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Effects of hyperosmotic medium on hepatocyte volume, transmembrane potential and intracellular K^+ activity

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Hepatocyte transmembrane potential (V_m) behaves as an osmometer and varies with changes in extracellular osmotic pressure created by altering the NaCl concentration in the external medium (Howard, L.D. and Wondergem, R. (1987) J. Membr. Biol. 100, 53). We now have demonstrated similar effects on V_m by increasing external osmolality with added sucrose and not altering ionic strength. We also have demonstrated that hyperosmotic stress-induced depolarization of V_m results from changes in membrane K^+ conductance, g_K, rather than from changes in the K^+ equilibrium potential. V_m and a_K^i of hepatocytes in liver slices were measured by conventional and ion-sensitive microelectrodes, respectively. Cell water vols. were estimated by differences in wet and dry weights of liver slices after 10-min incubations. Effect of hyperosmotic medium on membrane transference number for K^+, t_K, was measured by effects on V_m of step-changes in external $[K^+]$. Hepatocyte V_m decreased 34, 52 and 54% when tissue was superfused with medium made hyperosmotic with added sucrose (50, 100 and 150 mM). Correspondingly, a_K^i increased 10, 18 and 29% with this hyperosmotic stress of added sucrose. Tissue water of 2.92 ± 0.10 kg H_2O/kg dry weight in control solution decreased to 2.60 ± 0.05, 2.25 ± 0.06 and 2.22 ± 0.05 kg H_2O/kg dry weight with additions to medium of 50, 100 and 150 mM sucrose, respectively. Adding 50 mM sucrose to medium decreased t_K from 0.20 ± 0.01 to 0.05 ± 0.01. Depolarization by 50% with hyperosmotic stress (100 mM sucrose) also occurred in Cl-free medium where Cl^- was substituted with gluconate. We conclude that hepatocytes shrink during hyperosmotic stress, and the a_K^i increases. The accompanying decrease in V_m is opposite to that expected by an increase in a_K^i, and at least in part results from a concomitant decrease in g_K. Changes in membrane Cl^- conductance most likely do not contribute to osmotic stress-induced depolarization, since equivalent decreases in V_m occurred with added sucrose in cells depleted of Cl^- by superfusing tissue with Cl-free medium.

Introduction

The transmembrane potential, V_m, of mouse hepatocytes varies substantially with variations in external osmotic pressure produced by altering NaCl [1] or sucrose concentrations [2] in the external medium. Hyperpolarizations of V_m accompany hyposmotic stress and depolarizations occur during hyperosmotic stress [1,2]. These osmotic stress-induced changes in hepatocyte V_m are physiologically important when linked to homeostatic mechanisms that operate during changes in cell metabolism.

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Functional demands on the liver alter intracellular organic solute concentrations, which constitute a fluctuation in intracellular osmotic pressure. For example, Kristensen reported [3] steady state intracellular alanine concentrations of 93 mM resulted when 10 mM alanine was added to isolated hepatocytes whose alanine metabolism was inhibited by aminooxyacetate. The cells swelled as a result [3] accompanied by increases in membrane K^+ permeability [4] and hyperpolarization of V_m [5,6]. Reduction in intracellular cation concentrations partly compensated the increase in cell volume, but the membrane mechanisms controlling net ion efflux are unknown [3]. We have suggested that hepatocyte swelling may elicit adaptive increases in plasma membrane K^+ conductance, g_K. Corresponding hyperpolarization of V_m may provide electromotive force for redistribution of intracellular Cl^-,

which equilibrates according to hepatocyte V_m [7–9]. Thus, efflux of K^+ and Cl^- may compensate the increased intracellular osmotic pressure due to accumulation of organic solute.

Specific ions accounting for changes in hepatocyte V_m during amino acid uptake and osmotic stress are not fully known. Liver cell V_m derives substantially from the transmembrane K^+ concentration gradients, because the sinusoidal membrane K^+ conductance is higher than that for other ions [7]. Moreover, the V_m is considerably less negative than the hepatocyte K^+ equilibrium potential, E_K [7]. Consequently, passive transmembrane water fluxes may alter the driving forces for K^+ and change V_m. Khalbuss and Wondergem [2] reported that such volume changes along with partial volume regulation occur during osmotic stress to mouse hepatocytes. Thus, shrinking cells might be expected to increase intracellular K^+ activity and thereby hyperpolarize V_m. This, however, is contrary to the depolarization of V_m that occurred during hyperosmolality of external medium [1,2].

Results from ion substitution experiments suggested that hyposmotic stress-induced changes in hepatocyte V_m resulted from changes in membrane K^+ conductance, g_K [1,2]. Also, K^+ channel blockers, quinine and barium, inhibit hyperpolarization of hepatocyte V_m during hyposmotic stress [1,2]. Osmotic stress-induced changes in membrane g_K also have been suggested by a recent report on complementary changes in membrane resistance during osmotic stress for mouse hepatocytes in primary culture [10]. Finally, the hepatocyte plasma membrane has a substantial Cl^- conductance, g_{Cl} [7]. Notwithstanding, the transmembrane Cl^- distribution in hepatocytes is passive such that V_m equals the Cl^- equilibrium potential, E_{Cl} [7–9]. Therefore, changes in hepatocyte membrane g_{Cl} ostensibly do not effect changes in V_m and probably contribute little to osmotic stress-induced changes in V_m.

In the present study, we measured intracellular K^+ activity, a_K^i and V_m simultaneously during hyperosmotic stress to mouse hepatocytes in liver slices. In addition, changes in V_m during hyperosmotic stress were measured following external ion substitution of K^+ for Na^+ and gluconate for Cl^-. The purpose was to determine whether the changes in hepatocyte V_m with osmotic stress resulted principally from changes in membrane g_K or whether changes in driving forces for either K^+ or Cl^- might be involved.

