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Hepatocyte Water Volume and Potassium Activity during Hypotonic Stress

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Abstract. Hepatocytes exhibit a regulatory volume decrease (RVD) during hypotonic shock, which comprises loss of intracellular K^+ and Cl^- accompanied by hyperpolarization of transmembrane potential (V_m) due to an increase in membrane K^+ conductance, (G_K). To examine hepatocyte K^+ homeostasis during RVD, double-barrel, K^+-selective microelectrodes were used to measure changes in steady-state intracellular K^+ activity (a^i_K) and V_m during hyposmotic stress. Cell water volume change was evaluated by measuring changes in intracellular tetramethylammonium (TMA^+). Liver slices were superfused with modified Krebs physiological salt solution. Hyposmolality (0.8 × 300 mosm) was created by a 50 mM step-decrease of external sucrose concentration. Hepatocyte V_m hyperpolarized by 19 mV from -27 ± 1 to -46 ± 1 mV and a^i_K decreased by 14% from 91 ± 4 to 78 ± 4 mM when slices were exposed to hyposmotic stress for 4–5 min. Both V_m and a^i_K returned to control level after restoring isosmotic solution. In paired measurements, hypotonic stress induced similar changes in V_m and a^i_K in both control and added ouabain (1 mM) conditions, and these values returned to their control level after the osmotic stress. In another paired measurement, hypotonic shock first induced an 18-mV increase in V_m and a 15% decrease in a^i_K in control condition. After loading hepatocytes with TMA^+, the same hypotonic shock induced a 14-mV increase in V_m and a 14% decrease in a^i_{TMA}. This accounted for a 17% increase of intracellular water volume, which was identical to the cell water volume change obtained when a^i_K was used as the marker. Nonetheless, hyposmotic stress-induced changes in V_m and a^i_K were blocked partly by Ba^{2+} (2 mM). We conclude that (i) hepatocyte V_m increases and a^i_K decreases during hypotonic shock; (ii) the changes in hepatocyte V_m and a^i_K during and after hypotonic shock are independent of the Na^+-K^+ pump; (iii) the decrease in a^i_K during hypotonic stress results principally from hepatocyte swelling.

Key words: Osmotic stress — Intracellular K^+ — Ouabain — Membrane potential — Ion-sensitive microelectrodes

Introduction

Swelling-induced regulatory volume decrease (RVD) has been reported to occur in hepatocytes following anisosmotic stress accomplished by altering the external osmolality (Corasanti, Gleeson & Boyer, 1990). Hepatocyte volume regulation also occurs during the cell swelling that accompanies L-alanine transport and accumulation (Bakker-Grunwald, 1983; Kristensen, 1986). In both cases, cells swell initially, followed by compensatory restoration of volume. The general pattern of volume restoration in hepatocytes is thought to consist of cell swelling-induced loss of intracellular K^+ accompanied by anions, and water follows (Graf et al., 1988).

Hypotonic stress also hyperpolarizes the hepatocyte transmembrane potential, V_m (Howard & Wondergem, 1987) as does L-alanine accumulation (Fitz & Scharschmidt, 1987; Wondergem & Castillo, 1988). An increase in membrane K^+ conductance, G_K, accounts for both of these increases in transmembrane voltage based upon their inhibition by K^+ channel blockers, barium and quinine (Howard & Wondergem, 1987; Wondergem & Castillo, 1988; Khalbuss & Wondergem, 1990). Hypotonic stress-

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induced transient release of hepatocyte K^+ after hepatic water uptake into isolated perfused rat liver also is inhibited by barium and quinine (Haddad & Graf, 1989; Häussinger, Stehle & Lang, 1990). RVD in isolated rat hepatocytes also was blocked by barium and quinine as well as by a high concentration of extracellular K^+ (Corasanti et al., 1990). All these findings suggest that RVD in rat hepatocytes is mediated by efflux of intracellular K^+.

Intracellular K^+, nonetheless, is vital to cells. It is essential for protein synthesis and cell growth in eukaryotes and prokaryotes (Lubin, 1964, 1967). Moreover, a decrease in intracellular K^+ is a useful indicator of hepatocyte toxicity (Smith et al., 1987). From this point of view, any loss of cellular K^+ could impair cellular metabolism. Thus, the present study utilized electrophysiological techniques to determine (i) how much the hepatocyte intracellular K^+ decreases during hypotonic stress; (ii) what might cause this decrease in K^+; and (iii) what is the relationship between the change of intracellular K^+ and that of the cell water volume.

