reveal that also in HCO_3^--free solutions, hypertonic stress leads to a continuous reduction of specific cell membrane resistance which equalled 5.5 ± 1.6 k$\Omega \cdot cm^2$ ($n = 6$) in normosmotic solution and which decreased to 86.7 ± 2.7 and $77.2 \pm 4.2\%$ of control at 200 and 500 s in 400 mOsm/l, respectively (see also Section 4).

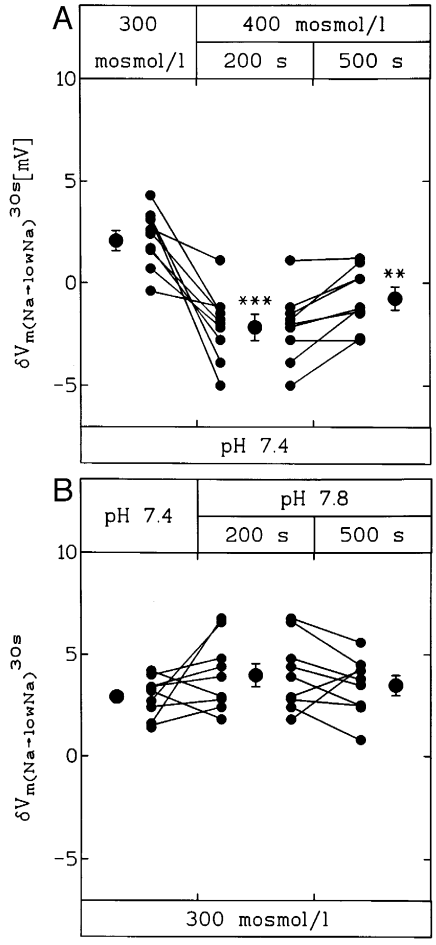

Fig. 4. Summary of the effects of increased osmolarity (A) and increased pH (B) on the voltage responses to low Na^+. Depicted are the changes in membrane voltage upon Na^+ substitution ($\delta V_{m(Na \rightarrow low\ Na)}$) at 30 s in low Na^+ solutions. Asterisks indicate significant difference from control: ** $P < 0.01$ and *** $P < 0.001$.

voltages in the presence of Ba^{2+} equalled -24.9 ± 1.9 mV ($n = 7$) at pH 7.4 and -25.2 ± 1.9 and -25.8 ± 2.0 mV at 200 and 500 s after change to pH 7.8, respectively, which is not significantly different to the preperiod ($P > 0.6$). The addition of Ba^{2+} increased cell input resistances by $12 \pm 2\%$ at pH 7.4 and this effect was increased to $20 \pm 2\%$ at pH 7.8, again in line with the notion that pH-induced membrane hyperpolarization is due to an increase in K^+ conductance. The apparently small effects of Ba^{2+} (and cell alkalinization) on cell input resistance are

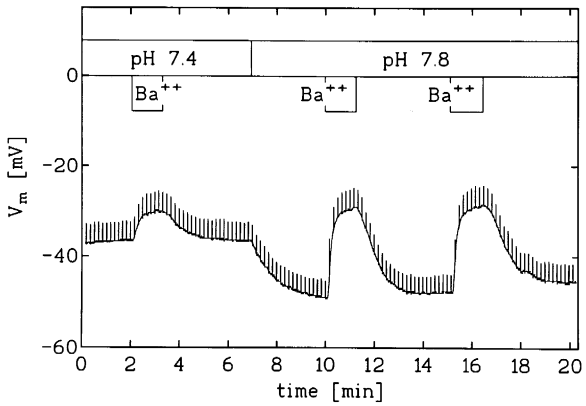

Fig. 5. Effects of an increased extracellular pH on membrane voltage and on the voltage response to 2 mmol/l Ba^{2+} (added to the superfusate for the times indicated). Current pulses of 1 nA.