Materials and Methods

Materials and animals

Adult male mice of ICR strain were purchased from Charles River Breeding Labs. (Charles River, MA) and were fasted 18–24 h 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 thick 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 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.

Liver slices for control V_m measurements were superfused with Krebs physiological solution. Temperature at the liver slice was monitored continuously with a thermistor positioned next to the tissue (Yellow Springs Instrument, Yellow Springs, OH), and it was maintained at 37°C by passing solution at 12–15 ml/min through a Graham condenser. This enabled countercurrent heat exchange to the medium from water circulating through the condenser jacket and a heater pump. Two separate condensers were used in which a solenoid valve was activated to switch between control and experimental solutions.

Solutions used in the experiments and osmotic control

Krebs physiological solution contained (in mM) 103 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 and it was equilibrated with 95% O_2–5% CO_2 (pH 7.46). Hyperosmotic conditions were created by adding sucrose of 50, 100 and 150 mM, respectively, to normal 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.). Gluconate salts were substituted for chloride salts for experiments requiring isosmotic, Cl-free medium and Ca^{2+} was increased to 5.1 mM to counter chelating effects of gluconate.

Fabrication of open-tip microelectrodes and ion-selective microelectrodes

Microfilament capillary glass (1.2 mm o.d., 0.68 mm i.d., A-M systems, Everett, WA) was cleaned first by boiling for 15 min in 500 ml of distilled water plus three drops of liquid detergent (Liquinox). After a 1-h

tap water rinse, the microfilament capillaries were boiled for 15 min in distilled water and then dried at 90°C in a forced-air oven. Open-tip microelectrodes were drawn in a horizontal puller (Industrial Scientific, Ridgewood, NY) from the cleaned capillaries and had tip-diameters of about 0.51 ± 0.08 μm ($n = 4$) measured by scanning electron microscopy. They were filled with 500 mM KCl, and they had tip resistances of 10–30 MΩ.

Double-barreled ion-selective microelectrodes were prepared from fiber-filled, borosilicate double-capillaries (1.2 mm o.d., 0.6 mm i.d., A-M Systems, Everett, WA) cleaned as described above. These were pulled in a vertical pipette puller (700D, D. Kopf, Instr., Tujunga, CA) and had tip-diameters of approx. 1.25 ± 0.07 μm ($n = 6$) measured by scanning electron microscopy. The tip of one barrel was filled with a drop of distilled water. The other was filled with 1 mm column of hexamethyldisilazane (H-4875, Sigma) and baked in a forced-air oven at 100°C for 2–5 h. The tip of the silanized barrel was filled with a 0.2–1.0 mm-column of potassium liquid ion-exchanger (477317, Corning Medical, Medfield, MA), which was introduced into the back of the microelectrode and filled the tip by capillary action along the groove formed by the microfilament against the tube wall. The microelectrode then was back-filled with 500 mM KCl. The reference barrel was filled with 500 mM sodium formate.

All microelectrodes were connected by Ag/AgCl half-cell to a high input impedance ($> 10^{14}\Omega$) preamplifier with unit gain (515L Analog Devices, Norwood, MA). A reference bath electrode consisted of a Ag/AgCl half-cell connected to the tissue 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), a Grass polygraph and a strip-chart recorder (Western Graphtec, Irvine, CA).

V_m measurements with open-tip microelectrodes

Criteria for valid cell 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 ms 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 [11,12], presumably through gap junctions. Thus, input resistance measurements 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 [11]. These factors disallowed 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 hyperosmotic stress, respectively. Hyperosmotic conditions were imposed onto the liver slices only after the membrane potentials became stable. The same applied when switching back to control solution from the hyperosmotic situation. Microelectrodes were repositioned after each impalement to ensure that consecutive measurements were not in the same cell.

Calibration of ion-selective microelectrodes and intracellular K^+ activity measurements

K^+-selective microelectrodes were calibrated in 100, 50, 20, 10, 5 and 1 mM KCl solutions. For electrode calibrations, the slopes of electrode voltage vs. K^+ activity ranged from 52 to 62 mV/10-fold change in K^+ activity, with a mean ± S.E. of 58 ± 0.9 ($n = 10$). Microelectrode selectivities for K^+ over Na^+, ranging from 20 to 47 with a mean ± S.E. of 29 ± 2.7 ($n = 9$) were determined by:

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

where k_{KNa} was the selectivity coefficient of the microelectrode for K^+ over Na^+, S was the slope of the microelectrode calibration in pure KCl and V_K and V_{Na} were the microelectrode voltages in pure, 100 mM solutions of the respective ions [13]. 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_K^i was computed by direct interpolation solving for:

$$a_K^i = 10^{(V_K - V_m - b)/S} \quad (3)$$

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 hyperosmotic stress, respectively. Hyper-

osmotic conditions were imposed onto 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 hyperosmotic 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 hyperosmotic solution.

K^+ transference number (t_K) as an index of membrane K^+ conductance

Transference number for K^+ (t_K) was used as a measure of the relative contribution of K^+ conductance (g_K) to overall plasma membrane conductance (g_{cell}) and V_m. It was calculated as follows:

$$t_K = \Delta V_m / \{(RT/zF) \ln(C_1/C_2)\} \quad (4)$$

where ΔV_m is the change in V_m in mV upon a step change in extracellular K^+ concentration from 5 mM (C_1) to 50 mM (C_2) for approx. 5 min or until a new steady state was achieved and RT/zF have their usual meanings and equal 26.7 mV at 37°C. This approach assumes neither voltage- nor concentration-dependence of membrane g_K, which cannot be completely ruled out. Hence, this technique is at best a first approximation in measuring t_K.