Materials and Methods

Materials and Animals

Adult, male mice (ICR strain) were purchased from Charles River Breeding Labs. (Charles River, MA) and were fasted 12–18 hr before experiments. The vivarium housing the mice met all standards of the American Association for Accreditation of Laboratory Animal Care. All inorganic chemicals were purchased from Fisher Scientific (Pittsburgh, PA), and organic chemicals were purchased from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO).

Liver Slice Preparation, Maintenance and Temperature Control

Mice were killed by cervical dislocation and the left-lateral or median lobe of the liver was removed quickly and placed on gauze, which was moistened with 0.9% NaCl. A glass microscope slide was pressed gently onto the lobe to keep it from moving, while a slice of 1 mm thickness was made by using a razor blade clamped in a hemostat. A slice of 5 × 5 mm surface area was placed into an acrylic tissue chamber (BSC-HT, Medical Systems, Greenvale, NY) and held down by a small, steel ring washer placed onto the slice. This ensured that microelectrode impalements, taken within the ring, were consistently of cells on the encapsulated, uncut surface of the slice.

Temperature at the slice was monitored continuously with a thermistor positioned next to the tissue, and it was maintained at 37°C by passing solution through tubing coiled within a temperature-controlled water jacket that formed the base of the tissue chamber. A solenoid valve controlled switching between control and experimental solutions.

Solutions Used in the Experiments and Osmotic Control

Modified Krebs physiological solution contained (in mM) 52.5 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.15 NaH_2PO_4, 2.8 glucose, 4.9 sodium pyruvate, 4.9 sodium glutamate, 2.7 sodium fumarate, 103 sucrose and it was equilibrated with 95% O_2–5% CO_2 (pH 7.46). The hyposmotic condition was created by reducing 80 mM sucrose from Krebs physiological solution. Osmolality of each experimental and control solution was measured with an automatic osmometer based on the principle of freezing point depression (Precision Systems, Natick, MA). For experiments involving measurement of tetramethylammonium ion (TMA^+), liver slices were loaded with TMA^+ chloride for 10 to 15 min by perfusing with Krebs solution in which 100 mM sucrose was replaced by 50 mM TMA^+ chloride. To maintain the intracellular TMA^+ concentration at constant level, 5 mM NaCl was replaced by 5 mM TMA chloride in both control and experimental Krebs solutions (Adorante & Miller, 1990).

Fabrication of Open-Tip Microelectrodes and Ion-Selective Microelectrodes

Double-barrel ion-selective microelectrodes were drawn in a vertical pipette puller (700D, D. Kopf, Instr., Tujunga, CA) from fiber-filled, borosilicate double-capillaries (1.2 mm OD, 0.6 mm ID, A-M Systems, Everett, WA). The latter were cleaned first by boiling them for 15 min in 500 ml of distilled water plus three drops of liquid detergent (Liquinox), rinsing for 1 hr with tap water, boiling for 15 min in distilled water, and drying at 90°C in a forced-air oven. Ion-selective microelectrodes were prepared as follows: one barrel from the blunt end of the double-barrel micropipette was ground 1 mm shorter than the other. The longer end was inverted and stuck into a patent, tapered hole in the lid of a Teflon jar. A few drops of N,N-dimethyltrimethylsilylamine (Fluka, Ronkonkoma, NY) were put into the jar, which then was covered with the lid holding the micropipettes. The jar was placed into a forced-air oven at 25°C for 25 min, allowing the compound in the jar to evaporate and coat the inside of each barrel inserted into the lid. The micropipettes then were put into a holder and dried in the oven at 100°C for 12 hr. Liquid ion-exchanger (477317, Ingold Electrodes, Wilmington, MA), was introduced into the back of the sialinized micropipette, filling the microelectrode tip with a column of exchanger ranging from 0.2–0.5 mm. The microelectrodes were backfilled with 0.5 M KCl. The open-tip (reference) barrel was filled with 1 M Na formate.

All microelectrodes were connected by Ag-AgCl half-cells to a high input impedance ($>10^{14}$ MΩ) preamplifier with unit gain (515L Analog Devices, Norwood, MA). Reference electrodes (bath) consisted of a Ag-AgCl half-cell connected to the cell chamber by an agar (4% in Krebs medium) bridge. Voltages were recorded by a digital voltmeter (Keithly Instr., Cleveland, OH), a storage oscilloscope (Tektronix, Beaverton, OR), and a strip-chart recorder (Western Graphtec, Irvine, CA).

