Hypertonic Stress Increases the Na⁺ Conductance of Rat Hepatocytes in Primary Culture

FRANK WEHNER, HEINRICH SAUER, and ROLF K. H. KINNE

From the Max-Planck-Institut für molekulare Physiologie, Abteilung Epithelphysiologie, 44026 Dortmund, Germany

ABSTRACT We studied the ionic mechanisms underlying the regulatory volume increase of rat hepatocytes in primary culture by use of confocal laser scanning microscopy, conventional and ion-sensitive microelectrodes, cable analysis, microfluorometry, and measurements of ⁸⁶Rb⁺ uptake. Increasing osmolarity from 300 to 400 mosm/liter by addition of sucrose decreased cell volumes to 88.6% within 1 min; thereafter, cell volumes increased to 94.1% of control within 10 min, equivalent to a regulatory volume increase (RVI) by 44.5%. This RVI was paralleled by a decrease in cell input resistance and in specific cell membrane resistance to 88 and 60%, respectively. Ion substitution experiments (high K⁺, low Na⁺, low Cl⁻) revealed that these membrane effects are due to an increase in hepatocyte Na⁺ conductance. During RVI, ouabain-sensitive ⁸⁶Rb⁺ uptake was augmented to 141% of control, and cell Na⁺ and cell K⁺ increased to 148 and 180%, respectively. The RVI, the increases in Na⁺ conductance and cell Na⁺, as well as the activation of Na⁺/K⁺-ATPase were completely blocked by 10⁻⁵ mol/liter amiloride. At this concentration, amiloride had no effect on osmotically induced cell alkalinization via Na⁺/H⁺ exchange. When osmolarity was increased from 220 to 300 mosm/liter (by readdition of sucrose after a preperiod of 15 min in which the cells underwent a regulatory volume decrease, RVD) cell volumes initially decreased to 81.5%; thereafter, cell volumes increased to 90.8% of control. This post-RVD-RVI of 55.0% is also mediated by an increase in Na⁺ conductance. We conclude that rat hepatocytes in confluent primary culture are capable of RVI as well as of post-RVD-RVI. In this system, hypertonic stress leads to a considerable increase in cell membrane Na⁺ conductance. In concert with conductive Na⁺ influx, cell K⁺ is then increased via activation of Na⁺/K⁺-ATPase. An additional role of Na⁺/H⁺ exchange in the volume regulation of rat hepatocytes remains to be defined.

This work was done with the technical assistance of Gabriela Beetz, Alexander Giffey, Sigrid Rosin-Steiner, and Hendrike Schütz.

Address correspondence to Dr. Frank Wehner at Max-Planck-Institut für molekulare Physiologie, Abteilung Epithelphysiologie, Postfach 10 26 64, 44026 Dortmund, Germany.
INTRODUCTION

Upon exposure to anisotonic conditions most cells, including hepatocytes, initially behave as osmometers and change their volumes according to the tonicity of the extracellular compartment (Corasanti, Gleeson, and Boyer, 1990). Many cell types, however, are capable to actively restore their volumes despite continuous hypotonic and hypertonic challenge, which is called regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively (see Lang, Ritter, Volkl, and Häussinger, 1993, for review).

Hepatocytes may, to a certain extent, undergo changes in cell volume due to absorptive processes in the small intestine (Haberich, Aziz, and Nowacki, 1965). Physiologically more relevant, however, are changes in hepatocyte volume occurring in the course of substrate transport and metabolism (Häussinger, Gerok, and Lang, 1993). Moreover, alterations of cell volume may represent a new principle of metabolic control in the liver (Häussinger and Lang, 1991).

In the perfused rat liver, hepatocytes exhibit a partial RVI under hypertonic conditions (Haddad, Thalhammer, and Graf, 1989; Lang, Stehle, and Häussinger, 1989). Isolated rat hepatocytes, in contrast, remain continuously shrunken for periods of up to 30 min when exposed to hypertonic solutions; in this model system, cells are only capable of a post-RVD-RVI: they exhibit a significant RVI only when transferred back from hypoosmotic to normosmotic solutions, i.e., after a preperiod of hypotonic stress in which partial RVD had occurred (Bakker-Grunwald, 1983; Corasanti et al., 1990). For mouse hepatocytes, indirect evidence for a RVI under hypertonic conditions has been reported (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990).

RVI is mediated by cellular salt and water uptake and generally occurs via Na⁺-K⁺-2Cl⁻ cotransport, Na⁺-Cl⁻ cotransport, or (parallel) activation of Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchange; cell Na⁺ may then be exchanged for K⁺ by activation of the Na⁺/K⁺-ATPase, resulting in a net increase in cellular K⁺ and Cl⁻ (Lang et al., 1993). Inhibition of K⁺ and Cl⁻ channels may support the net increase of cellular osmolytes in some systems (Graf, Haddad, Häussinger, and Lang, 1988; Stoddard and Reuss, 1989; Wang and Wondergem, 1991).

In the intact rat liver, an increase in perfusate osmolarity stimulates K⁺ uptake (Graf et al., 1988); this effect is inhibited by millimolar concentrations of ouabain and amiloride (Haddad and Graf, 1989; Haddad et al., 1989; Häussinger, Stehle and Lang, 1990) and is supposed to reflect accumulation of cell K⁺ by the Na⁺/K⁺-ATPase after an increase in cell Na⁺ via Na⁺/H⁺ exchange (Haddad and Graf, 1989; Häussinger et al., 1990), although Na⁺ entry mechanisms other than Na⁺/H⁺ exchange are not excluded (Graf et al., 1988; Wang and Wondergem, 1991). In support of the role of Na⁺/H⁺ exchange in RVI, Gleeson, Corasanti, and Boyer (1990) reported a significant increase in the activity of this transporter in subconfluent primary cultures of rat hepatocytes after change from 300 to 500 mosm/liter as well as after change back from 160 to 300 mosm/liter. In contrast to this, isolated rat hepatocytes exhibit post-RVD-RVI only (Corasanti et al., 1990). In mouse hepatocytes, hypertonic stress leads to a sustained depolarization of membrane voltage and a considerable increase in membrane resistance, which is interpreted as a decrease of
cell membrane K⁺ conductance (Graf et al., 1988; Khalbuss and Wondergem, 1990; Wang and Wondergem, 1991).

In the present study, we analyzed the mechanisms of RVI in confluent primary cultures of rat hepatocytes by means of confocal laser scanning microscopy, intracellular recordings with conventional and ion-sensitive microelectrodes, cable analysis, microfluorometry, and ⁸⁶Rb⁺ uptake experiments. We find that rat hepatocytes in this model system are capable of both RVI and post-RVD-RVI. We present evidence that in addition to activation of Na⁺/H⁺ exchange, hypertonic stress leads to a prominent increase in cell membrane Na⁺ conductance. In concert with conductive Na⁺ entry, Na⁺/K⁺-ATPase increases cell K⁺ to values as high as 170–180% of control. Contribution of Na⁺/H⁺ exchange to the RVI of rat hepatocytes appears to be minor and we have no evidence for a significant decrease of K⁺ conductance under hypertonic conditions. To our knowledge this is the first system, in which an increase in Na⁺ conductance significantly contributes to RVI.

METHODS

Isolation and Primary Culture of Hepatocytes

Isolation of hepatocytes and culturing of cells was the same as described previously (Petzinger, Föllmann, Acker, Hentschel, Zierold, and Kinne, 1988; Wehner and Guth, 1991). Briefly, after heparinization male Wistar rats (220 to 280 g body weight) were exsanguinized under urethane anaesthesia by in situ perfusion of the liver with nominally Ca²⁺-free Krebs-Henseleit solution (pH 7.4, 95% O₂/5% CO₂, 37°C). After removal, the liver was perfused for 20 min with 0.05% collagenase A, dissolved in the same buffer. Isolated cells were plated on collagen-coated Petriperm dishes and cultured in Dulbecco's Modified Eagles Medium (DMEM) fortified with 10% fetal bovine serum, 2 mmol/liter glutamine, penicillin-streptomycin (100 U/ml, 100 µg/ml), 10⁻⁶ mol/liter dexamethasone, 10⁻⁸ mol/liter triiodothyronine/thyroxine (T₃/T₄), and 5 µg/ml bovine insulin at 37°C in 5% CO₂/air. They form confluent monolayers within 24 h and were used at day 2 after preparation.

Petriperm dishes were bought from Bachofen (Reutlingen, Germany). DMEM, penicillin-streptomycin, and glutamine were purchased from Flow (Bonn, Germany). Collagenase and fetal bovine serum were obtained from Boehringer (Mannheim, Germany). All other components were purchased from Serva Chemical Company (Heidelberg, Germany).

Electrophysiological Techniques

Experimental setup and recording techniques are described in detail in previous publications (Wehner and Guth, 1991; Wehner, Beetz, and Rosin-Steiner, 1992). Briefly, sheets of gas-permeable membranes of ~1 cm² with confluent cell monolayers were cut from the bottom of the culture dishes and transferred to the superfusion chamber mounted on the stage of an inverted microscope (IM 35, Zeiss, Oberkochen, Germany). The total fluid volume above the tissue was 0.1 ml. Cells were continuously superfused at a rate of 4 ml/min; changes of experimental solutions were achieved by means of a four-way valve (ms-131 D; Whitey, Highland Heights, OH) close to the chamber. Storage vessels, superfusion lines and chamber were water-jacketed and maintained at 36 ± 0.5°C.

Two-channel microelectrodes were pulled from 1.5 mm OD Thick-Septum-Theta glass capillaries (WPI, New Haven, CT) on a Kopf vertical puller (750; David Kopf Instruments, Inc.).