Relative tissue water volume determination

Liver slices were prepared as described above except that the entire liver was used and the slices were ≈ 100 μm thick. These slices were distributed 5 each to two 25 ml flasks containing either 5 ml of Krebs solution or 5 ml of hyperosmotic Krebs solutions, respectively. Immediately after the distribution of the liver slices, they were incubated in a Dubnoff metabolic shaking incubator at 37°C for 10 min and were gassed throughout with 95% O_2–5% CO_2. Afterward, each slice was blotted on filter paper (Whatman #1) and weighed wet in a pre-weighed weighing bottle. Slices were then dried in a forced-air oven at 90°C for 12 h and weighed dry. Total water was the difference between wet and dry weights. Relative tissue water volume was expressed as total water (kg)/dry weight (kg).

Statistical analysis

Multiple comparisons of means were accomplished by Student-Newman-Keuls procedure [14]. Relative changes of intracellular K^+ activity were represented by the ratios of experimental/control values. Ratios of steady-state values were chosen because previous work demonstrated substantial variation of hepatocyte V_m and a_K^i between animals [15]. These ratios of a_K^i and changes in V_m between experimental and control conditions were plotted as functions of relative osmolality, experimental mOsm/control mOsm. Linear regression analysis of these plots was achieved by the least-squares computation and the significance of the regression coefficients was determined by the t-test at $n-2$ degrees of freedom and at $P < 0.05$.

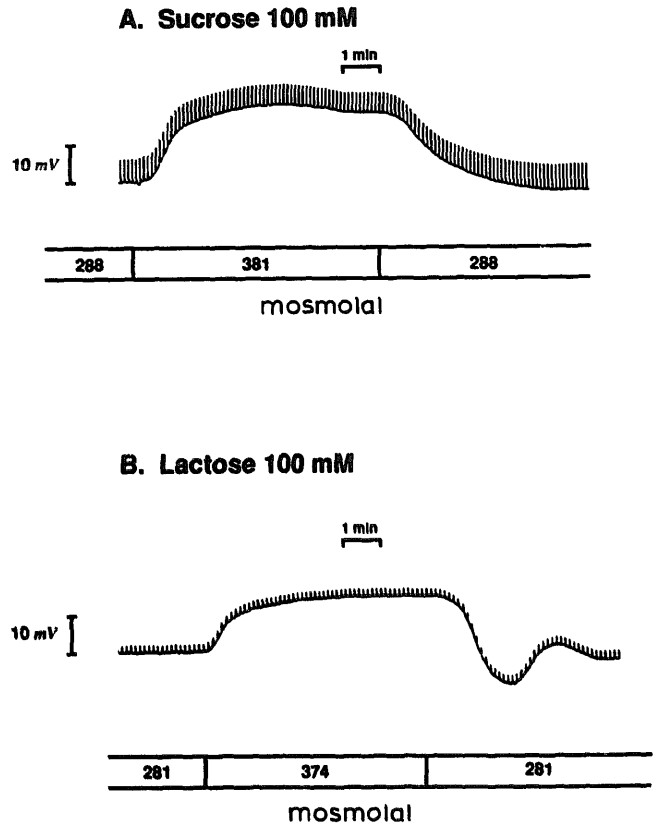

Fig. 1. Effect of hyperosmotic media on hepatocyte V_m in liver slices. (A). Hyperosmotic condition was created by switching from Krebs medium to Krebs medium plus 100 mM added sucrose. Control V_m before sucrose was −55 mV. (B). Hyperosmotic condition was created by switching from Krebs medium to Krebs medium plus 100 mM added lactose. Control V_m before sucrose was −36 mV. Similar decreases in V_m occur after switching to hyperosmotic conditions in (A) and (B). Periodic upward deflections in the voltage trace result from injection of 0.5 nA of intermittent current through the microelectrode to assess its resistance.

Results

Effects of hyperosmotic medium on the transmembrane potential, V_m, of mouse hepatocytes

Increasing osmolality of the external medium from 280 mOsm to 330 mOsm, 380 mOsm and 430 mOsm by adding 50 mM, 100 mM and 150 mM sucrose resulted in average decreases in V_m of 11 mV, 13 mV and 15 mV, respectively, over 4–5 min until a steady-state was reached. In all experiments, V_m was measured before, during and after the hyperosmotic stress and a representative trace of such a measurement is shown in Fig. 1A. Results of all measurements are summarized in

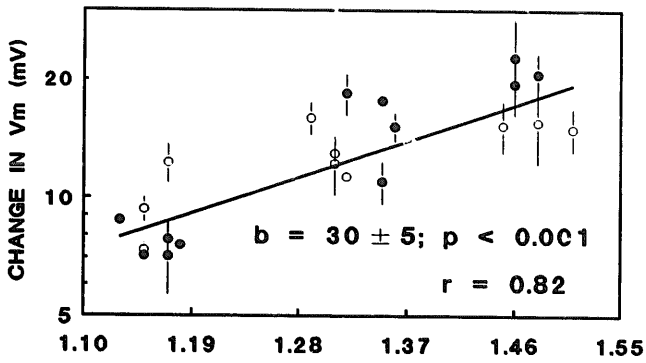

Fig. 2. The log value of the change in V_m (depolarization) plotted as a function of the ratio of experimental and control medium osmolality. Graph shows the decrease in steady-state hepatocyte V_m from control value with increases in medium osmolality above control. Each point represents the mean ΔV_m value of one animal. There are at least three measurements for each animal. The variation of the individual value is shown in the figure if its magnitude exceeds the size of the circle. Solid circles indicate data obtained from double barreled microelectrodes, while open circles indicate data obtained from single barreled microelectrodes. b = linear regression coefficient. r = correlation coefficient.

