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An electrophysiological technique to measure change in hepatocyte water volume

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We have applied an electrophysiologic technique (Reuss, L. (1985) Proc. Natl. Acad. Sci. USA 82, 6014) to measure changes in steady-state hepatocyte volume during osmotic stress. Hepatocytes in mouse liver slices were loaded with tetramethylammonium ion (TMA^+) during transient exposure of cells to nystatin. Intracellular TMA^+ activity (a^i_{TMA}) was measured with TMA^+-sensitive, double-barrelled microelectrodes. Loading hepatocytes with TMA^+ did not change their membrane potential (V_m), and under steady-state conditions a^i_{TMA} remained constant over 4 min in a single impalement. Hyperosmotic solutions (50, 100 and 150 mM sucrose added to media) and hyposmotic solutions (sucrose in media reduced by 50 and 100 mM) increased and decreased a^i_{TMA}, respectively, which demonstrated transmembrane water movements. The slope of the plot of change in steady-state cell water volume, $[(a^i_{TMA})_0/(a^i_{TMA})_{4min}] - 1$, on the relative osmolality of media, (experimental mosmol/control mosmol) − 1, was less than predicted for a perfect osmometer. Corresponding measurements of V_m showed that its magnitude increased with hyposmolality and decreased with hyperosmolality. When Ba^{2+} (2 mM) was present during hyposmotic stress of 0.66×286 mosmol (control), cell water volume increased by a factor of 1.44 ± 0.02 compared with that of hyposmotic stress alone, which increased cell water volume by a factor of only 1.12 ± 0.02, $P < 0.001$. Ba^{2+} also decreased the hyperpolarization of hyposmotic stress from a factor of 1.62 ± 0.04 to 1.24 ± 0.09, $P < 0.01$. We conclude that hepatocytes partially regulate their steady-state volume during hypo- and hyperosmotic stress. However, volume regulation during hyposmotic stress diminished along with hyperpolarization of V_m in the presence of the K^+-channel blocker, Ba^{2+}. This shows that variation in V_m during osmotic stress provides an intercurrent, electromotive force for hepatocyte volume regulation.

Introduction

The dynamics of cell metabolism, membrane transport and cell secretion constantly change the content and concentration of intracellular organic solutes. Mechanisms for cell volume regulation compensate corresponding changes in intracellular osmotic pressure, and they fulfull an important homeostatic function of maintaining steady-state cell water balance. These mechanisms vary with cell type and according to requirements for either volume regulatory increases or decreases [1].

Cell volume regulation may be singularly important for the liver, which as a transporting epithelium performs various organism-sustaining functions that alter cell metabolism, transport and secretion [2,3]. It is clear that changes in functional demand on the liver require mechanisms for cell volume control [2–6]. However, little is known about mechanisms involved in hepatocyte volume regulation.

We demonstrated recently that the hepatocyte transmembrane potential (V_m) varies with osmotic stress imposed by altering the ion concentration of the external medium, and V_m responds like an osmometer [2]. These shifts in V_m were linked to osmotically-sensitive variations in membrane K^+ conductance (Refs. 2,3; Wang and Wondergem, unpublished observations). Nonetheless, we do not know if these osmotic variations in V_m played any role in cell volume control.

We conducted the present study to determine whether variation in V_m with osmotic stress plays a role in hepatocyte volume regulation. To accomplish this we have adopted an electrophysiological technique to measure change in hepatocyte water volume [7,8]. Our results show that variations in hepatocyte V_m provide an intercurrent, electromotive force for control of hepato-

Abbreviation: TMA^+, tetramethylammonium ion.

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cyte volume. We postulate that the change in V_m is involved in hepatocyte volume regulation mediated by shifts in intracellular Cl^-.