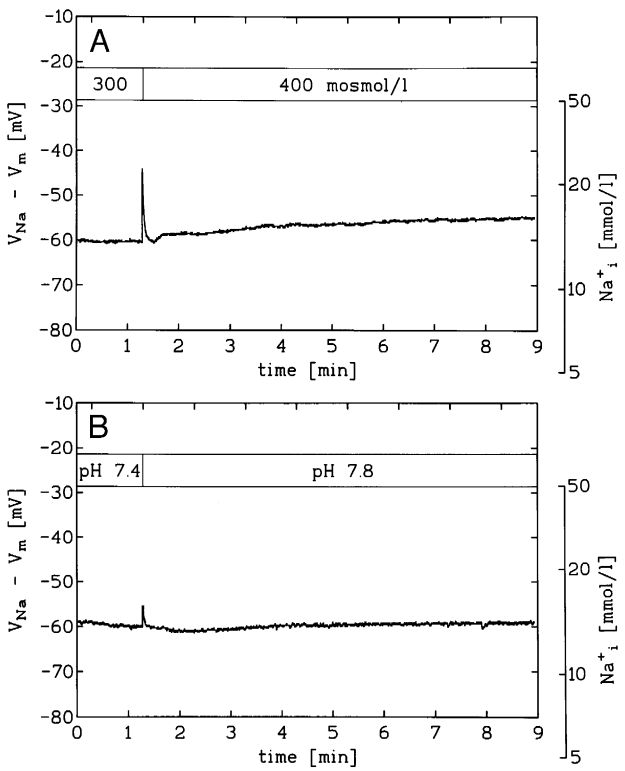

Fig. 6. Effects of increased osmolarity (A) and increased pH (B) on intracellular Na^+ activities (Na_i^+). Na_i^+ is calculated from the signal of a Na^+-sensitive microelectrode (V_{Na}) corrected for membrane voltage (V_m) which is determined in parallel by use of a conventional intracellular microelectrode (see Section 2: Material and methods). Voltage deflections are due to superfusion artifacts sensed by the high-impedance Na^+-sensitive electrodes.

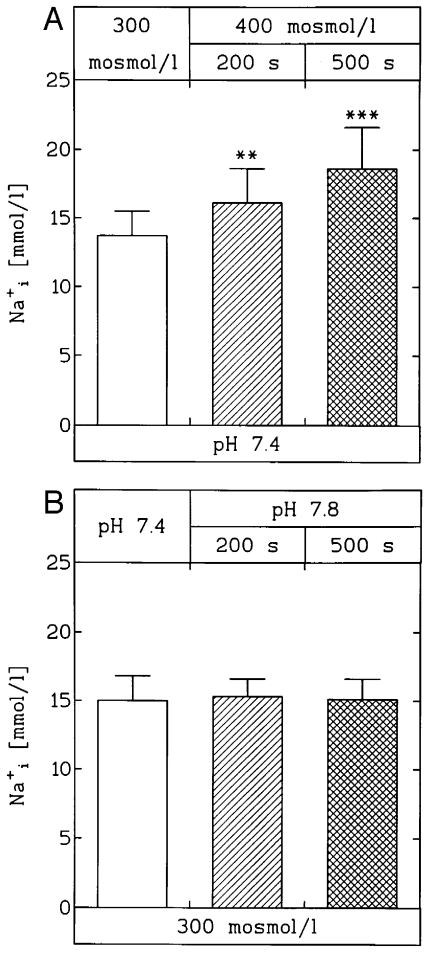

Fig. 7. Summary of the effects of increased osmolarity (A) and increased pH (B) on intracellular Na^+ activities (Na_i^+). $n = 9$ for each experimental condition. Asterisks indicate significant difference from control: ** $P < 0.01$ and *** $P < 0.001$.

due to the high degree of electric cell-to-cell coupling which is the main determinant of this parameter in confluent monolayers of rat hepatocytes [7,15–17].