Table I. The percent decreases in V_m were 34, 52 and 54%, respectively, during the experiments in the three hyperosmotic conditions. Similar depolarization of V_m occurred when hyperosmotic conditions were created by adding lactose to the external medium, Fig. 1B.

Switching back to control medium resulted in V_m returning to a steady-state that was 5, 3 and 2 mV higher than that of control value. We do not know the basis for these 'overshoots', but they are not accounted for by changes in a^i_K. Corresponding measurements of a^i_K following hyperosmotic stress were less than control values (Table I), which would predict a decrease in V_m. The change in V_m also varied with osmolality of the hyperosmotic medium. Results in Fig. 2 show the log value of the change in V_m (mV depolarization) plotted as a function of the ratio of experimental/control medium osmolality. The graph shows that hepatocyte V_m decreases from control value as a function of an increase in osmolality above that of the Krebs physiological medium.

Effects of hyperosmotic medium on the intracellular potassium activity, a^i_K, of mouse hepatocytes

Depolarization of V_m with hyperosmotic stress (above) is not consistent with the likelihood of cell shrinkage. Here, we might expect a^i_K to increase resulting in hyperpolarization of V_m. To begin exploring reasons for this apparent inconsistency, we measured a^i_K in hepatocytes during hyperosmotic stress identical to that above.

TABLE I

Effects of hyperosmotic conditions on hepatocyte membrane potential, V_m, and intracellular K^+ activity, a^i_K

V^c_m = hepatocyte transmembrane potential in control solution. V^e_m = transmembrane potential in hyperosmotic experimental solution. $V^{c'}_m$ = transmembrane potential in control solution after hyperosmotic stress. a^c_K = hepatocyte intracellular K^+ activity in control solution. a^e_K = intracellular K^+ activity in hyperosmotic experimental solution. $a^{c'}_K$ = intracellular K^+ in control solution after hyperosmotic stress. All values are averages ± S.E.; values for individual experiments are from at least three separate measurements, whereas the mean values are averages of the values from each animal. Control and Control' were obtained before and after experimental measurements, respectively, during continuous recording with microelectrodes

Exper. No.	Control (V^c_m)	Experimental (V^e_m)	Control' ($V^{c'}_m$)	Ratio (V^e_m / V^c_m)	Control (a^c_K)	Experimental (a^e_K)	Control' ($a^{c'}_K$)	Ratio (a^e_K / a^c_K)	π^e/π^c
50 mM sucrose									
1-5-1	−30 ± 1	−21 ± 1	−32 ± 3	0.69 ± 0.02	100 ± 2	104 ± 3	92 ± 11	1.05 ± 0.01	1.15
1-5-2	−27 ± 2	−20 ± 2	−37 ± 2	0.73 ± 0.03	83 ± 4	94 ± 4	82 ± 5	1.13 ± 0.01	1.15
1-21	−35 ± 2	−20 ± 1	−41 ± 2	0.56 ± 0.02	82 ± 5	91 ± 2	82 ± 4	1.11 ± 0.05	1.17
Mean	−31 ± 2	−20 ± 0 *	−37 ± 3	0.66 ± 0.05	88 ± 6	96 ± 4	85 ± 3	1.10 ± 0.02	
100 mM sucrose									
11-22	−22 ± 3	−12 ± 2	−27 ± 6	0.52 ± 0.04	109 ± 9	120 ± 10	106 ± 14	1.10 ± 0.01	1.32
11-24-1	−24 ± 1	−12 ± 1	−27 ± 2	0.49 ± 0.07	100 ± 8	120 ± 5	97 ± 6	1.22 ± 0.10	1.31
11-24-2	−26 ± 1	−13 ± 1	−30 ± 1	0.50 ± 0.01	85 ± 2	104 ± 8	88 ± 9	1.21 ± 0.60	1.31
12-6	−27 ± 2	−11 ± 1	−20 ± 4	0.40 ± 0.02	73 ± 11	110 ± 14	71 ± 12	1.19 ± 0.02	1.29
Mean	−25 ± 1	−12 ± 0 **	−26 ± 1	0.48 ± 0.03	92 ± 8	114 ± 14	90 ± 7	1.18 ± 0.03	
150 mM sucrose									
11-30	−28 ± 2	−13 ± 2	−28 ± 4	0.45 ± 0.07	126 ± 8	158 ± 21	115 ± 17	1.24 ± 0.09	1.45
12-2	−30 ± 2	−14 ± 1	−35 ± 4	0.49 ± 0.08	73 ± 3	100 ± 4	76 ± 3	1.37 ± 0.08	1.48
1-13	−27 ± 1	−12 ± 1	−28 ± 3	0.44 ± 0.06	91 ± 5	115 ± 7	83 ± 6	1.26 ± 0.02	1.51
Mean	−28 ± 1	−13 ± 1 **	−30 ± 3	0.46 ± 0.02	97 ± 16	124 ± 17	91 ± 12	1.29 ± 0.04	

* Differs from control, $P < 0.005$.
** Differs from control, $P < 0.001$.