Materials and Methods

Liver slice preparation, maintenance and temperature control

Adult, male mice of ICR strain were fasted 18–24 h before experiments, because variation in hepatocyte V_m between fasted and fed animals has been reported [9]. Mice were killed by cervical dislocation, and the left-lateral or median lobe of the liver was removed quickly and placed on gauze moistened with 0.9% NaCl. A glass microscope slide was pressed gently onto the lobe to keep it from moving while it was sliced using a razor blade clamped in a hemostat. A slice of about 1 mm thickness and of about 5×5 mm surface area was placed into a tissue slice chamber (BSC-HT, Medical System Co., Greenvale, NY). The liver slice was held in place by a small steel washer to insure that microelectrode impalements were always of cells on the encapsulated, uncut surface of the slice. Temperature of the liver slice was maintained at $37 \pm 0.3°C$, because changes in temperature alter hepatocyte V_m [10].

Solutions and control of osmolality

Physiological salt solution comprised (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, 103 sucrose, 4.9 sodium pyruvate, 4.9 sodium glutamate and 2.7 sodium fumarate and was equilibrated with 95% O_2/5% CO_2. Hypo- and hyperosmotic solutiuons for inducing osmotic stress were prepared by reducing or increasing medium sucrose concentrations, respectively. In some experiments medium osmolality was altered by changing the NaCl concentration. Here, the control solution contained 103 mM NaCl without sucrose. The osmolality of the solutions was measured before the experiments with an osmometer based on the principle of freezing point depression (Precision Systems, Natick, MA).

Loading hepatocytes with TMA^+

TMA^+-containing solution was made by replacing 20–50 mM of NaCl in medium with an isomolar concentration of TMA chloride. Nystatin (Sigma) at 45–180 U/ml or amphotericin B (Sigma) at 20 μM was dissolved in 0.08% dimethyl sulfoxide (DMSO) and was added to the TMA^+ solution. DMSO at concentrations used in these experiments has no effect on electrophysiology in epithelia [11]. Nonetheless, we added equal amounts of DMSO to control and experimental solutions. Liver slices were exposed to nystatin plus TMA^+ for 8–10 min to load hepatocytes with TMA^+. Then nystatin was washed from the slices for 40–60 min with TMA^+-containing solution.

Construction of 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 3 drops of liquid detergent (Liquinox) [12]. These were rinsed for 1 h in tap wayer, boiled again for 15 min in distilled water, and finally dried overnight at 90°C in a forced-air oven. Single-barrelled microelectrodes were drawn from these capillaries using a horizontal puller (Industrial Sci. Assoc., Ridgewood, NY). The microelectrodes were filled with 0.5 M KCl and had tip resistances in physiological medium of 10–30 MΩ and tip diameters of about 0.5 μm (Wang and Wondergem, unpublished observations by scanning electronmicroscopy). The microelectrodes were connected by Ag/AgCl half-cells to a high input impedance ($> 10^{14}$ Ω) preamplifier with unit gain (515L Analogue Devices, Norwood, MA). Reference electrodes consisted of a Ag/AgCl half-cell connected to the tissue chamber by an agar (4% in physiological medium) bridge. Voltages were recorded by a digital voltmeter (Keithly), an oscilloscope (Tektronix) and a rectilinear chart recorder.

Double-barrelled ion-selective microelectrodes were prepared from fiber-filled, borosilicate double capillaries (1.2 mm o.d., A-M Systems, Everett, WA), which were cleaned as described above. These were pulled by using a vertical puller (700D, David Kopf Instruments, Tujunga, CA), and the tip diameters were about 1 μm (Wang and Wondergem, unpublished measurements by scanning electronmicroscopy). K^+-selective exchanger (Corning 477317, Corning Medical, Medfield, MA) was used to determine the intracellular TMA^+ activity (a^i_{TMA}). This exchanger is more selective to quaternary ammonium ions such as TMA^+ than to K^+, and it calibrates as a Nernstian electrode for these ions [13]. Microelectrode tips were made hydrophobic by filling one barrel with a 1 mm column of hexamethyldisilazane (HMDS, Sigma). The reference barrel was filled first with a 1 mm column of distilled water. These were dried either by a hot air blower for at least 60 min or for 12 h at 110°C in a forced-air oven. The tip of the silanized barrel was filled with a 1 mm column of K^+-exchanger and was backfilled with 0.5 M KCl. The reference barrel was filled with 1 M sodium formate.