V_m Measurements with Open-Tip Microelectrodes

Criteria for valid impalements included: (1) rapid deflection of the voltage trace on advancing the microelectrode into the cell; (2) an intracellular voltage recording that was stable within 2 mV;

(3) return of the voltage trace to within 2 mV of baseline when the microelectrode was withdrawn; and (4) resting V_m for the control > 20 mV. Intermittent, constant current pulses (0.5 nA; 300 msec duration) were passed through the microelectrode to assess electrode resistance during the course of an intracellular measurement. Irreversible increases in microelectrode resistance indicated clogging of the microelectrode and results were disregarded. Hepatocytes show extensive, low-resistance intercellular communication, presumably through gap junctions. Thus, input resistance measurements by single-barrel microelectrodes comprise membrane resistance and intercellular resistance. Also, the cellular component of input resistance in mouse liver slices is often 10^2 less than microelectrode resistance (Graf & Petersen, 1978). These factors prevented inference about changes in membrane resistance from changes in input resistance.

At least three impalements were made for each individual animal (each liver slice). In each impalement, membrane potential was measured continuously and was recorded before, during and after the osmotic stress, respectively. Hyposmotic condition was imposed on the liver slices only after the membrane potentials became stable. The same applied when switching back to control solution from the anisosmotic situation. Microelectrodes were repositioned after each impalement to ensure that consecutive measurements were not in the same cell.

Calibration of K$^+$-selective Microelectrode and Intracellular K$^+$ Activity Measurement

K$^+$-selective microelectrodes were calibrated in 100, 50, 20, 10, 5 and 1 mM KCl solutions. K$^+$ activities in these calibration solutions were computed according to the Debye-Hückel equation as modified by Armstrong, Byrd and Hamang (1973) and according to the data of Conway (1969). For electrode calibrations, the slopes of voltage *vs.* K$^+$ activity ranged from 54 to 61 mV/10-fold change in K$^+$ activity, with a mean ± SE of 58.0 ± 0.5 (n = 15). Microelectrode selectivities for K$^+$ over Na$^+$, ranging from 29 to 70 with a mean ± SE of 43.0 ± 4.5 (n = 8) were determined by:

$$k_{KNa} = 10^{-(V_K - V_{Na})/S} \quad (1)$$

where k_{KNa} is the selectivity coefficient of the microelectrode for K$^+$ over Na$^+$, S is the slope of the microelectrode calibration in pure KCl and V_k and V_{Na} are the microelectrode voltages in pure, 100 mM solutions of the respective ions. The regression of the K-selective microelectrode potential, V_K, *vs.* log a_K in pure KCl is:

$$V_K = S \cdot \log a_K + b \quad (2)$$

Thus, a^i_K is computed by direct interpolation solving for:

$$a^i_K = 10^{(V - V_m - b)/S} \quad (3)$$

where V_K and V_m are potentials recorded in the intracellular space with K-selective microelectrodes and open-tip microelectrodes, respectively. S and b are slope and intercept of the regression line.

When measuring the K$^+$ activities with K$^+$-selective microelectrodes, in most cases at least three impalements were made in each liver slice, which was obtained consistently from a different animal. In each impalement, membrane potential and V_K were measured continuously and were recorded before, during and after the osmotic stress. The hyposmotic condition was imposed on the liver slices only after the membrane potentials and the V_K became stable. The same applied when switching back to control solution from the osmotic situations. In a few instances, either to reduce the risk of breaking the microelectrode tips or when experiencing difficulty finding a hepatocyte within a liver slice with a satisfactory V_m, microelectrodes were maintained in the same cell for a subsequent measurement after switching back to control solution from the anisosmotic solution.

Calibration of TMA$^+$-Selective Microelectrodes and Intracellular TMA$^+$ Activity Measurement

The calibrations of TMA$^+$-selective microelectrodes were carried out at room temperature in TMA$^+$ solutions whose concentrations covered the expected range of a^i_{TMA} for hepatocytes loaded with TMA$^+$. These solutions comprised 1, 5, 10, 20 and 50 mM TMA chloride, plus 125 mM KCl in each solution. The average slope of electrode voltage *vs.* TMA$^+$ for all experiments was 61 ± 1 mV/10-fold change in TMA activity (SE, n = 6). Intracellular TMA$^+$ activity was computed by direct interpolation solving for:

$$a^i_{TMA} = 10^{(V_{TMA} - V_o - V_m)/S} \quad (4)$$

where a^i_{TMA} = intracellular TMA$^+$ activity, V_{TMA} = intracellular voltage of the TMA$^+$-selective microelectrode. V_o = voltage at the intercept of the calibration curve, V_m = transmembrane potential, S = slope of the calibration curve.