1 Approved by Regierungspräsident Arnsberg and the Institute Animal Care Committee.
They were filled with 0.5 mol/liter KCl and had resistances of 80–130 MΩ when immersed in control Tyrode solution. One channel was used to measure voltage, the second to inject constant current pulses. The reference electrode was an Ag-AgCl wire in series with an agar-KCl (0.5 mol/liter bridge) except in Cl– substitution experiments, where a custom-made 5 mol/liter KCl flowing junction was used to avoid liquid junction potentials. The respective reference electrode was placed in an additional 1-ml compartment connected to the chamber via a hole 1.5 mm in diameter and 15 mm in length (through which all superfusates were removed) to exclude any contamination of the preparation by KCl leakage. In low Na+ solutions (see below) a liquid junction potential of 1.7 mV evolved at the agar-KCl bridge (measured with the KCl flowing junction as the reference). All membrane voltages in low Na+ were corrected by this amount (except those shown in the original tracings of Figs. 5, 7, and 8).

There were no measurable liquid junction potentials in the K+ substitution experiments.

Cell impalements were performed at an angle of 45° on a custom-made vibration-damped table under 320 × magnification by use of piezomanipulators (PM 500-20; Frankenberger, Germering, Germany). Cell membrane potentials and input resistances were determined by use of a high impedance electrometer equipped with a current injection unit (Frankenberger). Every 10 s current pulses of 1-s duration and of 1–5 nA were injected, as appropriate. Measured voltages were A/D converted (DT 2811; Data Translation, Inc., Marlboro, MA) and displayed and stored on a personal computer (PS 2/30; IBM). Criteria for successful impalements have been previously described (Wehner and Guth, 1991).

In the cable analysis, current pulses of 3–10 nA were injected into a cell and the resultant changes in membrane voltage were recorded in a second cell with a single-channel electrode either at 35, 100, 200, or 400 μm from the point of current injection. Single-channel electrodes were pulled from inner fiber borosilicate glass capillaries of 1.5 mm OD (Hilgenberg, Malsfeld, Germany) and had resistances of 50–70 MΩ. The voltage deflections in cell 2 (ΔV2) were normalized according to a standard current pulse of 10 nA (Wehner and Guth, 1991) and plotted against the distance between both electrodes (x). Data were fitted by use of the form

$$\Delta V_2 = A \cdot K_0 \left( \frac{x}{\lambda} \right)$$

(1)

(Frömter, 1972) and a computer-based least-square fit routine. $K_0$ is a zero-order Bessel function and $A$ and $\lambda$ are constants defining the function. From these constants cell coupling resistance ($R_c$) and specific cell membrane resistance ($R_m$) can be calculated according to

$$R_c = \frac{2 \pi A}{i_0}$$

(2)

and

$$R_m = R_c \lambda^2$$

(3)

respectively. $i_0$ is the total current applied.

Ion-sensitive microelectrodes were pulled from borosilicate filament glass capillaries of 1.0 mm OD and 0.5 mm ID (Hilgenberg) on a horizontal puller (PD-5, Narishige, Tokyo, Japan) and had resistances of 80–120 MΩ when filled with 0.5 mol/liter KCl. Electrodes were silanized by exposure to dimethyldichlorosilane vapor (Fluka, Neu-Ulm, Germany) at 200°C for 1 h. They were then back-filled with either Na-Ionophore 1-Cocktail A (Fluka, No. 71176) or K-Ionophore 1-Cocktail A (valinomycin; Fluka, No. 60031) and 0.5 mol/liter NaCl or 0.1 mol/liter KCl, respectively. The ion-sensitive microelectrodes were calibrated in mixed NaCl/KCl standard solutions, the sum of NaCl and KCl being 150 mmol/liter. The test solutions for Na+- and K+-sensitive electrodes contained 150, 50, 15, 5, and 1.5 mmol/liter NaCl and 150,
100, 10, 2.7, and 1 mmol/liter KCl, respectively, and were buffered to pH 7.4 with 5 mmol/liter tris-(hydroxymethyl)-aminomethane (Sigma, München, Germany). The activity coefficients were assumed to be constant and equal to 0.77 (Horisberger and Giebisch, 1988). The slope (S) and the selectivity coefficient for Na⁺ over K⁺ \( (K_{Na/K}) \) of Na⁺-sensitive electrodes were obtained by fitting the measured potential difference \( (V) \) and calculated Na⁺ activities \( (a_{Na}) \) of the test solutions by use of a nonlinear least-square fit routine to the Nicolsky equation (Edelman, Curci, Samarzija, and Frömter, 1978; Horisberger and Giebisch, 1988):

\[
\Delta V = S \left[ \log (a_{Na} + K_{Na/K}a_K) - \log (a_{Na,150}) \right],
\]

where \( a_{Na,150} \) is the Na⁺ activity of the 150 mmol/liter test solution. Electrodes were calibrated before and after use and the slopes and selectivity coefficients \( (1/K_{Na/K}) \) were 60.7 ± 3.5 mV/decade and 57 ± 29, respectively (mean values ± standard deviations). Intracellular Na⁺ activities were then calculated using the form

\[
a_{Na,i} = \frac{115.5 \left[ 10^{(V_m/Vm/s)} - K_{Na/K} \right]}{1 - K_{Na/K}}
\]

(Horisberger and Giebisch, 1988), where \( V_m \) is the voltage of a second intracellular microelectrode (see below). The slope of the K⁺-sensitive electrodes was determined according to the above procedure and was 55.6 ± 2.8 mV/decade (mean ± SD); \( K_{K/Na} \), however, was found to be virtually zero. The intracellular recordings were performed as follows: a single cell was first impaled with the internal reference electrode pulled from the same capillaries and with the same protocol as the ion-sensitive one, but filled with 0.5 mol/liter KCl. Once a stable registration of \( V_m \) was achieved the same cell was impaled with the ion-sensitive electrode. This was commonly accompanied by a transient positive deflection in \( V_m \) of some 5–8 mV. Only those experiments were accepted in which \( V_m \) was restored to within 2 mV of the original value. In all registrations a custom-made 0.5 mol/liter KCl flowing junction (outside the experimental chamber; see above) was used as the external reference.

**Confocal Laser Scanning Microscopy**

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) as previously described (Wehner, Rosin-Steiner, Beetz, and Sauer, 1993). Briefly, cells were exposed to the probe (in its acetoxymethyl ester form at a final concentration of ~10 μmol/liter) for 30 min and subsequently bathed in dye-free solution for at least 15 min before the experiment. Cell monolayers were then transferred to a Perspex chamber of 0.1 ml/vol and continuously superfused at a rate of 3 ml/min. Cell fluorescence was excited by use of the 488-nm band of an argon ion laser (Ion Laser Technology, Salt Lake City, UT) and the 442-nm band of a helium-cadmium laser (4310 N, Liconix, Santa Clara, CA) on a confocal laserscan unit (MRC-600, BioRad, Hemel Hempstead, UK). This device was coupled to a standard microscope (Diaphot, Nikon, Düsseldorf, Germany) with a 6.3 x Neofluar lens (Zeiss). Images were acquired every 60 s. Data were digitized and analyzed by use of a microcomputer (RM Nimbus AX/2, Oxford, UK) and cell pH was determined as the fluorescence ratio from both excitation wavelengths. Calibration of cell pH was performed as described elsewhere (Thomas, Buchsbaum, Zimniak, and Racker, 1979): Briefly, at the end of each experiment, monolayers were exposed to 140 mmol/liter KCl and 10 μmol/liter nigericin in HEPES-buffered solutions (10 mmol/liter) in the pH range of 6.4–7.8.

Cell volumes were determined by use of the confocal laser microscope with a 20 × objective (numerical aperture 0.65; Nikon) and the 488-nm band of the argon laser. Cells were loaded for 45 min with calcein (Molecular Probes, Inc.) in its acetoxymethyl ester form at a final
concentration of 10 μmol/liter and washed, thereafter, for ~5 min in dye-free solutions. Calcein is used as a volume marker of aqueous compartments and is (under physiological conditions) pH- and Ca²⁺ insensitive (Allen and Cleland, 1980; Kendall and MacDonald, 1983). The photobleaching of calcein was less than that of BCECF and, interestingly, calcein exhibits a high degree of fluorescence self quenching (see below; Kendall and MacDonald, 1983). Two methods were used for determination of cell volumes. First, cells were optically sectioned in 0.9-μm steps following the z axis and from the measured areas of slices (8–12/cycle) cell volumes were computed (Tinel, Wehner, and Sauer, 1994). This method yields absolute values of cell volume, however, the amount of photobleaching was still relatively high. Second, cell volumes were determined by quantifying calcein fluorescence in a single plane, which considerably reduced photobleaching of the dye. As mentioned above, calcein exhibits a pronounced self quenching of fluorescence so that fluorescence decreases upon concentration and increases upon dilution of the compound. Fluorescence was related to cell volume in a set of pilot experiments, in which osmolarities were increased from 300 to 400 mosm/liter, from 300 to 500 mosm/liter, from 300 to 600 mosm/liter, and from 220 to 300 mosm/liter (n = 8 for each protocol); after 1 min, i.e., at a time were RVI is still negligible, percent changes in calcein fluorescence were then compared to percent changes in cell volume determined with the first method. The two parameters were linearly correlated (r = 0.9987) with a slope of 0.601 ± 0.018 (95% confidence). In the experiments, there were no significant differences in the determination of relative cell volumes by either method.