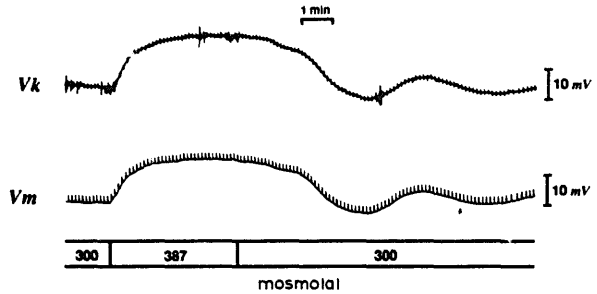

Fig. 3. Effect of hyperosmotic stress on intracellular voltages recorded with a double-barreled K^+-selective microelectrode. V_m shows voltages recorded with open-tip or reference microelectrode. Control V_m before sucrose was −30 mV. V_K shows voltage recorded with the liquid-exchanger K^+-selective microelectrode. Upward indicates decreases in potential for both voltage traces. a_K^i were calculated from the differences between steady-state V_K and V_m. Periodic upward deflections in the V_m trace are described in the legend of Fig. 1.

Intracellular K^+ activity (a_K^i) was measured before, during and after the hyperosmotic stress in all of our experiments. A representative trace obtained from a double-barreled microelectrode, which recorded intracellular voltages from a K-selective tip, V_K and from an open tip, V_m, is shown in Fig. 3. Increasing osmolality of the external medium from 270–280 mOsm to 330, 380 and 430 mOsm by adding 50, 100 and 150 mM sucrose resulted in an increase in a_K^i of 8, 22, 27 mM, respectively (Table I). These represented relative increases in a_K^i of 10, 18 and 29% of the control value.

Variation between animals in the magnitude of hepatocyte a_K^i resulted in no statistical significance for the increase in mean a_K^i with osmolality, Table I. To test whether hepatocyte a_K^i increased with increments in external osmolality, values of mean experimental a_K^i were normalized to the mean, paired control a_K^i by obtaining a ratio from each animal. The average ratios of steady-state experimental/control a_K^i were plotted as a function of the ratio of experimental/control medium osmolality, Fig. 4. The regression coefficient for this plot was significantly greater than zero, which shows that a_K^i increases from control value as a linear function of medium osmolality. This increase in a_K^i did not account for depolarization of the V_m during hyperosmotic stress, but it was consistent with loss of cell water.

Effects of hyperosmotic medium on the tissue water of the liver slices

The observation that hepatocyte a_K^i increases during hyperosmotic stress suggests indirectly that hepatocytes shrink. If that is true, a decrease in water content of the tissue during hyperosmotic stress would be expected. Our results on tissue water measurements confirmed this expectation. Liver slice water contents were measured after incubating the tissue in different osmotic medium at 37°C for 10 min. Hyperosmotic conditions were created by adding 50, 100 and 150 mM sucrose, respectively, to Krebs physiological solution. After 10 min incubation in hyperosmotic medium, tissue water of 2.92 ± 0.10 kg H_2O/kg dry weight in control solution decreased to 2.60 ± 0.05, 2.25 ± 0.06 and 2.22 ± 0.05 kg H_2O/kg dry weight in the respective hyperosmotic solutions. Differences between all values were significant except between the values at 100 and 150 mM added sucrose. The results in this experiment indicated that the tissue water decreased as the osmolality of the medium increased, at least up to 100 mM added sucrose.

Change in plasma membrane K^+ transference number, t_K, during hyperosmotic stress to hepatocytes

The transmembrane potential of the hepatocyte is determined partly by the transmembrane chemical gradient for K^+ because the membrane of the hepatocyte is much more selectively permeable to K^+ than any other cation [7]. According to the Nernst equation, an increase in intracellular potassium activity would result in an increase in V_m. Nevertheless, our results indicated a decrease in V_m corresponding to the increase in a_K^i during hyperosmotic stress on hepatocytes. The most reasonable explanation for this phenomenon is that the K^+ conductance, g_K, decreased during hyperosmotic stress on hepatocytes. To test this we compared membrane transference numbers for K^+ (t_K) measured at control osmolality with t_K measured in hyperosmotic medium of added sucrose (50 mM). A trace representative of 7 measurements to compute t_K in control medium and in hyperosmotic medium is shown in Fig. 5. Each paired comparison was made in one cell while continuously recording V_m with a single-barrel microelectrode. Summary of comparisons are shown in Table II. The t_K in hyperosmotic medium

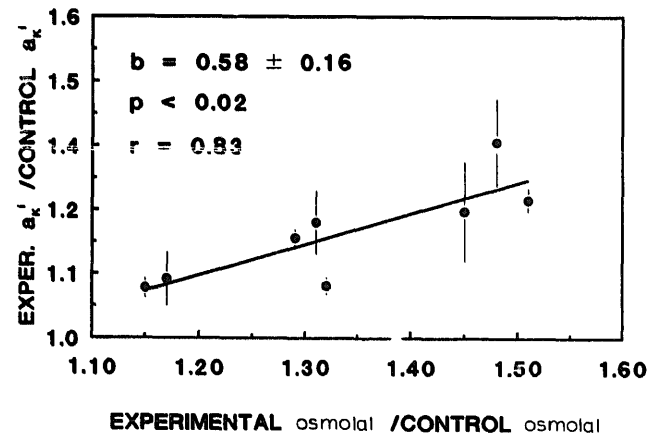

Fig. 4. Ratio of experimental and control steady-state intracellular K^+ activity (a_K^i) plotted as a function of the ratio of experimental and control medium osmolality. Graph shows the increase in hepatocyte a_K^i from control value plotted as a function of increases in osmolality above control value. b = linear regression coefficient. r = correlation coefficient.