Criteria for Effective Loading and Measurement of TMA$^+$ in Hepatocytes

To measure the change of cell volume with this TMA$^+$-loading technique, certain criteria must be met: (1) loading hepatocytes with TMA$^+$ must not permanently alter the transmembrane potential (V_m) of the hepatocyte; (2) the a^i_{TMA} in hepatocytes loaded with TMA$^+$ must remain constant during the intracellular recording period. The second point had been met practically by maintaining 5 mM TMA$^+$ in the perfusate (Adorante & Miller, 1990).

Computation for the Change in Cell Volume Based on Change in Cell a^i_{TMA}

Change in cell volume based on TMA$^+$ measurements is computed by Cotton, Weinstein and Reuss (1989) to be:

$$\Delta V = V_t - V_0 = V_0[(a^i_{TMA})_0/(a^i_{TMA})_t - 1] \quad (5)$$

where ΔV = change in cell water volume at time t, V_0 = initial cell water volume, $(a^i_{TMA})_0$ = intracellular TMA$^+$ activity at time zero, $(a^i_{TMA})_t$ = intracellular TMA$^+$ activity at time t. Nonetheless, mouse hepatocytes have a large range of initial volumes due to their polyploid nuclei, which range from 2 to 16n (Carriere, 1969). Consequently, we did not assign an initial cell volume to serve as a reference for all measurements and comparisons. Instead, we chose to compare the ratios of $(a^i_{TMA})_0/(a^i_{TMA})_t$ measured under control conditions with ratios obtained under experi-

mental conditions, where $(a^i_{TMA})_0$ = intracellular TMA^+ activity at time zero, and $(a^i_{TMA})_t$ = intracellular TMA^+ activity at time when the V_m became stable after onset of osmotic stress.

Statistical Analysis

Results were expressed as mean ± SE of different animals with the value for each animal comprising the mean of at least three repetitions. Significant differences for paired comparisons were determined by a paired t-test, $P < 0.05$. Multiple comparisons used the analysis of variance and significant differences determined by the method of least significant difference at $P < 0.05$.

Results

Effect of Hypotonic Shock on Hepatocyte Transmembrane Potential, V_m, and on Intracellular Potassium Activity, a^i_K

Reducing osmolality of the external medium from 300 to 250 mosm, by reducing medium sucrose, resulted in a 20-mV hyperpolarization of the hepatocyte V_m, from -27 ± 1 to -46 ± 1 mV ($n = 15$; $P < 0.001$), over 4–5 min until a steady-state was achieved. The hyperpolarized V_m returned to control value of -26 ± 1 mV within 3–4 min after switching back to control medium. Hypotonic shock also decreased hepatocyte a^i_K by 13 mM, from 91 ± 4 to 78 ± 4 mM ($n = 15$; $P < 0.001$), in the same cells. The corresponding K^+ equilibrium potentials, E_K, computed by the Nernst equation utilizing measured a^i_K and a^o_K were -86 and -81 mV, respectively. The hypotonic shock-induced decrease in a^i_K returned toward the original value within 3–4 min after switching back to control medium; however, the steady-state a^i_K at 88 ± 4 mM was 3 mM less than the original value ($P < 0.01$).

Effect of Hypotonic Shock on V_m and a^i_{TMA} in TMA^+-Loaded Hepatocytes

Reuss and colleagues developed the technique of loading cells with TMA^+ and used a cation-selective microelectrode to measure changes in a^i_{TMA} as an indicator of changes in cell water volume (Reuss, 1985; Cotton et al., 1989). Cells were treated with nystatin so they could be loaded with TMA^+. Nystatin was then washed from the cells. Khalbuss and Wondergem (1990) were the first to apply this technique to measure water volume changes in hepatocytes. Their results showed that the TMA^+-loading procedure had no effect on either cell viability or on the plasma membrane's relative ionic permeabilities as indicated by a stable V_m and its increase with hyposmotic stress (Khalbuss & Wondergem, 1990). We now have been able to show that pretreatment of hepatocytes with nystatin (1 mU) lowered a^i_K from 87 to 70 mM over 10 min; whereas, V_m remained unchanged at -24 mV. To avoid possible deleterious effects of nystatin on cell function, we loaded hepatocytes without nystatin, relying on the constituent, organic cation transporter found in hepatocyte plasma membrane (Moseley, Morrissette & Johnson, 1990).