To further validate our calcein experiments and to exclude artefacts due to photobleaching of the dye we performed measurements in which hepatocytes in subconfluent monolayers were superfused with 50 μmol/liter calcein in its free-acid (cell impermeant) form (Molecular Probes, Inc.). Under these conditions, there was no detectable fluorescence increase of intact cells within the experimental time frame of 30 min, whereas dead cells and cell fragments (occasionally present in primary cultures) immediately accumulated the dye. Cell volumes were then quantified by optical sectioning of the unstained image of hepatocytes. In 300 mosm/liter cell volumes determined by this method equalled 5.25 ± 0.49 μl (n = 6). After 1 min in 400 mosm/liter cells were shrunken to 85.3 ± 1.4% and, thereafter, continuously increased their volumes to 91.5 ± 1.6% of control, equivalent to a RVI of 42.0 ± 8.4%. These values are not significantly different from those obtained from calcein-stained cells by use of either optical sectioning or the calcein self quenching technique (c.f. Results).

Measurements of Rb⁺ Uptake

Circular sheets of 16-mm diam with confluent monolayers were cut from the bottom of the culture dishes, carefully washed, and transferred to standard scintillation vials (20 ml) filled with 5 ml of experimental solution. The sheets were fixed in a vertical position by use of fine tipped plastic forceps, which by themselves were clamped to the edges of the vials. Solutions were kept at 37°C and continuously gassed with humidified 95% O₂/5% CO₂. Rb⁺ uptake was determined by transferring monolayers for 2, 4, 6, 8, and 10 min to identical solutions labeled with 1–5 μCi/ml ⁸⁶Rb⁺. Uptake was measured in control solutions of 300 and 220 mosm/liter or 5 min after transfer to hypertonic solutions of 400 and 300 mosm/liter, respectively. Rb⁺ uptake in 300/400 mosm/liter and in 220/300 mosm/liter was determined in paired measurements, i.e., cell monolayers of one series were always derived from a single preparation of hepatocytes. Each experimental protocol was preceded by a preperiod of 10 min. In half of the measurements, 2 mmol/liter ouabain was present throughout the experiments. In an additional set of measurements, 10⁻⁵ mol/liter amiloride was added at the same time when osmolarity was increased from 300 to 400 mosmol/liter and Rb⁺ uptake was determined 5 min thereafter; here paired experiments with the same protocol but without amiloride served as the control. Influxes were terminated by removing the monolayers from the vials and washing them with the
ice-cold experimental solution of appropriate osmolarity. 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/liter ethylenedinitrilo-tetraacetic acid. In pilot experiments with ³⁵Cl-labeled sucrose as a second marker, we found no extracellular compartment that was not accessible to our washing and drying procedure. The whole procedure was completed within 10–15 min. After 60 min of cell lysis, aliquots were removed for liquid scintillation counting and determination of protein content by the method of Lowry, Rosebrough, Farr, and Randall (1951) in triplicate. Samples of experimental solutions were always counted for determination of specific ⁴⁰K⁺ activities.

**Solutions**

The normosmotic control tyrode solution (300 mosm/liter) contained (in millimoles/liter): NaCl, 124; KCl, 2.7; NaHCO₃, 25; NaH₂PO₄, 0.4; CaCl₂, 1.8, MgCl₂, 1.1, glucose, 5.6. In HCO₃⁻free solutions, HCO₃⁻ was replaced with Cl⁻ or its substitute (24 mmol/liter) and HEPES buffer (1 mmol/liter). HCO₃⁻-containing and HCO₃⁻-free solutions were gassed with 5% CO₂ in O₂ and pure O₂, respectively. pH was adjusted to 7.4 by addition of 1 mol/liter HCl (HCO₃⁻-containing solutions) or 4 mol/liter NaOH (HCO₃⁻-free solutions). In the hypoosmotic control solution (220 mosm/liter), the concentration of NaCl was decreased (by 40 mmol/liter) to 84 mmol/liter. In the experiments, increases in osmolarity from 300 to 400 mosm/liter and from 220 to 300 mosm/liter were achieved by addition of 100 and 80 mmol/liter sucrose, respectively. In ion substitution experiments, K⁺ was isoosmotically elevated 10-fold in exchange for Na⁺, Cl⁻ concentration was 100-fold reduced in exchange with gluconate (in HCO₃⁻-free solutions) and Na⁺ was 20-fold reduced with choline.²

HEPES was purchased 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).

**Statistical Analysis**

Mean values ±SE 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 for p < 0.05 was considered significant.

**RESULTS**

As confocal laser microscopy revealed, increasing extracellular osmolarity from 300 to 400 mosm/liter by addition of sucrose decreased the volumes of confluent rat hepatocytes in primary culture to 88.6 ± 0.5% within 1 min (from 5.92 ± 0.26 pl = 100%; n = 20; p < 0.001; Fig. 1 A). Thereafter, cell volumes gradually increased to 94.1 ± 1.1% of the control value within 10 min, i.e., hepatocytes exhibited a RVI by 44.5 ± 5.9% (p < 0.001). Upon return to 300 mosm/liter, cell volumes transiently increased to 107.0 ± 1.2% of control (p < 0.001) and, thereafter, slowly declined towards the baseline level. This overshoot substantiates the activation of volume regulatory mechanisms that are readjusted to initial values when cells return to isoosmotic conditions.

² The apparent K⁺ transference number calculated from these experiments (0.13) is not significantly different from the one reported in an earlier study from this laboratory, in which K⁺ was increased in exchange for tetramethylammonium (i.e., 0.16; Wehner and Guth, 1991).
When osmolarity was increased by changing from hypoosmotic to normosmotic conditions (from 220 to 300 mosm/liter, by readdition of sucrose after an incubation period of at least 15 min) hepatocyte volumes were decreased to 81.5 ± 0.9% (from 6.05 ± 0.15 pl = 100%; n = 20; p < 0.001; Fig. 1 B). Thereafter, cell volumes slowly increased to 90.8 ± 1.6% of the control value, which is equivalent to a RVI by 55.0 ± 6.4% (p < 0.001). After return to 220 mosm/liter cell volumes remained steadily increased at 114.4 ± 5.0% of control (p < 0.001). These measurements show that rat hepatocytes in primary culture exhibit both RVI as well as post-RVD-RVI.

To investigate the ionic mechanisms underlying RVI in rat hepatocytes various electrophysiological experiments were performed. In intracellular recordings with conventional microelectrodes, an increase in osmolarity from 300 to 400 mosm/liter led to a transient depolarization of hepatocyte membrane voltages from −38.9 ± 1.6 to −34.0 ± 1.6 mV (n = 16; p < 0.001) with the maximum effect occurring between 70 and 120 s (Fig. 2 A). This depolarization was followed by a slowly developing membrane hyperpolarization to −43.1 ± 1.7 mV at the end of a 10-min exposure to hypertonic solution, which is 4.2 ± 1.0 mV more negative than the control value.
In confluent primary cultures of rat hepatocytes, cell input resistance is largely determined by the high degree of electric cell-to-cell coupling (Wehner and Guth, 1991). Consequently, the observed decreases in input resistance under hypertonic stress could reflect increases in cell-to-cell coupling rather than increases in membrane conductance. To elucidate this point, cell-to-cell coupling and specific cell membrane conductances were quantified by means of cable analysis. As Table I summarizes, increases in osmolarity from 300 to 400 mosm/liter and from 220 to 300 mosm/liter decreased specific cell membrane resistances (within 10 min) to 60 and
80% of control values (the latter effect remaining short of significance), respectively, while there were no significant changes in electrical cell coupling.

The mechanism by which a hypertonic challenge increases the membrane conductance of rat hepatocytes was analyzed in ion substitution experiments. Ion substitutions were performed under control conditions and at 200 and 600 s after increases in osmolarity to differentiate between possible transient and steady state effects of hypertonic stress. Increasing K\(^+\) concentration from 2.7 to 27 mmol/liter (by isoosmotic replacement of Na\(^+\)) led to a depolarization of 7.8 ± 1.5 mV (n = 10; Fig. 3 A) under normosmotic conditions. In 400 mosm/liter, this voltage response was decreased to 2.2 ± 1.1 mV (p < 0.001) and 5.1 ± 1.0 mV (p < 0.05) at 200 and 600 s after osmolarity increase, respectively. In 220 mosm/liter high K\(^+\) depolarized membrane voltages by 9.6 ± 1.0 mV (n = 8; Fig. 3 B). After change to 300 mosm/liter this response was decreased to 3.5 ± 0.8 mV (p < 0.005) and 6.6 ± 1.6 mV (p < 0.02) at 200 and 600 s, respectively. Thus, under both experimental conditions, hypertonic stress decreases the voltage response to high K\(^+\). This could reflect a decrease in K\(^+\) conductance or activation of an alternative conductive pathway.