Calibration of TMA^+-selective microelectrode

TMA^+-selective microelectrodes were calibrated at room temperature in TMA^+ solutions whose concentrations covered the expected range of a^i_{TMA} for hepatocytes loaded with TMA^+. These solutions comprised 25, 10, 2.5 and 1 mM TMA chloride, plus 125 mM KCl in each solution. TMA^+ activities in these calibration solutions were computed according to the Debye-Hückel

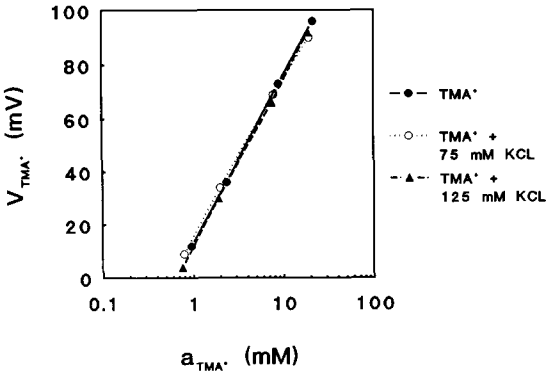

Fig. 1. Effect of K^+ on the calibration of a TMA^+-sensitive microelectrode. Voltage of the TMA^+-sensitive microelectrode is plotted vs. $\log_{10}$ of TMA^+ activity. The calibration plots are those obtained from one double-barrelled TMA^+-sensitive microelectrode in the presence of 0, 75, or 125 mM KCl. The average slope of the three plots is 61 ± 1.4 mV/10-fold change in a_{TMA^+}.

equation as modified by Armstrong et al. [14] and according to data of Conway [15]. The average slope of electrode voltage vs. TMA^+ for all experiments was 64 ± 2.1 (S.E., $n = 23$). Intracellular TMA^+ activity was computed by direct interpolation solving for:

$$a^i_{TMA} = 10^{(V_{TMA} - V_0 - V_m)/S} \quad (1)$$

where a^i_{TMA} = intracellular TMA^+ activity, V_{TMA} = intracellular voltage of the TMA^+-selective microelectrode. V_0 = voltage at the intercept of the calibration curve, V_m = transmembrane potential, S = slope of the calibration curve. Representative calibration curves for a TMA^+ electrode demonstrated the electrode's selectivity for TMA^+ over K^+, Fig. 1.

Criteria for valid impalements with open-tip or TMA^+-selective microelectrodes were: (1) rapid deplection of the voltage trace on advancing a microelectrode into the tissue; (2) an intracellular voltage recording that was stable within 2 mV for at least 10 s; (3) return of the voltage trace to within 5 mV of baseline when a microelectrode was withdrawn; and (4) resting V_m for control measurements above -20 mV.

Criteria for effective loading and measurement of TMA^+ in hepatocytes

An electrophysiological technique to measure change in cell volume [7,8] requires that certain criteria be met. For application to the liver we established that: (1) nystatin must form a non-selective cation pore in the hepatocyte plasma membrane to load cells with TMA^+, which must be reversible; (2) loading hepatocytes with TMA^+ must not permanently alter transmembrane voltage (V_m) of the hepatocyte plasma membrane; and (3) the a^i_{TMA} in hepatocytes loaded with TMA^+ must remain constant for a minimum period to measure accurately changes in a^i_{TMA} due to transmembrane water flux. This minimum period proved to be 4 min (see Results).

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 and co-workers [8] to be:

$$\Delta V_t = V_t - V_0 = V_0[(a^i_{TMA})_0/(a^i_{TMA})_t - 1] \quad (2)$$

where ΔV_t = 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 $2n$ to $16n$ [16]. Consequently, we did not assign an initial cell volume