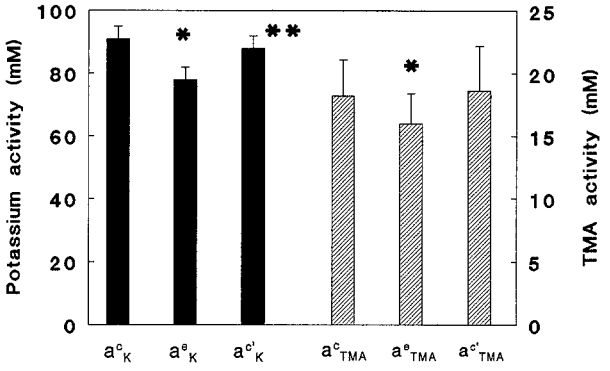

Fig. 1. Intracellular K^+ activity and intracellular TMA^+ activity before (a^c_K, a^c_{TMA}), during (a^e_K, a^e_{TMA}), and after ($a^{c'}_K$, $a^{c'}_{TMA}$) hyposmotic stress to mouse liver slices. Hyposmolality of 0.82 × control osmolality (300 mosm) was achieved by a 50 mM step-decrease in external sucrose concentration. Continuous, double-barrel microelectrode recordings were made, and the experimental values were obtained when steady-states were achieved 4–5 min after switching to hyposmotic solution. The second control values were obtained from continuous records when steady-state values were achieved 3–4 min after switching back to control solutions. All values are averages ± SE of 15 animals for K^+ and of 6 animals for TMA^+. The mean value for each animal was obtained from at least three separate measurements. * Differs from control (a^c), $P < 0.001$. ** Differs from a^c, $P < 0.01$.

The relative change in cell water volume was measured in hepatocytes loaded with TMA^+. Hypotonic shock (0.82 times control osmolality) resulted in a 16-mV increase in V_m, from -29 ± 2 to -45 ± 2 mV ($P < 0.001$), and a 12% decrease in hepatocyte a^i_{TMA}, from 18 ± 3 to 16 ± 2 mM ($n = 6$; $P < 0.01$). The latter accounted for a 14% increase of intracellular water volume. V_m and a_{TMA} (Fig. 1) returned to their control values

Table 1. Comparison of the effect of hypotonic stress on intracellular a^i_K and a^i_{TMA} ($n = 3$)

Experimental condition	V^c_m (−mV)	V^e_m (−mV)	V^e_m/V^c_m	$V^e_m - V^c_m$ (−mV)	a^c_K (mM)	a^e_K (mM)	a^e_K/a^c_K	$a^c_K/a^e_K - 1$	π^e/π^c
K^+	26 ± 0	44 ± 2[c]	1.71 ± 0.10	18 ± 2	88 ± 2	75 ± 3[b]	0.85 ± 0.01	0.17 ± 0.01	0.83
	V^c_m (−mV)	V^e_m (−mV)	V^e_m/V^c_m	$V^e_m - V^c_m$ (−mV)	a^c_T (mM)	a^e_T (mM)	a^e_T/a^c_T	$a^c_T/a^e_T - 1$	π^e/π^c
TMA^+	32 ± 1[d]	46 ± 1[c]	1.45 ± 0.08	14 ± 2	23 ± 3	20 ± 3[a]	0.86 ± 0.01	0.17 ± 0.01	0.82

V^c_m = hepatocyte transmembrane potential. V^e_m = transmembrane potential in hyposmotic experimental solution. a^c_K = hepatocyte intracellular K^+ activity in isosmotic control solution. a^e_K = intracellular K^+ activity in hyposmotic experimental solution. a^c_T = hepatocyte intracellular TMA^+ activity in isosmotic control solution. a^e_T = intracellular TMA^+ activity in hyposmotic experimental solution. $a^c_T/a^e_T - 1$ and $a^c_K/a^e_K - 1$ represent the relative intracellular water volume change while intracellular TMA^+ or K^+ was used as the marker. All values are averages ± SE; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. [a] Differs from control, $P < 0.05$. [b] Differs from control, $P < 0.01$. [c] Differs from control, $P < 0.001$. [d] Differs from corresponding value in K^+ experimental condition, $P < 0.05$.

after switching back to the isotonic condition. This indicated that no leakage of TMA^+ occurred during the measurements.