To investigate the role of Cl\(^-\) in the membrane response to hyperosmotic solutions, we performed ion substitution experiments in which 99% of Cl\(^-\) was replaced with gluconate. These sets of measurements were carried out in HCO\(_3\)-free solutions to avoid secondary effects of Cl\(^-\) substitution on membrane voltage via the Cl\(^-\)/HCO\(_3\)-exchanger recently described for primary cultures of rat hepatocytes (Benedetti, Strazzabosco, Corasanti, Haddad, Graf, and Boyer, 1991) and the dependence of K\(^+\) conductance on cell pH (Bear, Davison, and Shaffer, 1988; Fitz, Trouillot, and Scharschmidt, 1989b; Henderson, Krumpholz, Boyer, and Graf, 1988). Under normosmotic conditions, Cl\(^-\) substitution depolarized membrane voltages by 16.0 ± 2.1 mV (n = 12; Fig. 4 A). Although there was a clear tendency towards re-

### Table I

Electrophysiological Variables of Rat Hepatocyte Monolayers

|                     | 300 mosm/liter | 400 mosm/liter | 400 mosm/liter | 220 mosm/liter | 300 mosm/liter | 300 mosm/liter |
|---------------------|---------------|---------------|---------------|---------------|---------------|---------------|
|                     | 120 s         | 600 s         | 120 s         | 600 s         | 120 s         | 600 s         |
| \( \lambda \) [\( \mu \)m] | 321           | 306           | 248           | 213           | 212           | 188           |
| A [mV]              | 7.4           | 7.2           | 7.6           | 7.7           | 8.4           | 8.3           |
| \( R_x \) [M\Omega] | 4.7           | 4.5           | 4.8           | 4.9           | 5.3           | 5.2           |
| \( R_z \) [k\Omega\cm²] | 4.8           | 4.2           | 2.9           | 2.2           | 2.4           | 1.8           |

Data obtained from cable analysis. Confluent monolayers were impaled with two microelectrodes, the first for current injection and the second to monitor the resultant deflections in membrane voltage either at 35, 100, 200, or 400 \( \mu \)m from the first impalement. Fits of experimental data were performed by use of zero-order Bessel functions. Due to the experimental design multiple monolayers were necessary to generate a Bessel function. As a consequence, only single Bessel function parameters were obtained for each experimental condition. \( \lambda \) and A are defining the Bessel function, \( R_x \) and \( R_z \) represent electric coupling between cells and specific cell membrane resistance, respectively (see Methods for further details). Measurements were performed before and after change to hyperosmotic solutions at times indicated in continuous intracellular recordings. \( n = 16 \) (four for each distance between electrodes) and \( n = 20 \) (five for each distance) for RVI and post-RVD-RVI, respectively.
Hypertonic Stress Increases Rat Hepatocyte Na⁺ Conductance

**Figure 3.** Effects of increases in osmolarity on K⁺-induced membrane depolarizations. At the times indicated, K⁺ concentration was isoosmotically increased from 2.7 to 27 mmol/liter in exchange for Na⁺. Current pulses of 4 and 1 nA in A and B, respectively.

**Figure 4.** Effects of increases in osmolarity on membrane depolarizations elicited by Cl⁻ removal. Cl⁻ concentrations were 100-fold reduced with gluconate as the substitute. HCO₃⁻-free solutions. Current pulses of 1 nA in A and B.
duced voltage responses in 400 mosmol/liter these changes remained short of significance: voltage changes averaged 14.1 ± 2.1 mV and 12.9 ± 1.9 mV at 200 and 600 s after osmolarity increase, respectively. In 220 mosmol/liter, low Cl− depolarized membrane voltages by 17.8 ± 2.7 mV (n = 11; Fig. 4 B). After change to 300 mosmol/liter, membrane depolarizations decreased to 13.1 ± 1.9 mV and 13.4 ± 2.1 mV at 200 and 600 s, respectively (p < 0.05). These decreases in the voltage response to low Cl− solutions could be due to an actual decrease in Cl− conductance. Equally well, however, they may reflect the activation of an alternative conductive pathway.

A quantitative analysis of rat hepatocyte Na+ conductance in ion substitution experiments is complicated by the presence of Na+-coupled transport mechanism namely Na+-HCO3− cotransport and Na+/H+ exchange that directly or indirectly (via changes in cell pH) contribute to membrane voltage (Fitz, Persico, and Schardt, 1989a; Fitz et al., 1989b; Henderson et al., 1987). Probably due to these transport pathways Na+ substitution in primary cultures of rat hepatocytes does not readily lead to the membrane hyperpolarization expected for a sizable Na+ conductance (Wehner, 1993; Wehner and Guth, 1991). Fig. 5 depicts experiments in which Na+ was reduced 20-fold in exchange for choline. Under normosmotic conditions, this maneuver slowly depolarized membrane voltages by 8.0 ± 0.6 mV (n = 38) within 128 ± 1 s. In 400 mosmol/liter, this voltage change was significantly reduced to 4.6 ± 0.8 mV and 5.5 ± 1.3 mV (p < 0.001) at 200 and 600 s after osmolarity increase, respectively (Table II). Moreover, in 400 mosmol/liter, these slow depolarizations were commonly preceeded by transient membrane hyperpolarizations (Fig. 5 A), which were maximal after 25 ± 2 s. Consequently, at 25 s in low Na+ solution, membrane voltages were hyperpolarized by 2.8 ± 0.6 mV (at 200 s in 400

![Figure 5](Image)

**Figure 5.** Effects of increases in osmolarity on the membrane response to low Na+ (20-fold reduced with choline as the substitute). Current pulses of 1 and 2 nA in A and B, respectively. No correction for liquid junction potentials (see Methods).
mosm/liter; \( p < 0.001 \)) and 
\[ 4.4 \pm 0.9 \text{ mV (at 600 s; } p < 0.001) \], on the average (Table II). When compared with the membrane voltages in 300 mosm/liter at the same time after \( Na^+ \) substitution, these hyperpolarizations augment to 
\[ 4.7 \pm 0.5 \text{ mV and } 6.3 \pm 0.7 \text{ mV } (p < 0.001) \], respectively (Fig. 6 A).

In 220 mosm/liter, low \( Na^+ \) slowly depolarized the cells by 
\[ 9.4 \pm 0.8 \text{ mV within 2 min } (n = 21) \]. At 200 s after change to 300 mosm/liter, this depolarization was reduced to 
\[ 5.7 \pm 1.1 \text{ mV } (p < 0.005) \]; at 600 s membrane depolarizations equalled 
\[ 9.3 \pm 0.8 \text{ mV, which is not significantly different from control. In addition to this temporary reduction of steady state membrane depolarizations, the increase in } \]

### Table II

| \( Na^+ \) Substitution | 300 mosm/liter | 400 mosm/liter | 400 mosm/liter |
|--------------------------|---------------|---------------|---------------|
| Control                  | 1.9 ± 0.4/8.0 ± 0.6 | -2.8 ± 0.6*/4.6 ± 0.8 \( * \) | -4.4 ± 0.9*/5.5 ± 1.3 \( * \) |
| Amiloride (10 \( \mu \text{mol/liter} \)) | 2.4 ± 0.4/8.0 ± 0.8 | 1.2 ± 0.5*/9.0 ± 0.8 \( * \) | 0.1 ± 0.6*/10.4 ± 1.0 \( * \) |
| Quinine (0.5 mmol/liter) | -7.1 ± 0.8 | -10.1 ± 1.9 * | -16.1 ± 2.1 * |
| 220 mosm/liter            | 5.2 ± 0.4/9.4 ± 0.8 | -1.3 ± 1.1*/5.7 ± 1.1 * | -1.7 ± 0.9*/9.3 ± 0.8 |
| Amiloride (10 \( \mu \text{mol/liter} \)) | 4.5 ± 0.4/8.8 ± 0.4 | 2.8 ± 0.4*/7.6 ± 0.4 * | 2.6 ± 0.5*/9.4 ± 0.5 |
| Quinine (0.5 mmol/liter) | -0.8 ± 0.4 | -4.8 ± 0.7 * | -5.2 ± 0.8 * |

\( Na^+ \) substitutions were carried out in 300 and 220 mosm/liter and after increases of osmolarity to 400 and 300 mosm/liter, respectively, at 120 and 600 s as indicated. For control experiments and for the experiments with amiloride, the changes at 25 s/128 s after \( Na^+ \) substitution are compared. For the experiments with quinine, the maximal voltage changes are given. \( * \), \( * \), and \( * \) Significantly different from the membrane response before osmolarity increase with \( p < 0.05 \), \( p < 0.01 \), and \( p < 0.001 \), respectively. \( * \) and \( * \) Significantly different from the experiment in the absence of amiloride with \( p < 0.01 \) and \( p < 0.001 \), respectively.

osmolarity led to a considerable decrease in the rate of voltage changes (Fig. 5 B): In 220 mosm/liter, at 25 s after \( Na^+ \) substitution (i.e., at the time peak hyperpolarizations occur after change from 300 to 400 mosm/liter; see above), membrane depolarizations equalled 
\[ 5.2 \pm 0.4 \text{ mV. In 300 mosm/liter, this voltage change } \]
(measured at the same time in low \( Na^+ \) solution) was significantly reduced to 
\[ -1.3 \pm 1.1 \text{ mV and } -1.7 \pm 0.9 \text{ mV } (p < 0.001; \text{Table II}, \text{i.e., membrane voltages were shifted to more negative values by } 6.4 \pm 1.0 \text{ mV and } 6.8 \pm 0.9 \text{ mV, respectively, at } 200 \text{ and } 600 \text{ s after osmolarity increase (Fig. 6 B).} \]

These experiments show that increases in osmolarity significantly shift the voltage response of rat hepatocytes to low \( Na^+ \) solutions to more negative values. In principle, this could reflect an increase in \( Na^+ \) conductance, an inactivation of an
alternative conductive pathways (namely K⁺ and Cl⁻ conductances) by which the effects of Na⁺ conductance are unmasked, or both. The decreases in cell input resistance and specific cell membrane resistance (see cable analysis above) under hypertonic stress strongly support the former assumption.