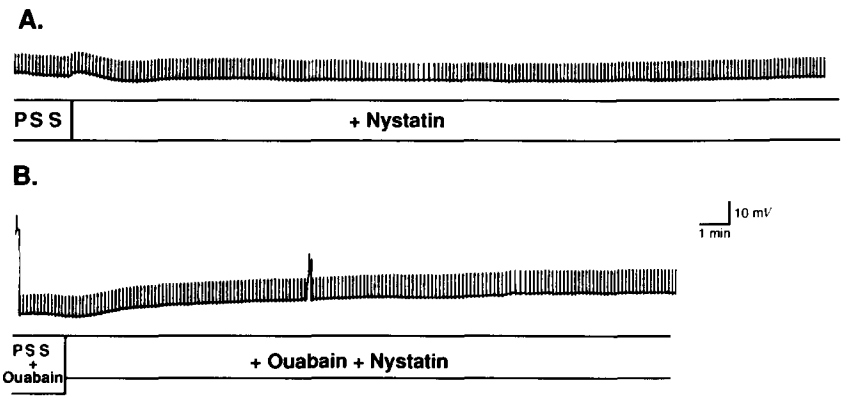

Fig. 2. Effect of nystatin and ouabain on hepatocyte transmembrane potential (V_m). (A) Effect of nystatin (270 U/ml) on hepatocyte V_m. Hepatocyte V_m in physiologic salt solution (PSS) was -30 mV. (B) Effect of nystatin (180 U/ml) on hepatocyte V_m measured after addition of ouabain (1 mM for 15 min). V_m was -35 mV when nystatin was added with ouabain already present. It decreased to -24 mV 20 min after addition of nystatin. After this impalement, ouabain and nystatin were washed out with PSS for 15 min, and the V_m after wash was -32 ± 0.7 mV ($n = 6$). Time-voltage calibration applies to all traces. Rapid downward deflection at the beginning of trace B indicates microelectrode impalement of the cell. Intermittent upward voltage deflection throughout result from 0.5 nA of constant current to assess microelectrode input resistance.

to serve as a reference for all measurements and comparisons. Instead, we chose to compare the ratios of $(a^i_{TMA})_0/(a^i_{TMA})_{4min}$ measured under control conditions with ratios obtained under experimental conditions, where $(a^i_{TMA})_0$ = intracellular TMA^+ activity at time zero, and $(a^i_{TMA})_{4min}$ = intracellular TMA^+ activity 4 min after onset of osmotic stress. 4 min proved to be the maximum period that a^i_{TMA} remained constant without decrement during a single, continuous measurement. Paired measurements were made in the same liver slices. Results are expressed as mean ± S.E. Analysis of variance was applied to test significant differences, $P < 0.05$. Linear regression and correlation coefficients were determined by the least-squares computation.

Results

Effect of nystatin and amphotericin B on hepatocyte V_m

Hepatocyte V_m results principally from selective conductance of the sinusoidal plasma membrane to K^+ [10,17]. Thus, we expected pore-forming antibiotics to increase non-selective, cation conductance through the membrane and decrease hepatocyte V_m. However, neither nystatin at 45–270 U/ml (Fig. 2A) nor amphotericin B at 20 μM (not shown) changed hepatocyte V_m. Either these agents had no effect on the cells, or some compensatory mechanism maintained V_m notwithstanding insertion of non-selective cation pores into the membrane.

We surmised that an electrogenic Na^+-K^+ pump in hepatocyte plasma membrane [17] may have compensated the depolarizing effects of polyene antibiotics. To test this, liver slices were superfused with ouabain (1 mM) for a minimum of 10 min to allow ouabain binding. Then nystatin was added at 180 U/ml. The results in Fig. 2B show that nystatin plus ouabain decreased

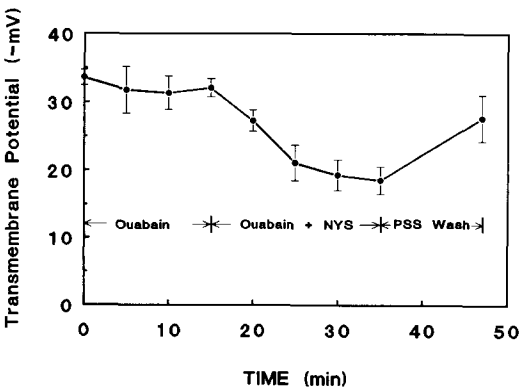

Fig. 3. Effect of nystatin (NYS; 180 U/ml) on hepatocyte V_m in the presence of ouabain (1 mM). Data are averages of V_m obtained from four continuous impalements, each in different slices from four mice ($n = 4$). The combination of ouabain and nystatin was washed out with physiologic salt solution (PSS).