Comparison of the Hypotonic Shock-Induced Changes in Hepatocyte V_m and a^i_K with the Changes of V_m and a^i_{TMA} in TMA^+-Loaded Hepatocytes

During hypotonic stress, we also compared in the same liver slices changes in a^i_K with changes in a^i_{TMA} (Table 1). In paired measurements, hypotonic shock (0.83 times control osmolality) induced an 18-mV increase in V_m, from −26 ± 0 to −44 ± 2 mV, and a 15% decrease in hepatocyte a^i_K, from 88 ± 3 to 75 ± 3 mM in control condition. If we use intracellular K^+ as a marker to calculate the cell water volume change, then this accounted for a 17 ± 1% increase in cellular water. After loading the hepatocytes with TMA^+, the same hypotonic shock induced a 14-mV increase in V_m, from −32 ± 1 to −46 ± 1 mV and a 14% decrease in a^i_{TMA}, from 23 ± 3 to 20 ± 3 mM ($n = 3$). This accounted for a 17 ± 1% increase of intracellular water volume.

Effect of Ouabain on the Hypotonic Shock-Induced Changes in Hepatocyte V_m and a^i_K

Hypotonic stress also was performed in the presence of ouabain (1 mM) to determine if the hyperpolarization of V_m resulted in part from activation of the electrogenic Na^+-K^+ pump. In paired measurements (Table 2), hypotonic shock (0.81 times control osmolality) to untreated hepatocytes induced a 20-mV increase in V_m, from −24 ± 1 to −44 ± 0 mV, and an 11-mM decrease in hepatocyte a^i_K, from 81 ± 4 to 70 ± 5 mM ($n = 5$). Treating the same tissues with ouabain (1 mM) for at least 30 min did not alter the control V_m, but the control a^i_K decreased by 17 mM (Table 2). Moreover, hypotonic shock induced an 18-mV increase in V_m, from −22 ± 2 to −40 ± 1 mV, and an 8-mM decrease in a^i_K, from 64 ± 3 to 56 ± 3 mM ($n = 4$). The magnitude of these changes was comparable to that in the untreated cells (Table 2). Both the V_m and the a^i_K returned to their original levels after switching back to isosmotic perfusate (Table 2).

Effect of Barium on the Hypotonic Shock-Induced Changes in Hepatocyte V_m and a^i_K

Hypotonic stress in the presence of Ba^{2+} (2 mM) was performed to assess the effect of blocking K^+ channels on the changes in V_m, a^i_K, and cell water volume. In paired measurements (Table 3), hypotonic shock (0.81 times control osmolality) to untreated hepatocytes caused an 18-mV increase in V_m from −28 ± 1 to −46 ± 2 mV, and a 13-mM decrease in a^i_K from 86 ± 6 to 73 ± 5 mM ($n = 5$). Treating the same tissue with barium (2 mM) did not alter either the control V_m or a^i_K. Hypotonic shock now caused only a 5-mV increase in V_m from −24 ± 2 to −29 ± 2 mV, and a^i_K decreased only 6 mM from 88 ± 7 to 82 ± 6 mM. Thus, hypotonic shock resulted in smaller decrease in a^i_K when Ba^{2+} was added. In contrast, cell water volume increased by 10 ± 2% in untreated cells and by 12 ± 2% in Ba^{2+}-treated

Table 2. Effect of ouabain (1 mM) on hypotonic stress induced changes of V_m and a^i_K ($n = 4$)

Experimental condition	V^c_m (−mV)	V^e_m (−mV)	$V^{c'}_m$ (−mV)	V^e_m/V^c_m	a^c_K (mM)	a^e_K (mM)	$a^{c'}_K$ (mM)	a^e_K/a^c_K	π^e/π^c
Untreated	24 ± 1	44 ± 0[c]	25 ± 1	1.82 ± 0.07	81 ± 4	70 ± 5[b]	80 ± 4	0.87 ± 0.02	0.82
Ouabain	22 ± 2	40 ± 1[c,d]	23 ± 2	1.82 ± 0.09	64 ± 3[e]	56 ± 3[a,e]	63 ± 4[e]	0.87 ± 0.02	0.82

V^c_m = hepatocyte transmembrane potential. V^e_m = transmembrane potential in hyposmotic experimental solution. $V^{c'}_m$ = transmembrane potential after hyposmotic stress. a^c_K = hepatocyte intracellular K^+ activity in isosmotic control solution. a^e_K = intracellular K^+ activity in hyposmotic experimental solution. $a^{c'}_K$ = intracellular K^+ activity after hyposmotic stress. All values are averages ± SE; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. [a] Differs from control, $P < 0.05$. [b] Differs from control, $P < 0.01$. [c] Differs from control, $P < 0.001$. [d] Differs from untreated value, $P < 0.05$. [e] Differs from untreated value, $P < 0.001$.