![Graph showing changes in membrane voltage at 25 s after Na⁺ substitution and at different times in hypertonic conditions.](image)

**Figure 6.** (A) Changes in membrane voltage at 25 s after Na⁺ substitution (ΔVm) in 300 mosm/liter and after different times in 400 mosm/liter as indicated. ΔVm values in 400 mosm/liter at 200 s and at 600 s are significantly different from control (p < 0.001). (B) ΔVm at 25 s after Na⁺ substitution in 220 mosm/liter and after different times in 300 mosm/liter as indicated. ΔVm values in 300 mosm/liter at 200 s and at 600 s are significantly different from control (p < 0.001).

In an additional set of experiments, Na⁺ substitutions were carried out in the presence of 0.5 mmol/liter quinine, an effective blocker of K⁺ conductance in hepatocytes (Bear et al., 1988; Wehner et al., 1992). In the continuous presence of the drug, Na⁺ substitution under isoosmotic conditions led to distinct membrane
hyperpolarizations of $7.1 \pm 0.8$ mV ($n = 6$; Fig. 7 A), supporting the notion that the observed slow membrane depolarizations in the absence of the drug are, in fact, due to indirect effects via changes in $K^+$ conductance. In 400 mosm/liter, membrane hyperpolarizations were increased to $10.1 \pm 1.9$ mV ($p < 0.05$) and $16.1 \pm 2.1$ mV ($p < 0.005$) at 200 and 600 s after osmolarity increase, respectively (Table II). In 220 mosm/liter (with quinine present) $Na^+$ substitution hyperpolarized membrane voltages by $0.8 \pm 0.4$ mV (remaining short of significance; $n = 8$). In 300 mosm/liter, however, low $Na^+$ elicited distinct membrane hyperpolarizations of $4.8 \pm 0.7$ mV and

![Diagram](image)

**FIGURE 7.** Same experiments as in Fig. 5 but in the continuous presence of 0.5 mmol/liter quinine. Current pulses of 1 and 3 nA in A and B, respectively. No correction for liquid junction potentials.

$5.2 \pm 0.8$ mV at 200 and 600 s after osmolarity increase, respectively (Fig. 7 B), which is significantly different from control ($p < 0.005$; Table II).

The conclusion that hypertonic stress increases the $Na^+$ conductance of rat hepatocytes is corroborated by $Na^+$ substitution experiments in the continuous presence of $10^{-5}$ mol/liter amiloride. At this concentration, amiloride is an effective inhibitor of epithelial $Na^+$ channels (see Kleyman and Cragoe, Jr., 1988, for review): Under normosmotic conditions as well as after change to 400 mosm/liter at 200 and 600 s, $Na^+$ substitution led to slowly developing membrane depolarizations of $8.0 \pm 0.8$ mV, $9.0 \pm 0.8$ mV, and $10.4 \pm 1.0$ mV, respectively ($n = 10$; Fig. 8 A, Table II).
In 220 mosm/liter and after change to 300 mosm/liter at 200 and 600 s, membrane depolarizations by low Na⁺ equalled 8.8 ± 0.4 mV, 7.6 ± 0.4 mV, and 9.4 ± 0.5 mV, respectively (n = 10; Fig. 8 B, Table II). In none of the experiments with amiloride, there was any transient membrane hyperpolarization upon Na⁺ substitution under hypertonic stress. With respect to membrane voltages at 25 s after Na⁺ substitution (see above), amiloride significantly reduced the differences in the voltage responses occurring between 300 and 400 mosm/liter to 1.2 ± 0.4 mV (4.7 ± 0.5 mV without amiloride; p < 0.001) and 2.3 ± 0.6 mV (6.3 ± 0.7 mV without amiloride; p < 0.01) at 200 and 600 s, respectively. Likewise, amiloride reduced the corresponding voltage differences between 220 and 300 mosm/liter to 1.7 ± 0.4 mV (6.4 ± 1.0 mV without amiloride; p < 0.005) and 1.9 ± 0.4 mV (6.8 ± 0.9 mV without amiloride; p < 0.001) at 200 and 600 s after osmolarity increase, respectively.

In the Na⁺ substitution experiments, increasing osmolarity under any experimental condition always led to initial membrane depolarizations of comparable size. After change from 300 to 400 mosm/liter, membrane voltages depolarized by 4.1 ± 0.6 mV, 4.1 ± 0.3 mV, and 5.0 ± 0.4 mV, in the absence of a drug and in the presence of quinine and amiloride, respectively (p < 0.001 for each set of experiments). After change from 220 to 300 mosm/liter voltages depolarized by 4.1 ± 1.0 mV, 5.3 ± 1.1 mV, and 7.1 ± 1.1 mV, in the absence of a drug and in the presence of quinine and
amiloride, respectively ($p < 0.001$ for each experimental group). In contrast, both drugs had significant effects on the membrane voltages at the end of a 10-min exposure to hypertonic solutions. In the absence of a drug, voltages in 400 and 300 mosm/liter were hyperpolarized by $2.4 \pm 1.0$ mV and $3.8 \pm 1.6$ mV, respectively ($p < 0.025$). In the presence of quinine, membrane voltages were depolarized by $7.2 \pm 0.9$ mV (400 mosm/liter; $p < 0.001$) and $5.5 \pm 1.7$ mV (300 mosm/liter; $p < 0.02$). In the presence of amiloride, 10 min hypertonic stress led to intermediate voltage levels, i.e., to $0.5 \pm 1.3$ mV (n.s. with respect to the control voltage) and $2.3 \pm 1.3$ mV (n.s.) in 400 and 300 mosm/liter, respectively.

Isolated rat hepatocytes transferred from hypoosmotic to normosmotic solutions exhibit a post-RVD-RVI, whereas cells remain continuously shrunk for periods of 30 min and more after change from normosmotic to hyperosmotic conditions (Corasanti et al., 1990). In contrast, activation of Na$^+$/H$^+$ exchange (claimed to be the central mechanism of RVI in rat liver cells) occurs under both experimental protocols (Gleeson et al., 1990). To elucidate the role of Na$^+$/H$^+$ exchange in the RVI of confluent rat hepatocyte monolayers intracellular pH was monitored by use of the fluorescent dye BCECF. In HCO$_3^-$-containing solutions, increasing osmolarity from 300 to 400 mosm/liter and from 220 to 300 mosm/liter reversibly increased cell pH from $7.34 \pm 0.01$ to $7.54 \pm 0.01$ ($n = 6; p < 0.005$) and from $7.39 \pm 0.01$ to $7.65 \pm 0.01$ ($n = 6; p < 0.001$), respectively (Fig. 9). Increasing osmolarity in HCO$_3^-$-free solutions, reversibly increased cell pH from $7.12 \pm 0.02$ to $7.47 \pm 0.04$ ($n = 40; p < 0.001$) and from $7.05 \pm 0.03$ to $7.44 \pm 0.10$ ($n = 7; p < 0.001$), respectively (Fig. 10). Thus, during RVI and during post-RVD-RVI hypertonic stress leads to similar increases of cell pH both, in the presence and in the absence of HCO$_3^-$. This similarity strongly argues against the involvement of pH-regulating mechanisms other than Na$^+$/H$^+$ exchange in the RVI of rat hepatocytes, namely Na$^+$/HCO$_3^-$ cotransport and Cl$^-$/HCO$_3^-$ exchange. In principle, activation of Na$^+$/H$^+$ exchange will participate in the increase of intracellular Na$^+$ under hyperosmotic stress (see Discussion).

Haddad et al. (1989) and Graf et al. (1988) demonstrated an ouabain-sensitive net K$^+$ influx in isolated rat livers that were perfused with hypertonic solutions. In many systems exhibiting RVI, an increase in Na$^+$ influx (via Na$^+$/H$^+$ exchange or Na$^+$/K$^+$/2Cl$^-$ cotransport) and an increasing activity of Na$^+$/K$^+$-ATPase in concert augment the intracellular activity of K$^+$ (Lang et al., 1993). If such a mechanism holds for the RVI of rat hepatocytes and if a considerable amount of Na$^+$ influx is, in fact, mediated via Na$^+$ conductance, hypertonic stress should increase the amount of ouabain-sensitive Rb$^+$ uptake and this effect should be inhibitable by micromolar concentrations of amiloride. To elucidate these points we performed experiments in which the activity of rat hepatocyte Na$^+$/K$^+$-ATPase was monitored by measuring $^{86}$Rb$^+$ uptake over time. In pilot experiments, Rb$^+$ uptake was quasi linear for the first 20 min (data not shown), which is in line with the findings of Renner, Lake, Cragoe Jr., and Scharschmidt (1988; rat hepatocyte primary cultures). Data were obtained in paired experiments, i.e., all monolayers that are compared were taken from the very same preparations. In normosmotic solutions, Rb$^+$ uptake equalled $5.0 \pm 0.1$ nmol/mg protein-min ($n = 4$). At the same osmolarity but in the continuous presence of 2 mmol/liter ouabain, Rb$^+$ uptake was reduced to $0.9 \pm 0.1$
nmol/mg protein·min (i.e., to ~18%) so that the ouabain-insensitive fraction equalled 4.1 ± 0.1 nmol/mg protein·min. In 400 mosm/liter, total uptake was stimulated whereas uptake in the presence of ouabain remained the same. Thus, ouabain-sensitive Rb⁺ uptake was significantly increased to 5.8 ± 0.2 nmol/mg protein·min (n = 4; p < 0.02) which is 141 ± 8% of control. Changing osmolarity from 220 to 300 mosm/liter increased ouabain-sensitive Rb⁺ uptake from 3.8 ± 0.6 to 4.5 ± 0.7 nmol/mg protein·min (n = 4), i.e., to 120 ± 13%; however, this did not reach statistical significance.