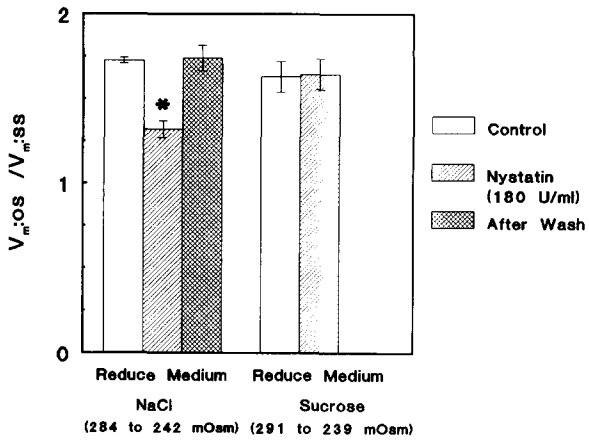

Fig. 4. Effect of nystatin (180 U/ml) on hyposmotic stress-induced increases of hepatocyte V_m in mouse liver slices. Hyposmotic conditions result either from reducing external NaCl concentration to lower external osmolality from 284 to 242 mosmol, or by reducing external sucrose concentration to lower external osmolality from 291 to 239 mosmol. V_m:ss is the steady state V_m before osmotic stress, and V_m:os is the maximum V_m after osmotic stress. Control and added nystatin (mean ± S.E.) were obtained as paired measurements in each of four liver slices for reduced NaCl and for reduced sucrose. Washout of nystatin were done in only three liver slices. * Significant difference between control and nystatin treatment, $P < 0.001$.

hepatocyte V_m. Results from four liver slices (Fig. 3) demonstrate the depolarizing effect of ouabain plus nystatin, and its reversibility. To rule out that the depolarizing effects of ouabain plus nystatin resulted from a direct effect of ouabain on transmembrane ion gradients, we exposed the hepatocytes to ouabain (1 mM) for 30–40 min during continuous recording of V_m. Here, ouabain reduced V_m by only 4 mV after 40 min, from -30 ± 0.4 mV to -26 ± 1.8 mV, $n = 4$.

Effect of nystatin on the increase in hepatocyte V_m during hyposmotic stress

Howard and Wondergem [2] reported that hepatocyte V_m increases substantially during hyposmotic stress, and this results primarily from an increase in membrane K^+ conductance. By utilizing this phenomenon, we were able to establish further the effectiveness of nystatin's capability to form pores in the hepatocyte plasma membrane. Nystatin added to cells forms single-length, monocation-selective channels in the plasma membrane [18]. Thus, we predicted that with added nystatin osmotic stress induced by altering external NaCl concentration may affect V_m to lesser degree than that induced by altering external sucrose. Nystatin partially inhibited the increase in V_m during hyposmotic stress due to lowering the external NaCl concentration, and this effect was reversible, Fig. 4. However, nystatin did not inhibit this increase in V_m if hyposmotic stress resulted from lowering external sucrose concentration, Fig. 4.