Table 3. Effect of barium (2 mM) on hypotonic stress induced changes of V_m and a^i_K ($n = 5$)

Experimental condition	V^c_m (−mV)	V^e_m (−mV)	V^e_m/V^c_m	a^c_K (mM)	a^e_K (mM)	a^e_K/a^c_K	π^e/π^c
Untreated	28 ± 1	46 ± 2[b]	1.64 ± 0.08	86 ± 6	73 ± 5[b]	0.84 ± 0.01	0.81
Barium	24 ± 2	29 ± 2[b,d]	1.19 ± 0.02	88 ± 7	82 ± 6[a,c]	0.92 ± 0.01	0.81

V^c_m = hepatocyte transmembrane potential. V^e_m = transmembrane potential in hyposmotic experimental solution. a^c_K = hepatocyte intracellular K^+ activity in isosmotic control solution. a^e_K = intracellular K^+ activity in hyposmotic experimental solution. All values are averages ± SE; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. [a] Differs from control, $P < 0.005$. [b] Differs from control, $P < 0.001$. [c] Differs from untreated value, $P < 0.05$. [d] Differs from untreated value, $P < 0.001$.

cells; however, this difference was not significant at $P < 0.05$ ($n = 10$).

Discussion

These results show that a 4–5 min hypotonic shock to mouse heptocytes at 82% of control osmolality causes a 14% decrease in intracellular K^+ activity. We attribute this decrease in a^i_K to cell swelling for three reasons. Firstly, the percent decreases in a^i_K and a^i_{TMA} during hypotonic stress are nearly equal, which leads to identical increases in cell water whether the change in activity of either cation is used for the computation of change in water volume. Secondly, the a^i_K rapidly returns to near control values following hypotonic stress, which shows that little net loss of cell K^+ occurs during cell swelling. Thirdly, ouabain has no effect on the change of a^i_K during or after hypotonic stress, which suggests that active pumping of K^+ has no role in compensating changes in cell K^+ during increases in cell water volume. This is most evident by complete restoration of control value for a^i_K following hyposmotic stress in the presence of ouabain. This would not have occurred had there been significant K^+ efflux during cell swelling and inhibition of compensatory pumping on return to control osmolality.

The decreases in either a^i_K or a^i_{TMA} and the corresponding increases in cell water volume are less than predicted by the decrease in external osmotic pressure. This is consistent with cell volume regulation that compensates cell swelling (Corasanti et al., 1990; Khalbuss & Wondergem, 1990). Efflux of cell K^+ is an important ionic component for cell volume regulatory mechanisms for hepatocytes (Bakker-Grunwald, 1983; Kristensen & Folke, 1984) and many other cell types (Chamberlin & Strange, 1989). What has not been measured, however, is the extent of change in hepatocyte a^i_K during this process. Our findings show that the decrease in a^i_K is less than might be expected based upon measured changes in rate of efflux of K^+ (Bakker-Grunwald, 1983; Kristensen & Folke, 1984), by changes in V_m and G_K (Howard & Wondergem, 1987; Khalbuss & Wondergem, 1990), or by changes in effluent K^+ concentration in the hypotonically stressed perfused rat liver (Haddad & Graf, 1989; Lang, Stehle & Häussinger, 1989). Since K^+ is vital to cell function, regulation of intracellular K^+ is perhaps more important to maintaining hepatic function as is the need to maintain hepatocyte volume.

Hepatocytes nonetheless lose some K^+ under the present hyposmotic condition as is evident by the smaller decrement in cell a^i_K when Ba^{2+} was added during the hyposmolality. Here, we infer that sufficient block of K^+ channels inhibits hyperpolarization of V_m and prevents efflux of cell K^+. The corresponding cell swelling was slightly greater than in the untreated cells, which is consistent with previous reports that blocking hyposmotic stress-induced hyperpolarization of V_m inhibits volume regulation and leads to greater swelling (Khalbuss & Wondergem, 1990; Corasanti et al., 1990). It remains to be explained, however, why cell a^i_K remains stable in hepatocytes under control and hyposmotic conditions (even in the presence of ouabain) when under both conditions the E_K is far greater than V_m. This could result for various reasons alone or in combination, such as a membrane G_K that is relatively low compared to other ions; membrane K^+ pumps in addition to the ouabain-sensitive Na^+-K^+ ATPase; or chemical and structural compartmentation of cell K^+ that is in equilibrium with the a^i_K of aqueous cytoplasm.