In an additional set of (paired) experiments, ouabain-sensitive Rb⁺ uptake after change from 300 to 400 mosm/liter equalled 8.7 ± 0.4 nmol/mg protein·min. Under the same experimental conditions but in the presence of 10⁻⁵ mol/liter amiloride (added at the time when osmolarity was increased), Rb⁺ uptake was reduced to 6.4 ± 0.4 nmol/mg protein·min (n = 4; p < 0.005), i.e., to 73 ± 3% or in other words, by a factor of 1.37. With respect to the experiments without the drug, in which an increase in osmolarity from 300 to 400 mosm/liter augmented Rb⁺ uptake by a factor

**Figure 9.** Effects of increases in osmolarity on intracellular pH. HCO₃⁻-containing solutions; n = 6 in A and B. For the times indicated 25 mmol/liter Na⁺ was exchanged by NH₄⁺ as a positive control.
of 1.41 (see above), $10^{-5}$ mol/liter amiloride is apparently sufficient to almost completely block the effects of hypertonic stress on Na\textsuperscript{+}/K\textsuperscript{+}-ATPase.

The results presented so far suggest that conductive Na\textsuperscript{+} entry plays a prominent role in the RVI of rat hepatocytes. This is corroborated by experiments in which the effects of hypertonic stress on cell volume were determined in the presence of $10^{-5}$ mol/liter amiloride. As Fig. 11 clearly shows amiloride completely blocked the RVI as well as the post-RVD-RVI of rat hepatocytes in primary culture.

We performed intracellular recordings with ion-sensitive microelectrodes to directly monitor the changes in cell Na\textsuperscript{+} and K\textsuperscript{+} upon hypertonic stress. The relative increases in Na\textsuperscript{+} and K\textsuperscript{+} activities expected to occur solely via cell shrinkage (cf. to Fig. 1) are given in square brackets. In 300 mosm/liter, the intracellular Na\textsuperscript{+} activity equalled $17.9 \pm 2.2$ mmol/liter ($n = 6$). After change to 400 mosm/liter, cell Na\textsuperscript{+} increased to $23.0 \pm 3.8$ and $27.1 \pm 5.0$ mmol/liter ($p < 0.05$) at 200 and 600 s, respectively (Fig. 12A), which is $126 \pm 6$ and $148 \pm 15\%$ of control ($112$ and $106\%$). In 220 mosm/liter, the intracellular Na\textsuperscript{+} activity was $14.5 \pm 2.0$ mmol/liter ($n = 6$).
After change to 300 mosm/liter, cell Na\(^+\) equalled 15.7 ± 2.9 mmol/liter (n.s.) and 25.0 ± 4.9 mmol/liter \((p < 0.025)\) at 200 and 600 s, respectively (Fig. 12 B), which is 106 ± 5 and 170 ± 22\% of control (120 and 110\%). In 300 mosm/liter, the intracellular K\(^+\) activity equalled 75.0 ± 11.0 mmol/liter \((n = 4)\). After change to 400 mosm/liter, cell K\(^+\) increased to 107.4 ± 12.0 mmol/liter \((p < 0.02)\) and 127.7 ± 10.4 mmol/liter \((p < 0.05)\) at 200 and 600 s, respectively (Fig. 13 A), which is 146 ± 12 and 180 ± 27\% of control [112 and 106\%]. In 220 mosm/liter, the intracellular K\(^+\) activity was 81.4 ± 10.2 mmol/liter \((n = 4)\). After change to 300 mosm/liter at 200 and 600 s, cell K\(^+\) was increased to 110.2 ± 11.3 mmol/liter \((p < 0.005)\) and 138.3 ± 19.1 mmol/liter \((p < 0.02)\), respectively (Fig. 13 B), which is equivalent to 137 ± 5 and 170 ± 11\% of control (120 and 110\%).
FIGURE 12. Effects of increasing osmolarity on intracellular voltages recorded with a Na⁺-sensitive microelectrode (V₉Na) and an open tip (KCl-filled) reference microelectrode (Vₘ). V₉Na - Vₘ depicts the differential voltage of both traces. Calibration slopes of Na⁺-sensitive electrodes were 52 and 61 mV/decade in A and B, respectively.
FIGURE 13. Effects of increasing osmolarity on intracellular voltages recorded with a K⁺-sensitive microelectrode \( (V_K) \) and an open tip reference \( (V_m) \). \( V_K - V_m \) is the differential voltage of both channels. Calibration slopes of K⁺-sensitive electrodes were 56 and 54 mV/decade in A and B, respectively.
DISCUSSION

In the perfused rat liver, hepatocytes under hyperosmotic conditions are capable of a partial RVI (Haddad et al., 1989; Lang et al., 1989). In contrast, isolated rat hepatocytes in hyperosmotic solution remain continuously shrunken for periods of up to 30 min (Bakker-Grunwald, 1983; Corasanti et al., 1990) and only exhibit RVI when transferred back from hypoosmotic to normosmotic solutions, i.e., after a preperiod, in which they underwent hypotonic stress and partial RVD (post-RVD-RVI; Corasanti et al., 1990). For mouse liver slices and for mouse hepatocytes in primary culture indirect evidence for a RVI in hyperosmotic solutions has been reported (Howard and Wondergem, 1987; Khalbuss and Wondergem, 1990). In the present paper, we could demonstrate that rat hepatocytes in confluent primary cultures exhibit RVI as well as post-RVD-RVI. In this preparation, cells are readily accessible for confocal laser scanning microscopy (quantification of cell volume), intracellular recordings with conventional and ion-sensitive microelectrodes (determination of membrane voltage and conductance, ion selectivity, and intracellular ion activities), microfluorometry (measurement of cell pH), as well as flux measurements (determination of K⁺ transport). Taken together, this renders primary cultures a suitable in vitro model for the study of RVI in rat hepatocytes.

In the ion substitution experiments, we found that hypertonic stress decreases the apparent transference numbers for K⁺ and Cl⁻. The apparent \( t_K \) and \( t_{CI} \) values decreased from 0.13 in 300 mosm/liter to 0.04 and 0.12, and to 0.09 and 0.11 at 200 and 600 s in 400 mosm/liter, respectively; in 220 mosm/liter, the apparent \( t_K \) and \( t_{CI} \) values equalled 0.16 and 0.15 and decreased to 0.06 and 0.11, and to 0.11 at 200 and 600 s in 300 mosm/liter, respectively. This could reflect a decrease in cell K⁺ and Cl⁻ conductances, but it could equally well be due to the activation of an alternative conductive pathway. The decrease in cell input resistance measured under both experimental protocols strongly supported the latter hypothesis and this could also be confirmed in the cable analysis. In the Na⁺ substitution experiments under control conditions and in the presence of quinine and amiloride, it became evident that this increase in overall membrane conductance is due to a sustained increase in rat hepatocyte Na⁺ conductance.

The apparently low absolute values for transference numbers in our confluent monolayer system are readily explained by the geometry of the system because only approximately one half of the total cell surface is directly accessible to changes of the superfusate (Wehner and Guth, 1991). For the same reason cell shrinkage after change to hyperosmotic conditions may be attenuated to some extent. On the other hand, the decrease in cell volume to 88.6% 1 min after change from 300 to 400 mosm/liter is in good agreement with a shrinkage to 83.5% for the same change in osmolarity, that can be calculated from the data of Corasanti et al. (1990) obtained from isolated rat hepatocytes.

In isolated mouse hepatocytes, hypertonic stress (+80 mmol/liter sucrose) leads to a sustained membrane depolarization of 13 mV and a concomitant doubling of membrane resistance (Graf et al., 1988); a comparable depolarization is also found in mouse liver slices (Khalbuss and Wondergem, 1990; Wang and Wondergem, 1991). These effects were attributed to a decrease in mouse hepatocyte K⁺ conductance.
From our Na⁺ substitution experiments in the presence of amiloride we conclude that rat hepatocytes under hypertonic stress do not exhibit sustained changes in membrane-conductive properties other than those attributable to cell Na⁺ conductance. However, based on our experiments, we cannot exclude a transient decrease in K⁺ conductance upon hypertonic stress. In this respect, it is notable that increasing osmolarity under any experimental condition, i.e., also in the continuous presence of amiloride and quinine, always led to initial membrane depolarizations of similar size. If, in fact, both activation of Na⁺ conductance and inactivation of K⁺ conductance together were responsible for this effect, the efficiency of each mechanism in depolarizing the cells would be relatively enlarged if its counterpart was blocked (due to an increase in its own transference number) so that there may be little if any changes in the overall membrane response. In addition, Cl⁻ may fill the gap because a 20% shrinkage of a cell will decrease $E_{Cl}$ by 6 mV, an effect that will also tend to depolarize membrane voltage and that may as well become more efficient under conditions where the Cl⁻ transference number is increased, i.e., in the presence of quinine or amiloride. The activation of Na⁺/H⁺ exchange will not participate in the initial membrane depolarization, because this effect alkalizes the cell and due to the pH dependence of rat hepatocyte K⁺ conductance (Fitz et al., 1989b) would rather tend to hyperpolarize membrane voltages.

In our Na⁺ substitution experiments (in the absence of a drug) hypertonic stress shifted the voltage response to low Na⁺ solutions to more negative values. Although this effect is obvious in every single experiment, its variance is remarkable (see Fig. 6, A and B). The effect became statistically significant after some five to eight experiments; however, we increased the number of measurements considerably to compare different experimental groups and to ensure its reproducibility. The high degree of variability was typical for each group of measurements and to date we have no explanation for this phenomenon. Because Na⁺ substitution experiments (without and with amiloride) were conducted over a period of two years we can exclude any seasonal variability.