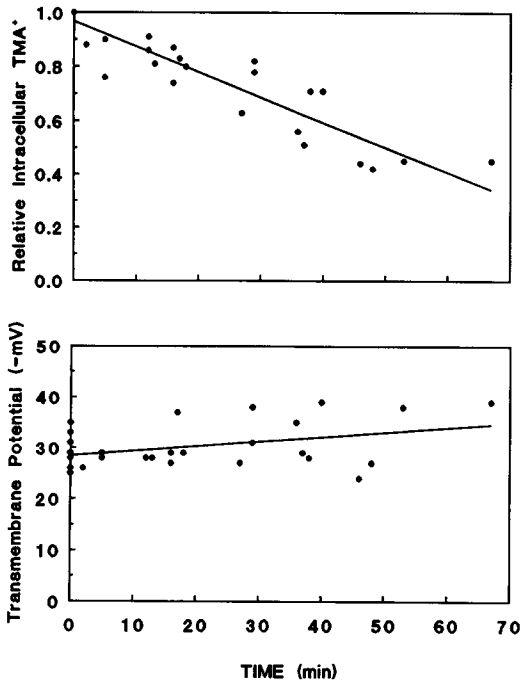

Fig. 5. Hepatocyte TMA^+ activity and transmembrane potential (V_m) plotted vs. time. Data were obtained with double-barrelled microelectrodes from 29 cells in eight liver slices. (Upper) Hepatocyte relative intracellular TMA^+ (ratio of intracellular TMA^+ activity at time x/intracellular TMA^+ activity of first impalement in liver slice at time zero) vs. time. Regression coefficient (b) = -0.009 ± 0.001/min, $P < 0.001$; correlation coefficient (r) = -0.92. (Lower) Hepatocyte transmembrane potential (V_m from the same impalements above) vs. time. $b = 0.09 \pm 0.04$ mV/min, $P < 0.05$; $r = 0.40$.

Time-dependent decrease in a^i_{TMA} in TMA^+-loaded hepatocytes

Average a^i_{TMA} immediately after loading hepatocytes in liver slices was 22 ± 2 mM ($n = 28$ measurements in 10 liver slices) and ranged for all measurements from 4 to 46 mM. For ten liver slices the average maximum value for $a^i_{TMA} = 23 \pm 4$ mM and the average minimum value for $a^i_{TMA} = 16 \pm 4$ mM. The large range for control values of a^i_{TMA}, along with polyploid mouse hepatocytes [16] with various normal volumes, prompted us to compare ratios of experimental a^i_{TMA} and control a^i_{TMA} obtained from different cells or slices.

Intracellular TMA^+ activity (a^i_{TMA}) remains constant over 3 h in TMA^+-loaded epithelial cells of *Necturus* gallbladder [7]. In contrast, hepatocyte a^i_{TMA} decreased steadily when measured by serial sampling of different cells over 1 h, Fig. 5. Paired measurements of V_m by the double-barrelled microelectrode showed that V_m did not decrease but increased slightly during this same period, Fig. 5. This suggests that the time-related decrease in a^i_{TMA} did not result from ions leaking from the cell around the microelectrode. However, a^i_{TMA} always remained constant for at least 4 min, and in some cases up to 11 min, when a^i_{TMA} was measured continuously in single cells (not shown). Consequently, measurements of a^i_{TMA} to asses changes in hepatocyte volume (see below) were taken within 4 min after osmotic stress.

To exclude further the possibility that decrement of a^i_{TMA} with time resulted from TMA^+ leaking around the microelectrode at the impalement site, we first measured the time-course of TMA^+ electrode voltage changes on addition of 1 mM TMA^+ to the bath with electrodes positioned in the medium above the tissue, Fig. 6A. This was repeated with addition of 5 mM TMA-Cl (substituted for 5 mM NaCl) after a double-barrelled microelectrode impaled the cell, Fig. 6B. The latter demonstrated no change in a^i_{TMA} for up to 20