The variation in hepatocyte V_m plays an important role in hepatocyte volume regulation (Howard & Wondergem, 1987; Khalbuss & Wondergem, 1991; Wang & Wondergem, Wang & Wondergem, 1991, 1992); however, the mechanisms are more complex than we previously envisioned them. We had thought that transmembrane flux of K^+ during osmotic stress was both conductive, changing V_m, and osmotic, changing internal K^+ concentration while offsetting water volume changes. Our present findings are inconsistent with the latter conclusion, since the change in hepatocyte a^i_K equaled that of the change in cell water volume. Thus, it seems that osmotic stress-induced changes in membrane K^+ are principally conductive changes with little corresponding change in cell K^+ per se. The resultant changes in V_m provide electromotive force that drives transmembrane Cl^- fluxes (Haddad et al., 1991; Wang & Wondergem, 1992). This leads to substantial changes in hepatocyte Cl^- activity, which is osmoregulatory (Wang & Wondergem, 1992).

The 13-mM loss of a^i_K during hyposmotic stress was reduced to a 6-mM loss with added Ba^{2+}. The difference of 7 mM approximately equals the loss of cell Cl^-, which also is inhibited by Ba^{2+}, during a comparable hyposmotic stress to liver slices (Wang & Wondergem, 1992). This accounts for electroneutral transport of these ionic osmolytes. We do not know if additional cations accompany Cl^- to preserve electroneutrality under these conditions. Van Rossum and Russo (1984) suggest that cotransport of NaCl plays an important role in the mechanism for ouabain-resistant water extrusion from hepatocytes recovering at 38°C from a period of swelling at 1°C. This and our findings differ from the conclusions of other investigators who predicted substantial osmoregulatory K^+ fluxes based upon tracer permeability changes (Bakker-Grunwald, 1983; Kristensen & Folke, 1984) or upon changes in effluent K^+ concentrations in perfused liver (Haddad & Graf, 1989; Lang et al., 1989). It is noteworthy that the transmembrane osmotic pressure differentials in these studies were much larger than in ours.

Zeuthen (1982) measured steady-state a^i_K in *Necturus* gallbladder epithelium equilibrated across the mucosal membrane with solutions of low osmolarity. In spite of transmembrane water influx along with decreases of Na^+ and Cl^-, intracellular K^+ remained surprisingly high. He attributed this to K^+ influx of unknown mechanism, along with the influx of water (Zeuthen, 1982). MacKnight and Leaf (1977) were skeptical of inferences about the physiologic importance of K^+ in cell volume regulation drawn from the results of experiments in which cells had been exposed to anisosmotic media. They concluded that it was "wrong to regard such potassium loss from cells incubated in very dilute media as in any way a reflection of a cellular homeostatic mechanism regulating, or attempting to regulate, cellular volume." We feel that this caveat is particularly meaningful in light of our present findings and the overall importance of cell K^+ in maintaining hepatocyte function. Furthermore, any adaptive response involving loss of substantial cell K^+ into the extracellular compartment by an organ as large as the liver would have deleterious effects on excitable cells, such as those in the heart, and, therefore, could not sustain the organism.

The only other direct evidence that hepatocytes lose intracellular K^+ in hyposmotic solution comes from an electron probe microanalysis of cultured rat hepatocytes (Cohen & Lechene, 1990). In their study, rat hepatocyte intracellular K^+ fell by 18% after 15 min exposure to hypotonic solution where NaCl concentration was reduced by 57 mM. Part of this loss in K^+ could have resulted from low extracellular Na^+ concentration rather than the hyposmotic condition alone. Lowering the extracellular Na^+ concentration could lower intracellular Na^+ and inhibit the Na^+-K^+ pump. This, in turn, could decrease intracellular K^+. The effects of ouabain on a^i_K reported here show how Na^+-K^+ pump inhibition decreases a^i_K.

In summary, mouse hepatocyte V_m hyperpolarized and intracellular a^i_K decreased during short-term hypotonic shock. This change in a^i_K can be explained largely as a result of cell swelling. The disagreement between measured cell water volume

change and the expected value (assuming hepatocytes respond as perfect osmometers) is consistent with an RVD mechanism in hepatocytes during hypotonic stress. The negative effect of ouabain on changes of both V_m and a^i_K induced by hypotonic stress suggests the independence of hepatocyte RVD from Na^+-K^+ pump activity. This study also suggests that an increase in hepatocyte K^+ conductance is not necessarily accompanied by a measurable loss of intracellular K^+, as has been demonstrated in Na^+-alanine cotransport- or hypotonic stress-induced loss of K^+. However, the increase in K^+ conductance is necessary for setting up a more negative V_m, which may be important for maintaining hepatocyte metabolic function during the RVD process. Here, the increased electromotive force could redistribute cell Cl^-, and accompanying cation to facilitate the adaptive response to change in cell water volume.

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