In addition to our measurements on the effects of hypertonic stress, we frequently performed control experiments in which Na⁺ was repetitively substituted under normosmotic conditions (i.e., in 300 and 220 mosm/liter without increasing osmolarity). We never detected significant changes in the voltage response to low Na⁺ solutions (three consecutive ion substitutions) in such experiments (data not shown).

In our experiments, we used $10^{-5}$ mol/liter amiloride as a selective blocker of cell membrane Na⁺ conductance. While the efficiency of amiloride to block epithelial Na⁺ channels at this concentration is generally accepted (Kleyman and Cragoe, Jr., 1988), we performed two sets of pilot experiments to exclude additional effects on Na⁺/H⁺ exchange. First, in normosmotic solutions (300 mosm/liter), the activity of Na⁺/H⁺ exchange was quantified as pH recovery from an acid load after exposure to 25 mmol/liter NH₄⁺ for 2 min (HCO₃⁻-free conditions). This process could readily be fitted by use of the form

$$ y = A(1 - e^{-t/\tau}) + B $$

where $B$ and $A$ are the pH value at maximum acidification and the amount of pH recovery, respectively. $\tau$ represents the time constant of the recovery process. In the
absence of amiloride, B, A, and τ were 6.45 ± 0.05, 0.57 ± 0.03, and 239 ± 31 s, respectively (n = 10). In the presence of 10⁻⁵ mol/liter amiloride, B, A, and τ equalled 6.45 ± 0.02, 0.58 ± 0.04, and 296 ± 29 s, respectively (n = 15), which is not significantly different from control (p > 0.2). Second, under control conditions, increasing osmolarity from 300 to 400 mosm/liter alkalinized the cells by 0.35 ± 0.03 pH units (HCO₃⁻-free conditions; n = 40; Fig. 11 A). In the presence of 10⁻⁵ mol/liter amiloride the same maneuver led to an alkalinization by 0.37 ± 0.01 pH units (n = 14; Fig. 14). From these experiments we conclude that amiloride at 10⁻⁵ mol/liter has no significant effect on the activity of Na⁺/H⁺ exchange in rat liver. In particular this holds for the volume-sensitive portion of the cotransport.

Our measurements of intracellular pH are in good agreement with data reported by Gleeson et al. (1990) who found increases in cell pH from 7.14 to 7.31 and from 6.86 to 7.15 when rat hepatocytes were transferred from 160 to 300 mosm/liter in HCO₃⁻-containing and in HCO₃⁻-free solutions, respectively. From these data and from determinations of total intracellular H⁺ buffering capacity, Gleeson et al. (1990) calculated total H⁺ effluxes (via Na⁺/H⁺ exchange) that would tend to increase cell Na⁺ by 1.70 (with HCO₃⁻ present) and 2.31 mmol/liter·min (HCO₃⁻-free conditions). In our cable analysis, increasing osmolarity from 300 to 400 mosm/liter decreases the specific cell membrane resistance R₂ from 4.8 to 2.9 kΩ cm². Assuming an average surface area of a single cell of 19.3 · 10⁻⁶ cm² (from a mean cell diameter of 35 μm [Wehner and Guth, 1991]) this is equivalent to a change in membrane resistance from 249 to 150 MΩ. If this decrease in membrane resistance is solely due to an increase in Na⁺ conductance and if one assumes a driving force of -95 mV for conductive Na⁺ entry (Vₘ - E₉Na), hypertonic stress will cause a Na⁺ inward current of 0.26 nA equivalent to a Na⁺ influx of 0.16 pmol/min (0.26 · 10⁻⁹ C · s⁻¹ · 96500 C · V⁻¹).
mol⁻¹ · 60 s⁻¹). Because the average cell volume in 300 mosm/liter is 5.9 pl, as was determined by confocal laser scanning microscopy, this influx would tend to increase cell Na⁺ by 27 mmol/min. Thus, under the assumptions made, cell Na⁺ conductance would be 10–15 times more effective in increasing cell Na⁺ under hypertonic stress than Na⁺/H⁺ exchange.

In our experiments, changing osmolarity from 300 to 400 mosm/liter augmented ouabain-sensitive ⁸⁶Rb⁺ uptake by a factor of 1.41. It is of note that 10⁻⁵ mol/liter amiloride reduced ⁸⁶Rb⁺ uptake in 400 mosm/liter by almost the same degree, i.e., by a factor of 1.37. This provides further evidence for the prominent role of rat hepatocyte Na⁺ conductance in the elevation of cell Na⁺ under hypertonic stress. Moreover, 10⁻⁵ mol/liter amiloride completely blocked the RVI as well as the post-RVD-RVI of rat hepatocytes (Fig. 11). In preliminary experiments with ion-sensitive microelectrodes, hypertonic stress in the continuous presence of 10⁻⁵ mol/liter amiloride did not significantly change cell Na⁺ which was 20.4 ± 6.0 mmol/liter in 300 mosm/liter (n = 4), and 23.6 ± 8.0 and 23.2 ± 7.0 mmol/liter at 200 and 600 s in 400 mosm/liter, respectively. The values in 400 mosm/liter reflect 113.7 ± 9.3 (115%) and 115.7 ± 10.7 (117%) of the control period. Thus, amiloride confines Na⁺ activities in 400 mosm/liter to the values expected to occur solely via cell shrinkage (given in square brackets as percentage of control, cf. to Fig. 11 A).

In the perfused rat liver, an increase in extracellular osmolarity leads to a distinct stimulation of K⁺ uptake, which is inhibited by 1 mmol/liter ouabain and 1 mmol/liter amiloride (Graf et al., 1988; Haddad and Graf, 1989; Haddad et al., 1989; Häussinger et al., 1990). Amiloride (1 mmol/liter) as well as Na⁺ substitution inhibits post-RVD-RVI in isolated rat hepatocytes (Corasanti et al., 1990) and, in subconfluent primary cultures, increasing osmolarity from hypoosmotic to normosmotic as well as from normosmotic to hyperosmotic solutions activates Na⁺/H⁺ exchange (Gleeson et al., 1990). In confluent primary cultures of rat hepatocytes, the activity of Na⁺/K⁺-ATPase is regulated by the intracellular Na⁺ concentration with a Kₘ of 17.8 mmol/liter (van Dyke and Scharschmidt, 1983). Taken together, it is generally accepted that activation of Na⁺/H⁺ exchange and Na⁺/K⁺-ATPase in concert augment the intracellular K⁺ concentration of rat hepatocytes under hypertonic stress (Häussinger et al., 1993). The data presented in this paper, however, suggest a considerable role of cell membrane Na⁺ conductance in the RVI of rat hepatocytes, whose contribution to the increase in cell Na⁺ probably exceeds the part of Na⁺/H⁺ exchange. The effects of amiloride and Na⁺ substitution reported so far may equally well be explained in terms of an inhibition of Na⁺ conductance instead of Na⁺/H⁺ exchange. One has to keep in mind, however, that primary cultures are an experimental system that may to some extent differ from intact liver or from freshly isolated cells. On the other hand, primary cultures as used in this study in some respect more closely resemble the in vivo situation than isolated cells and subconfluent primary cultures because the formation of primary bile (Petzinger et al., 1988) and the development of cell-to-cell coupling (Wehner and Guth, 1991) have been observed and (most important for this study) because these cells exhibit a distinct RVI under hypertonic stress.

In rat inner medullary collecting duct (ADH dependent) RVI is inhibited by substitution of Na⁺ and HCO₃⁻, and by 0.1 mmol/liter 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS); these results are interpreted in terms of a parallel
activation of Na\(^+/\)H\(^+\) exchange and a HCO\(_3^-\)-dependent pathway, however, activation of a luminal nonselective cation channel is not excluded (Sun and Hebert, 1989). The cation channel in this system appears to be equally permeable to Na\(^+\) and K\(^+\) (Light, McCann, Keller, and Stanton, 1988). In human airway epithelial cells, shrinkage activates a nonselective cation channel which exhibits a permeability ratio of \(P_K/P_{Na}\) of 1.1 and which is regulated by external Cl\(^-\) (Chan and Nelson, 1992). In principle, the increases in cell Na\(^+\) found in our studies could occur via activation of a nonselective cation channel as well. In the ion substitution experiments, however, we observe a decrease in the voltage response to high K\(^+\) whereas the opposite is to be expected from a parallel increase in Na\(^+\) and K\(^+\) conductance.

For conductive Na\(^+\) entry to mediate RVI, a parallel electrogenic anion uptake is to be expected. Although \(E_{Cl}\) in rat hepatocytes is close to membrane voltage (Graf, Henderson, Krumpholz, and Boyer, 1987) and we do not have evidence for an increase in Cl\(^-\) conductance, a conductive Cl\(^-\) entry may be the most likely counterpart to Na\(^+\) inward current, because Cl\(^-\) is the major charge-carrying anion of the sinusoidal membrane (Graf and Petersen, 1978; Graf et al., 1987). The transient depolarization of membrane voltage upon change to hyperosmotic conditions may initiate Cl\(^-\) entry, which could then be followed by quasi-electroneutral cotransport of Na\(^+\) and Cl\(^-\). Moreover, the sizeable intrinsic Cl\(^-\) conductance of hepatocytes may be sufficient to balance electrogenic Na\(^+\) entry under hypertonic stress. A comparable model was proposed for the RVD of rat hepatocytes. Under hypotonic stress, the cells exhibit a transient increase in K\(^+\) conductance while Cl\(^-\) conductance most probably does not change (Wehner et al., 1992).