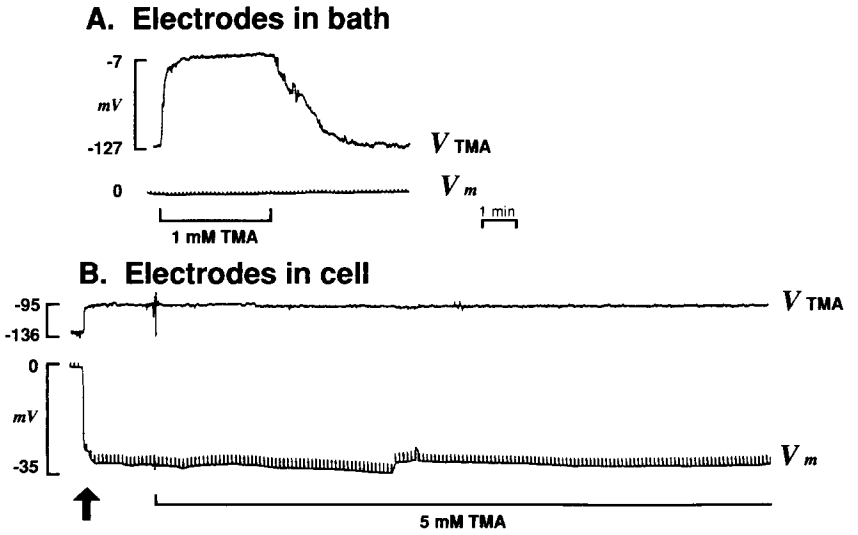

Fig. 6. (A) Effect on TMA^+ electrode voltage (V_{TMA}) and on reference electrode voltage (V_m) of superfusing liver slice with 1 mM TMA-Cl with electrodes in the bath above the liver slice. (B) Effect on TMA^+ electrode voltage (V_{TMA}) and on reference electrode voltage (V_m) of superfusing liver slice with 5 mM TMA^+ with electrodes in a cell. Arrow indicates time of impalement. Time base applies to all traces. Intermittent upward voltage deflections throughout recordings result from 0.5 nA of constant current through the open-tip (reference) microelectrode to assess its input resistance. Apparent shift in V_m is balance voltage adjustment.

TABLE I

Effect of loading hepatocytes with TMA^+ on hepatocyte transmembrane potential (V_m) and on the increase in V_m during hyposmotic stress

Effect on V_m shows averages from four liver slices ($n = 4$ animals) representing 20 impalements (8 control: before loading with TMA^+; 12 experimental: after loading with TMA^+). Effect on V_m during hyposmotic stress shows averages from three liver slices ($n = 3$ animals) representing 12 impalements (5 control; 7 experimental). Control osmolality was 284 mosmol and experimental osmolality was 202 mosmol. V_m: os = maximum value of V_m during osmotic stress. V_m: ss = steady-state V_m. n.s., not significant.

Variable	Before loading with TMA^+	After loading with TMA^+
V_m (mV)	-30 ± 1	-31 ± 3 n.s.
V_m: os/V_m: ss	1.97 ± 0.13	1.99 ± 0.23 n.s.

min, which excluded passive entry of TMA^+ around the microelectrode.

Effect of loading the hepatocytes with TMA^+ on V_m and the increase in V_m during hyposmotic stress

Loading hepatocytes with TMA^+ had no effect on V_m or on the increase in V_m during hyposmotic stress, Table I. This indicated that the loading procedure had no permanent effects on either cell viability or on the plasma membrane's relative ion permeabilities as indicated by the V_m and its increase with hyposmotic stress.

Effect of osmotic stress on a^i_{TMA} and V_m in hepatocytes loaded with TMA^+

Osmotic stress on hepatocytes was achieved by altering external sucrose concentrations, and the accompanying changes in V_m and a^i_{TMA} were measured with double-barreled microelectrodes, Fig. 7. The a^i_{TMA} was directly proportional to the differential voltage of the ion-selective (V_{TMA}) and open-tip (V_m) electrodes (Eqn. 1, above). Hyposmotic stress decreased a^i_{TMA}, which indicated an increase in cell water content and implied swelling of hepatocytes (Fig. 7A). Conversely, hyperosmotic solutions increased a^i_{TMA}, which showed a decrease in cell water and implied cell shrinkage, Fig. 7B. Increases and decreases in hepatocyte V_m also accompanied hypo- and hyperosmotic stress, respectively, Fig. 7A and B, and these were similar to those reported by Howard and Wondergem [2].

Variation in V_m and a^i_{TMA} with external osmolality

To determine whether volume regulation occurs in hepatocytes in liver slices, we plotted the change in steady-state cell water volume, $[(a^i_{TMA})_0/(a^i_{TMA})_{4min}] - 1$, as a function of relative osmolality ((experimental mosmol/control mosmol) $- 1$; Fig. 8, upper. Fitting these data to a linear function gave a regression coefficient of -0.34 ± 0.03 ($P < 0.001$), which showed significant change in volume with external osmolality. However, these points did not fit the plot predicted for