We then determined the effects of hypertonic stress and cell pH on intracellular Na^+ activities and took these as an indirect measure of changes in Na^+ conductance (see below). Increases in Na^+ activities expected to occur solely via cell shrinkage (cf. Fig. 2A and B) are given in square brackets. In the control experiments, cell Na^+ increased from 13.7 ± 1.8 mmol/l in 300 mOsm/l ($n = 9$) to 16.1 ± 2.5 mmol/l [15.3 mmol/l] (n.s., $P < 0.1$, taking the expected changes via cell shrinkage into account) and 18.6 ± 3.0 mmol/l [14.9 mmol/l] ($P < 0.01$) at 200 and 500 s in hypertonic solution, respectively, equivalent to increases to $114.7 \pm 3.5\%$ [111.7%] and

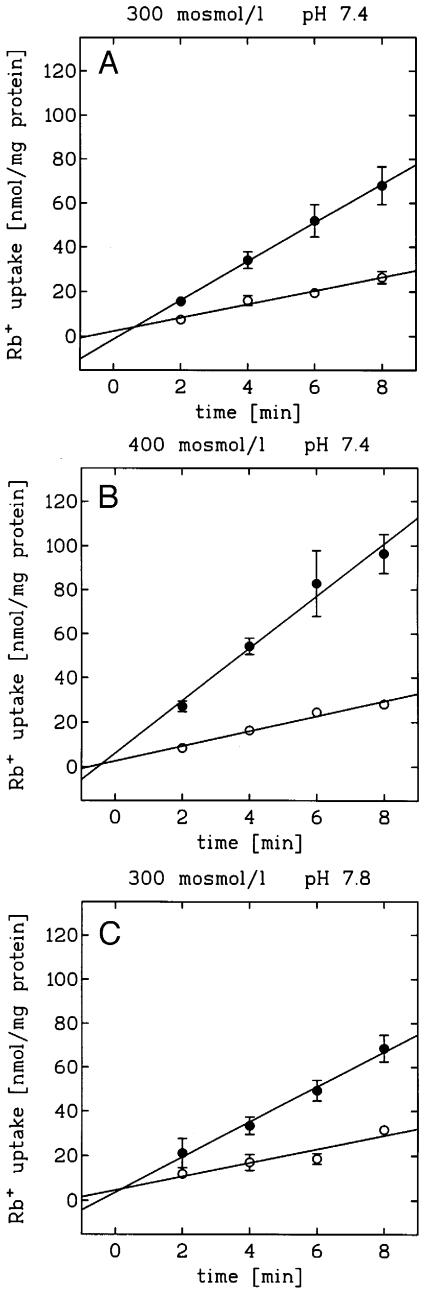

Fig. 8. Rb^+ uptake of rat hepatocyte monolayers in 300 mOsm/l, pH 7.4 (A), in 400 mOsm/l, pH 7.4 (B), and in 300 mOsm/l, pH 7.8 (C), under control conditions (filled circles) and in the presence of 2 mmol/l ouabain (open circles). $n = 4$ for each datapoint. In some cases, the error bars are smaller than the symbols used.

132.2 ± 8.5% [108.8%] (Fig. 6A and Fig. 7A). In the presence of 10^{-5} mol/l amiloride, cell Na^+ equalled 12.7 ± 1.8 mmol/l under normosmotic conditions ($n = 9$) and 13.4 ± 2.0 mmol/l [14.5 mmol/l] and 13.2 ± 1.8 mmol/l [14.5 mmol/l] at 200 and 500 s after change to 400 mOsm/l, respectively, which is not significantly different. This further supports the notion that (also in HCO_3^--free solutions conductive) Na^+ entry rather than Na^+ transport via Na^+/H^+ exchange plays the key role in the increase of cell Na^+ under hypertonic stress. In the pH experiments performed in normosmotic solutions, cell Na^+ equalled 15.0 ± 1.8 mmol/l at pH 7.4 and 15.3 ± 1.3 and 15.1 ± 1.5 mmol/l at 200 and 500 s after change to pH 7.8, respectively (Fig. 6B and Fig. 7B). Thus, an intracellular alkalinization by 0.3 pH units has virtually no effect on the intracellular Na^+ activity of rat hepatocytes.