Our recordings with ion-sensitive microelectrodes show an increase in cell Na\(^+\) plus cell K\(^+\) by 50 to 60 mmol/liter within 10 min of hypertonic stress (the proportion due to cell shrinkage not included). Because parallel electrogenic anion uptake is to be expected (see above) the total gain of cellular osmolytes via such pathways may well account for the observed RVI and post-RVD-RVI.

In conclusion, rat hepatocytes in confluent primary culture exhibit a RVI as well as a post-RVD-RVI under hypertonic conditions. We find that in this system hypertonic stress leads to a significant increase of cell membrane Na\(^+\) conductance. Most likely, this increase in Na\(^+\) conductance represents the main mechanism by which cell Na\(^+\) increases to some 150–170\%, whereas the contribution of Na\(^+\)/H\(^+\) exchange appears to be minor. This increase in cell Na\(^+\) in turn activates Na\(^+\)/K\(^+\)-ATPase which results in an increase in cell K\(^+\) to some 170–180\% of control. To the best of our knowledge, rat hepatocytes in primary culture are the first system in which hypertonic stress increases Na\(^+\) conductance.

The authors wish to thank Mrs. Daniela Mägdefessel for skillful secretarial assistance.

Original version received 30 September 1994 and accepted version received 9 January 1995.

References

Allen, T. M., and L. G. Cleland. 1980. Serum-induced leakage of liposome contents. Biochimica et Biophysica Acta. 597:418–426.

Bakker-Grunwald, T. 1983. Potassium permeability and volume control in isolated rat hepatocytes. Biochimica et Biophysica Acta. 731:239–242.
Bear, C. E., J. S. Davison, and E. A. Shaffer. 1988. Intracellular pH influences the resting membrane potential of isolated rat hepatocytes. Biochimica et Biophysica Acta. 944:113–120.

Benedetti, A., M. Strazzabosco, J. G. Corasanti, P. Haddad, J. Graf, and J. L. Boyer. 1991. Cl−-HCO3− exchanger in isolated rat hepatocytes: role in regulation of intracellular pH. American Journal of Physiology. 261:G512–G522.

Chan, H. C., and D. J. Nelson. 1992. Chloride-dependent cation conductance activated during cellular shrinkage. Science. 257:669–671.

Corasanti, J. G., D. Gleeson, and J. L. Boyer. 1990. Effects of osmotic stresses on isolated rat hepatocytes. I. Ionic mechanisms of cell volume regulation. American Journal of Physiology. 258:G290–G298.

Edelman, A., S. Curci, I. Samarizija, and E. Frömter. 1978. Determination of intracellular K+ activity in rat kidney proximal tubular cells. Pflügers Archiv. 378:37–45.

Fitz, J. G., M. Persico, and B. F. Scharschmidt. 1989a. Electrophysiological evidence for Na+-coupled bicarbonate transport in cultured rat hepatocytes. American Journal of Physiology. 256:G491–G500.

Fitz, J. G., T. E. Trouillot, and B. F. Scharschmidt. 1989b. Effect of pH on membrane potential and K+ conductance in cultured rat hepatocytes. American Journal of Physiology. 257:G961–G968.

Frömter, E. 1972. The route of passive ion movement through the epithelium of Necturus gallbladder. Journal of Membrane Biology. 8:259–301.

Gleeson, D., J. G. Corasanti, and J. L. Boyer. 1990. Effects of osmotic stresses on isolated rat hepatocytes. II. Modulation of intracellular pH. American Journal of Physiology. 258:G299–G307.

Graf, J., and O. H. Petersen. 1978. Cell membrane potential and resistance in liver. Journal of Physiology. 284:105–126.

Graf, J., P. Haddad, D. Häussinger, and F. Lang. 1988. Cell volume regulation in liver. Renal Physiology and Biochemistry. 35:202–220.

Graf, J., R. M. Henderson, B. Krumpholz, and J. L. Boyer. 1987. Cell membrane and transepithelial voltages and resistances in isolated rat hepatocyte couplets. Journal of Membrane Biology. 95:241–254.

Haberich, F. J., O. Aziz, and P. E. Nowacki. 1965. Über einen osmoreceptorisch tätigen Mechanismus in der Leber. Pflügers Archiv. 285:73–89.

Haddad, P., and J. Graf. 1989. Volume-regulatory K+ fluxes in the isolated perfused rat liver: characterization by ion transport inhibitors. American Journal of Physiology. 257:G357–G363.

Haddad, P., T. Thalhammer, and J. Graf. 1989. Effect of hypertonic stress on liver cell volume, bile flow, and volume-regulatory K+ fluxes. American Journal of Physiology. 256:G565–G569.

Häussinger, D., and F. Lang. 1991. The mutual interaction between cell volume and cell function: a new principle of metabolic regulation. Biochemistry and Cell Biology. 69:1–4.

Häussinger, D., W. Gerok, and F. Lang. 1995. Cell volume and hepatic metabolism. Advances in Comparative and Environmental Physiology. 14:53–65.

Häussinger, D., T. Stehle, and F. Lang. 1990. Volume regulation in liver: Further characterization by inhibitors and ionic substitutions. Hepatology. 11:243–254.

Henderson, R. M., J. Graf, and J. L. Boyer. 1987. Na-H exchange regulates intracellular pH in isolated rat hepatocyte couplets. American Journal of Physiology. 252:G109–G113.

Henderson, R. M., B. Krumpholz, J. L. Boyer, and J. Graf. 1988. Effect of intracellular pH on potassium conductance in liver. Pflügers Archiv. 412:334–335.

Horisberger, J.-D., and G. Giebisch. 1988. Intracellular Na+ and K+ activities and membrane conductances in the collecting tubule of Amphiuma. Journal of General Physiology. 92:643–665.

Howard, L. D., and R. Wondergem. 1987. Effects of anisomotic medium on cell volume, transmembrane potential and intracellular K+ activity in mouse hepatocytes. Journal of Membrane Biology. 100:53–61.
Kendall, D. A., and R. C. MacDonald. 1983. Characterization of a fluorescence assay to monitor changes in the aqueous volume of lipid vesicles. *Analytical Biochemistry.* 134:26–33.

Khalbuss, W. E., and R. Wondergem. 1990. An electrophysiological technique to measure change in hepatocyte water volume. *Biochimica et Biophysica Acta.* 1029:51–60.

Kleyman, T. R., and E. J. Cragoe, Jr. 1988. Amiloride and its analogs as tools in the study of ion transport. *Journal of Membrane Biology.* 105:1–21.

Lang, F., M. Ritter, H. Völkl, and D. Häussinger. 1993. Cell volume regulatory mechanisms—an overview. *Advances in Comparative and Environmental Physiology.* 14:2–31.

Lang, F., T. Stehle, and D. Häussinger. 1989. Water, K⁺, H⁺, lactate and glucose fluxes during cell volume regulation in perfused rat liver. *Pflügers Archiv.* 413:209–216.

Light, D. B., F. V. McCann, T. M. Keller, and B. A. Stanton. 1988. Amiloride-sensitive cation channel in apical membrane of inner medullary collecting duct. *American Journal of Physiology.* 255:F278–F286.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry.* 193:265–275.

Petzinger, E., W. Föllmann, H. Acker, J. Hentschel, K. Zierold, and R. K. H. Kinne. 1988. Primary liver cell cultures grown on gas permeable membrane as source for the collection of primary bile. *In Vitro Cellular & Developmental Biology.* 24:491–499.

Renner, E. L., J. R. Lake, E. J. Cragoe, Jr., and B. F. Scharschmidt. 1988. Amiloride and amiloride analogs inhibit Na⁺/K⁺-transporting ATPase and Na⁺-coupled alanine transport in rat hepatocytes. *Biochimica et Biophysica Acta.* 938:386–394.

Stoddard, J. S., and L. Reuss. 1989. Electrophysiological effects of mucosal Cl⁻ removal in *Necturus* gallbladder epithelium. *American Journal of Physiology.* 257:C568–C578.

Sun, A., and S. C. Hebert. 1989. Rapid hypertonic cell volume regulation in the perfused inner medullary collecting duct. *Kidney International.* 36:831–842.

Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 18:2210–2218.

Tinel, H., F. Wehner, and H. Sauer. 1994. Intracellular Ca²⁺ release and Ca²⁺ influx during regulatory volume decrease in IMCD cells. *American Journal of Physiology.* 267:F130–F138.

van Dyke, R. W., and B. F. Scharschmidt. 1983. (Na,K)-ATPase-mediated cation pumping in cultured rat hepatocytes. Rapid modulation by alanine and taurocholate transport and characterization of its relationship to intracellular sodium concentration. *Journal of Biological Chemistry.* 258:12912–12919.

Wang, K., and R. Wondergem. 1991. Effects of hyperosmotic medium on hepatocyte volume, transmembrane potential and intracellular K⁺ activity. *Biochimica et Biophysica Acta.* 1069:187–196.

Wehner, F. 1993. Taurocholate depolarizes rat hepatocytes in primary culture by increasing cell membrane Na⁺ conductance. *Pflügers Archiv.* 424:145–151.

Wehner, F., G. Beetz, and S. Rosin-Steiner. 1992. Osmolarity reduction transiently increases K⁺ conductance of confluent rat hepatocytes in primary culture. *American Journal of Physiology.* 263:G913–G919.

Wehner, F., and D. Guth. 1991. Electric properties of rat liver cell cultures on gas-permeable membranes. *American Journal of Physiology.* 261:G407–G416.

Wehner, F., S. Rosin-Steiner, G. Beetz, and H. Sauer. 1993. The anion transport inhibitor DIDS increases rat hepatocyte K⁺ conductance via uptake through the bilirubin pathway. *Journal of Physiology.* 471:617–635.