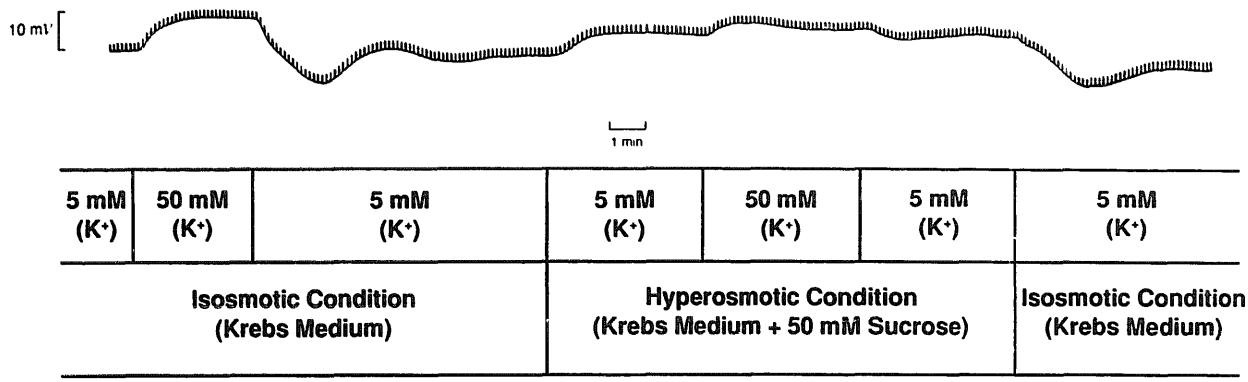

Fig. 5. A continuous recording of V_m from one cell showing decreases in V_m in response to step-changes in extracellular K^+ concentration from 5 mM to 50 mM, first done in isosmotic medium and then repeated in hyperosmotic medium. Control V_m in 5 mM K^+ was −32 mV. Hyperosmotic condition was created by adding 50 mM sucrose to Krebs solution. Results were used to compute hepatocyte t_K for isosmotic and hyperosmotic conditions. Periodic upward deflections in the voltage trace are described in the legend to Fig. 1.

(added 50 mM sucrose) was 0.25 times t_K in control osmolality. This is consistent with a decrease in membrane g_K with hyperosmotic stress, which may account for the corresponding decreases in V_m.

Effects of hyperosmotic, Cl-free medium on V_m of mouse hepatocytes

Depolarizations of hepatocyte V_m with hyperosmotic stress may be attributable to decreases in membrane g_K, which override increases in a^i_K and the tendency for V_m to hyperpolarize. Shrinking of cells also may increase intracellular Cl^- activity, a^i_{Cl}, and thereby decrease the Cl^- equilibrium potential. Since the hepatocyte plasma membrane has a relatively high Cl^- conductance [7,11], this shift in E_{Cl} also could account for the depolarization. To test this, the hyperosmotic stress was repeated after cells were depleted of Cl^- by substituting external Cl^- with an impermeable anion, gluconate. Lyall et al. [8] have shown that such substitution rapidly depletes mouse hepatocytes of cytoplasmic Cl^-, and accordingly the cells' V_m depolarizes with this loss of anion. Results of a typical trace are shown in Fig. 6. Gluconate/Cl substitution resulted in a slow depolarization of V_m with effluence of Cl^- from the cell; however, the difference here in mean V_m from three animals was not significant. Once a new steady-state V_m was achieved, hyperosmotic sucrose (100 mM) still rapidly depolarized V_m, Fig. 6. Results of repetitive measurements as in Fig. 6 are summarized in Table III. The absolute change in V_m

TABLE II

Effect of hyperosmotic medium on transference number for K^+ (t_K)

Numbers indicating potential are −mV. V_m = transmembrane potential of hepatocyte. ΔV_m = transmembrane potential change in mV in response step-changes in extracellular K^+ concentration from 5 mM to 50 mM. t_K = transference number for K^+. Hyperosmotic condition was created by adding 50 mM sucrose to the Krebs solution. No. 4–7 are measurements at different cells within the same liver slice. Data obtained from a total of four liver slices

Exper. No.	Isosmotic medium				Hyperosmotic medium			
$[K^+]_0$:	5 mM	50 mM			5 mM	50 mM		
	V_m (mV)	V_m (mV)	ΔV_m (mV)	t_K	V_m (mV)	V_m (mV)	ΔV_m (mV)	t_K
1	39	29	10	0.16	29	28	1	0.02
2	42	28	14	0.23	39	36	3	0.05
3	36	26	10	0.16	32	28	4	0.07
4	27	16	11	0.18	21	20	1	0.01
5	35	22	13	0.21	33	25	8	0.13
6	40	24	16	0.26	29	26	3	0.05
7	32	20	12	0.20	26	24	2	0.03
Mean	36±2	24±2	12±1	0.20±0.01	30±2	27±2	3±1	0.05±0.01 *

* Value differs from that obtained in isosmotic medium, $P < 0.001$.

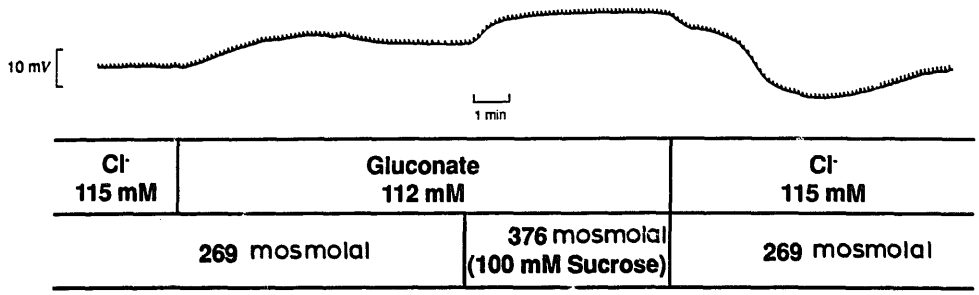

Fig. 6. A continuous recording of V_m from one cell showing consecutive decreases in V_m, first with substitution of external Cl^- with gluconate and second with hyperosmotic stress (100 mM sucrose) concurrent with the Cl-free condition. Control V_m with 115 mM Cl^- was −33 mV. Periodic upward deflections in the voltage trace are described in the legend to Fig. 1.

with hyperosmotic stress (100 mM sucrose) in Cl^--free medium was comparable to that in Cl^--containing medium, Table I, as were the ratios of V_m^c/V_m^c.