Taken together, the Na^+-substitution experiments, the measurements with Ba^{2+} pulses, and the recordings with Na^+-sensitive microelectrodes clearly show that, in rat hepatocytes under hypertonic stress, cell alkalinization is not the triggering mechanism for activation of Na^+ conductance.

In an additional set of experiments, we tested the hypothesis that intracellular alkalinization may serve as an activator of Na^+,K^+-ATPase. In 300 mOsm/l, the ouabain (2 mmol/l)-sensitive portion of $^{86}Rb^+$ uptake equalled 5.5 ± 1.1 nmol mg protein^{-1} min^{-1} (Fig. 8A). In 400 mOsm/l, this $^{86}Rb^+$ uptake was significantly increased to 8.5 ± 1.6 nmol mg protein^{-1} min^{-1} ($P < 0.01$; Fig. 8B). In contrast, $^{86}Rb^+$ uptake in 300 mOsm/l at pH 7.8 equalled 4.9 ± 1.6 nmol/mg protein^{-1} min^{-1} which is not significantly different to the control value (Fig. 8C). The results from this series of measurements are summarized in Table 1.

4. Discussion

In the present study, we investigated the role of cell alkalinization as a possible triggering mechanism in the activation of Na^+ conductance and Na^+,K^+-ATPase in rat hepatocytes. Intracellular alkalinization becomes evident as the response of Na^+/H^+ exchange to hypertonic stress [2] and equals approximately 0.3 pH units upon change from 300 to 400 mOsm/l [7]. We mimicked this cell alkalinization on base of a series of calibration experiments in which we find that pH_i is a linear function of pH_e with a slope of approximately 0.7. This behavior of cell pH is in good agreement with the findings of Samuelson et al. [31] who reported that in primary cultures of rat hepatocytes, pH_i follows pH_e in the range of 6.2 to 7.6 in a linear fashion and with a slope of one in the presence of HCO_3^- and with a considerably lower slope in its absence. The above experimental approach differs from the volume-induced cell alkalinization where solely intracellular pH is changing and extracellular pH remains constant. Given the complete absence of any effect of alkalinization on Na^+ conductance as well as Na^+,K^+-ATPase in our measurements, however, we do not consider significant effects of these differences in extracellular pH to be likely. In addition, we are not aware of an experimental protocol that would allow us to stably alkalinize hepatocytes for time periods as long as 8–13 min while leaving the extracellular pH unchanged.

As confocal laser microscopy revealed, hypertonicity-induced changes in cell volume, both in the absence and in the presence of 10^{-5} mol/l amiloride, are similar in HCO_3^--free (this study) and HCO_3^--containing solutions [7]. This finding per se does not lead us to conclude that Na^+–HCO_3^- cotransport is *not* involved in the RVI of rat hepatocytes, because if the

Table 1
Effects of increases in osmolarity and pH on $^{86}Rb^+$ uptake

	300 mOsm/l, pH 7.4	400 mOsm/l, pH 7.4	300 mOsm/l, pH 7.8
Control	8.3 ± 1.1	11.8 ± 1.6 **	7.9 ± 1.5
Ouabain	2.8 ± 0.1	3.4 ± 0.2 **	3.0 ± 0.2
Control minus ouabain	5.5 ± 1.1	8.5 ± 1.6 **	4.9 ± 1.6

Data are given in nmol $^{86}Rb^+$ mg protein^{-1} min^{-1}. Measurements were performed under control conditions and in the presence of 2 mmol/l ouabain.
** Significantly different to 300 mOsm/l, pH 7.4 with $P < 0.01$. Paired experiments; $n = 4$ for each experimental condition.

initial increase in cell Na^+ under hypertonic conditions is achieved by combination of different Na^+ entry pathways, namely Na^+ conductance, Na^+/H^+ exchange, and Na^+–HCO_3^- cotransport, then blockage of one of these transporters will result (via cell volume) in an increased activation of the others [3]. Such a mechanism could explain the somewhat slower onset of RVI in this study when compared to HCO_3^--containing conditions (cf. [7]), although these differences are not statistically significant. Nevertheless, the use of HCO_3^--free solutions was essential to achieve stable intracellular alkalinizations for reasonable experimental time frames and will not interfer with our analysis of Na^+ conductance and Na^+,K^+-ATPase.