There is considerable heterogeneity of hepatocyte V_m between animals reported herein and elsewhere [15]. This unexplained phenomenon impacts on comparison of results shown in Tables I and III, since the baseline V_m of the latter were greater. We, therefore, repeated these measurements with an experimental design utilizing multiple impalements. Effect on V_m of added 100 mM sucrose to Cl^--containing medium was first measured in different cells (three repetitions). The measurements were repeated in the same liver slice with a Cl^--free medium after the tissue had equilibrated with the gluconate medium for 20–30 min. Hyperosmotic stress by 100 mM sucrose in Cl^- medium decreased V_m from -42 ± 1.8 mV to -21 ± 2.6 mV ($P < 0.001$; n = three animals). The same hyperosmotic condition in gluconate medium decreased V_m from -45 ± 3.3 mV to -23 ± 3.2 mV ($P < 0.001$; n = 3 animals). Again, the absolute change in V_m with hyperosmotic stress (100 mM sucrose) in Cl^--free medium equaled that in Cl^--containing medium

Discussion

We have measured the effect on hepatocyte V_m of a pure osmotic stress created by adding sucrose to Krebs physiological salt medium. A previous report on the impermeability of hepatocyte plasma membrane to sucrose provided the basis for this design [16]. Our results demonstrated an inverse relationship between hepatocyte V_m and medium osmolality. These effects were not specifically due to sucrose because lactose had a similar effect. Thus, altering medium osmolality without altering ionic strength changes hepatocyte V_m.

This change in transmembrane potential represents a difference in electrochemical ionic equilibrium across the plasma membrane. The most predictable explanation for hyperosmotic stress-induced change in V_m is a passive phenomenon involving cell volume change. Here, we would expect cells to shrink, which would

TABLE III

Effect on hepatocyte transmembrane potential, V_m, of hyperosmotic stress (added sucrose, 100 mM) following chloride-free medium

V_m^c = hepatocyte transmembrane potential in control solution. V_m^{gluc} = hepatocyte steady-state transmembrane potential following replacement of all external Cl^- with gluconate. V_m^c = hepatocyte steady-state transmembrane potential in hyperosmotic solution created by adding 100 mM sucrose to chloride-free medium (gluconate substitution). All values are shown as averages ± S.E.; values for individual experiments were obtained from at least three separate, continuous measurements as shown in Fig. 6, whereas the mean values are averages for the values from each animal

Exper. No.	Control (V_m^c)	Cl^--free (V_m^{gluc}) **	Hyperosm$^{Cl\text{-free}}$ (V_m^c) **	Ratio (V_m^c / V_m^{gluc})	$\pi^{hyper}/\pi^{Cl\text{-free}}$
10-18-2	−46 ± 2	−40 ± 2	−19 ± 2	0.47 ± 0.02	1.40
10-18-3	−38 ± 4	−32 ± 7	−19 ± 5	0.58 ± 0.05	1.40
11-18-1	−37 ± 2	−25 ± 3	−12 ± 2	0.47 ± 0.01	1.40
Mean	−40 ± 3	−32 ± 4	−16 ± 2 *	0.51 ± 0.04	1.40

* Differs from V_m^{gluc}, $P < 0.05$; differs from V_m^c, $P < 0.005$.

** All steady-state values of V_m in Cl-free medium were corrected for artifactual electronegative potentials shifts caused by junction potentials formed at the agar bridge of the reference electrode when gluconate was substituted for Cl^- in the medium. The magnitude of these stable shifts in junction potentials was −6 mV as determined by Cl/gluconate substitution with glass microelectrode and agar bridge in the bath.

increase a^i_K and accordingly increase V_m, since diffusive forces for cell K^+ predominate among all the ions contributing to hepatocyte V_m [7,15]. Instead, we observed that hepatocyte V_m depolarized with hyperosmotic stress, which differs from the depolarization of V_m that accompanies hyposmotic stress in Ehrlich ascites tumor cells, Madin-Darby canine kidney cells and proximal tubule cells [17]. The polarity of the change in hepatocyte V_m with osmotic stress suggests adaptive regulatory mechanisms involving the plasma membrane in addition to changes in the K^+ equilibrium potential.