In our ion substitution experiments, increases in Na^+ conductance were monitored as negative shifts in the voltage response to a low Na^+ solution [7,29]. This approach is valid because cable analysis in HCO_3^--containing [7] as well as in HCO_3^--free solutions (F. Wehner, unpublished results; see footnote 2) reveal a continuous decrease of specific cell membrane resistance under hypertonic stress which excludes a possible decrease in K^+ conductance as the source of the above-mentioned negative voltage shifts. Moreover, in rat hepatocytes, the equilibrium potential for Cl^- is known to strictly follow membrane voltage [33] so that possible changes in Cl^- conductance will not become evident as part of the voltage response to low Na^+. In an earlier report from this laboratory [7], we succeeded in unmasking Na^+ conductance in HCO_3^--containing solutions by the addition of quinine, which is an effective blocker of K^+ conductance in hepatocytes [30], and thus eliminates secondary effects of Na^+ substitution via this pathway. In the absence of HCO_3^-, however, this approach was not feasible: successive Na^+ substitutions carried out in the continuous presence of 0.5 mmol/l quinine (or 1 mmol/l Ba^{2+}) led to progressive decreases in membrane voltage and input resistance indicative of a slow deterioration of cells.

As could be shown for frog skin epithelium, toad urinary bladder, A6 cells, as well as for rat cortical collecting duct, changes in cell pH may be of considerable importance for the regulation of conductive Na^+ entry [8,10–12]. In contrast, the Na^+ substitution experiments, the quantification of K^+ conductance by means of Ba^{2+} pulses, and the determination of intracellular Na^+ activities performed in this study clearly show that, in rat hepatocytes, the activation of Na^+/H^+ exchange and the resultant alkalinization of the cytosol is not the triggering mechanism for the increase of rat hepatocyte Na^+ conductance under hypertonic conditions. We, therefore, conclude that activation of Na^+ conductance and Na^+/H^+ exchange together, but to a markedly different extent, serve to gain Na^+ as the initial osmolyte during RVI with Na^+ conductance playing the prominent role. It should be kept in mind, however, that we focussed our analysis of the RVI in rat hepatocytes on one particular osmotic step so far and that the relative contribution of transporters to this process may vary according to the degree of hypertonicity because there is no reason to assume that they exhibit identical set-points for activation.

With respect to Na^+,K^+-ATPase, our $^{86}Rb^+$ uptake experiments reveal virtually no influence of cell alkalinization on the activity of this enzyme. Thus, short-term regulation of Na^+,K^+-ATPase which in primary cultures of rat hepatocytes exhibits a K_m value of 17.8 mmol/l for intracellular Na^+ [34] will mainly occur via changes in cell Na^+ activity. The above value is well within the range of Na^+ activities found in 300 mOsm/l and at 500 s after change to 400 mOsm/l which were 13.7 and 18.6 mmol/l, respectively.

In conclusion, we used confocal laser scanning microscopy, intracellular recordings with conventional and Na^+-sensitive microelectrodes, as well as measurements of time-dependent $^{86}Rb^+$ uptake to assess a possible role of cell alkalinization in the RVI of rat hepatocytes. Our results indicate that an intracellular alkalinization comparable in size to that following stimulation of Na^+/H^+ exchange under hypertonic conditions is not responsible for the activation of Na^+ conductance and probably does not participate in the stimulation of Na^+,K^+-ATPase. Based on our experimental strategy, however, we cannot exclude a modulatory role of intracellular pH in the activation of both pathways under hypertonic stress.

[3] A comparable cross-talk between different transport mechanisms has also been postulated for the release of organic osmolytes in rat inner medullary collecting duct cells under *hypo*tonic stress [32].

Acknowledgements

Without the invaluable technical assistance of G. Beetz, S. Rosin-Steiner, and A. Giffey this study would not have been possible. We are also grateful to D. Mägdefessel for excellent secretarial help.

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