We postulated that membrane K^+ conductance, g_K, decreases during hyperosmotic stress and that this accounts for depolarization of V_m. To test this, the transference number for K^+ (t_K) was determined before and after hyperosmotic stress. The t_K value deceased from 0.20 ± 0.01 in isosmotic condition to 0.05 ± 0.01 in hyperosmotic condition. This means a 50 mOsm increase in medium osmolality resulted in a 4-fold decrease in the relative contribution of g_K to overall membrane conductance, g_{cell}, compared with that in isosmotic medium. This is consistent with our postulate that changes in membrane g_K account for osmotic stress-induced changes of hepatocyte V_m. In related studies of the rabbit proximal tubule, Welling and O'Neil reported that basolateral t_K increased from 0.16 to 0.34 on cell swelling due to hypotonic medium [18]. Donaldson and Lewis [19] reported a depolarization of basolateral V_m of rabbit urinary bladder immediately after hyperosmotic addition of mannitol to the serosal medium. This depolarization was attributable to decrease in Ba^{2+}-sensitive K^+ conductance. We have reported recently that extra- and intracellular Ca^{2+} may be involved in mechanisms controlling osmotic stress-induced changes in V_m and controlling cell volume [20]. This implies a role for Ca^{2+}-activated K^+ channels in these events; however, their functional existence in hepatocytes is not unequivocal. They are not in chicken hepatocytes [21] and negative findings were also reported in some studies on rat hepatocytes [22,23]. Others, however, have reported that they exist in rat hepatocytes [24] and in rabbit [22] and guinea-pig hepatocytes [25].

Our values for t_K were low considering that mouse hepatocyte E_K ranges from -85 to -90 mV (Ref. 15, and computed from data in Table I) Various explanations may account for this. Firstly, other specific ion conductances or nonspecific ion leak conductances may contribute to the total conductance of the plasma membrane. Secondly, depolarization by high external K^+ or the concentration shift alone may alter membrane g_K and the membrane conductances of other ions. Thirdly, diffusion delays in liver slices plus the complex lobular geometry may result in mixing and prohibit virtual step-changes in external K^+ concentration. Thus, increments of external K^+ may have been less than predicted and may have resulted in a protracted decreases in steady-state V_m, over a period of min, Fig. 5. The latter two situations might have underestimated the magnitude of t_K. Notwithstanding, the decrease in t_K with hyperosmotic stress provides an accurate accounting for the corresponding decrease in hepatocyte V_m. Values of t_K for control and hyperosmotic conditions resulted from measurements taken during continuous recordings in the same cell. Thus, any underestimation of t_K applied equally to both conditions.

Since t_K is a relative value, an increase of other ionic-current through the membrane could have resulted in an apparent decrease of t_K as well. Other contributing ions most likely are Na^+ and Cl^-, but we have excluded them based on our findings and those of others. Most Na^+ entering hepatocytes during hyperosmotic stress results from the basolateral, electroneutral Na-H exchange mechanism [26,27]. This alone would not alter V_m. The only change in V_m attributable to increases in Na^+ influx might be a small hyperpolarization of V_m secondary to stimulation of the electrogenic Na-K pump. Instead, we observed marked depolarization of V_m with hyperosmolality. This might result also from an increase in a^i_{Cl} with cell shrinkage, which would decrease E_{Cl} and thus contribute to hyperosmotic stress-induced depolarization of V_m. Hyperosmolality from addition of 100 mM sucrose increases a^i_{Cl} from 18 mM to 36 mM (Wang and Wondergem, unpublished observations). However, the effect on V_m of this shift in E_{Cl} seems minimal since equivalent depolarization of V_m with hyperosmotic stress occurred in cells equilibrated with either Cl-containing or Cl-free medium.

The physiologic significance of the change in V_m during osmotic stress seems related to the volume regulatory responses of hepatocytes [2]. For example, V_m decrease with hyperosmotic stress obviously facilitates the entrance of Cl^-, since transmembrane Cl^- distribution in hepatocytes is passive with respect to V_m [7,8,9]. Such Cl^- entry into hepatocytes may increase intracellular osmolality, resist cell shrinking, and thereby contribute to cell volume regulation during hyperosmotic stress.

Increases in a^i_K during hyperosmotic shock could result from combined events. The reduction in g_K will have the primary function of reducing exit of K^+ in order to maintain cell volume. In addition, activation of the Na^+-K^+ pump and redistribution of intracellular K^+ along with cell shrinkage during hyperosmotic stress could contribute to the increase in a^i_K. Isolated rat hepatocytes remained shrunken for at least 15 min following hypertonic shock [4]. Corasanti, et al. [26] reported that regulatory volume increases in isolated rat hepatocytes occurred only during hypertonic stress in cells previously swollen but did not occur following

hypertonic stress *per se*. This, however, does not preclude a physiological role for regulatory volume increases in hepatocytes, particularly that which operates under small increases in external osmolality [1,2]. Further experimentation is necessary to determine whether discrepancies of regulatory volume increases in hepatocytes results from differences in function of isolated cells in suspension compared with cells in situ or from differences among species.

In these experiments we assume rapid diffusion of sucrose throughout the extracellular space. This most likely is a valid assumption for the surface cells in which a_K^i was recorded, but this assumption may not hold true for cells in the center of the slice considering the greater distance. This may account for some discrepancy between the percent tissue water loss and the percent increase in a_K^i. The former ranged from 11 to 24%, and the latter ranged from 10 to 29% over the range of osmolalities studied. So there is fair agreement in the percent changes between these two measurements. Nonetheless, differences in diffusion time between cells on the surface of a liver slice compared with cells in the interior preclude exact comparisons of results obtained by different methods. This may explain the mismatch between the obvious increase in a_K^i and the very small decrease in tissue water content when hyperosmotic stress was increased from that of 100 mM added sucrose to that of 150 mM added sucrose.

In conclusion, osmotic stress to hepatocytes triggers adaptive changes in V_m. We have shown that hyperosmotic stress by added sucrose without altering ionic strength depolarizes V_m and accompanies decreases in tissue water and increases in a_K^i, consistent with cell shrinking. Corresponding decreases in membrane t_K and equal V_m depolarization in Cl-free and Cl-containing medium suggest that changes in membrane g_K account for shifts in hepatocyte V_m during osmotic stress instead of changes in transmembrane driving forces for either K^+ or Cl^-.

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