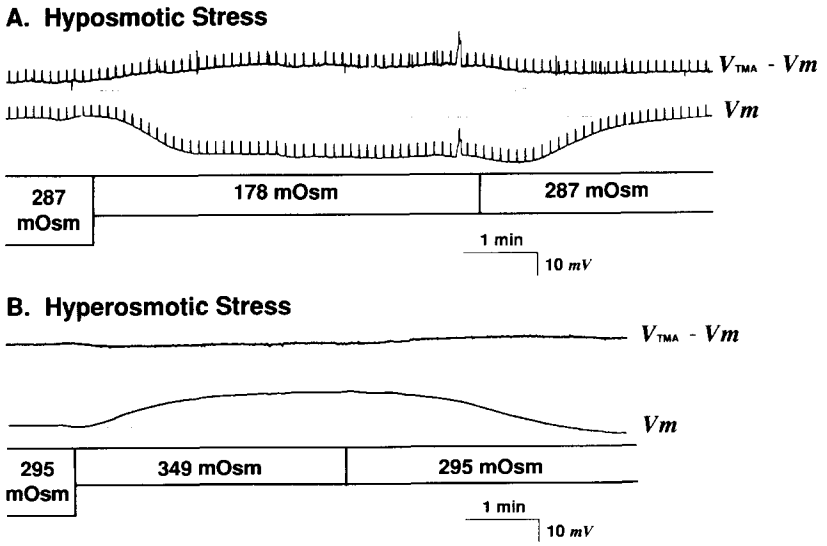

Fig. 7. Effects of osmotic stress on intracellular TMA^+ activity and transmembrane potential (V_m) of hepatocytes loaded with TMA^+. (A) Effect of hyposmotic stress. V_m was -25 mV and increased during hyposmotic stress to -43 mV. The a^i_{TMA} was 42 mM and decreased within 3 min during hyposmotic stress to 36 mM. Control a^i_{TMA}/experimental $a^i_{TMA} = 1.17$. Relative osmolality: experimental osmol/control osmol = 0.62. (B) Effect of hyperosmotic stress. V_m was -34 mV and decreased during hyperosmotic stress to -27 mV. The a^i_{TMA} was 15 mM and increased without 3 min during hyperosmotic stress to 16 mM. Control a^i_{TMA}/experimental $a^i_{TMA} = 0.94$. Relative osmolality = 1.2. Time and voltage calibration applies to both traces in A and B. Downward is greater electronegativity for traces of V_m. Downward is more electropositive for traces of $V_{TMA} - V_m$. Intermittent upward voltage deflections throughout some recordings result from 0.5 nA of constant current through the open-tip microelectrode to assess its input resistance.

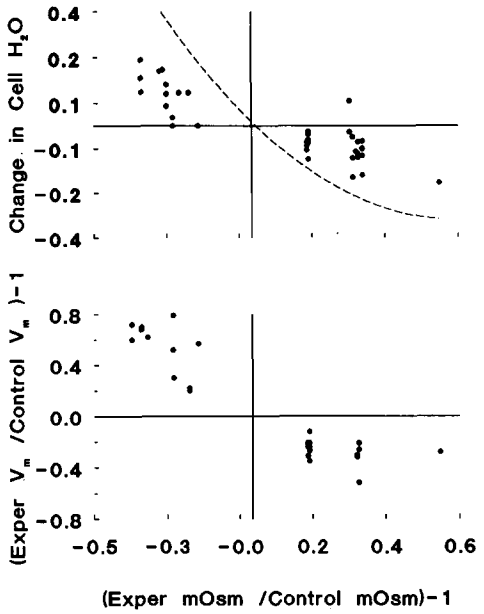

Fig. 8. Effects of osmotic stress on change in cell water and transmembrane potential (V_m) of hepatocytes loaded with TMA^+. (Upper) Change cell water volume, $[(a^i_{TMA})_0/(a^i_{TMA})_{4min}]-1$, vs. relative osmolality, [(experimental mosmol/control mosmol)]−1. Dashed line shows predicted osmometric response of the cells to changes in external osmolality, [(control mosmol)/(experimental mosmol)]−1 vs. [(experimental mosmol/control mosmol)]−1. (Lower) (Experimental V_m/control V_m)−1 vs. relative osmolality. Data were obtained from 39 recordings as shown in Fig. 7 representing 30 impalements from 13 liver slices. The average a^i_{TMA} and V_m in control PSS were 18±1.8 mM and −32±0.9 mV, respectively. Osmolalities of PSS were obtained by altering external sucrose concentrations, and they ranged from 178 to 459 mosmol.

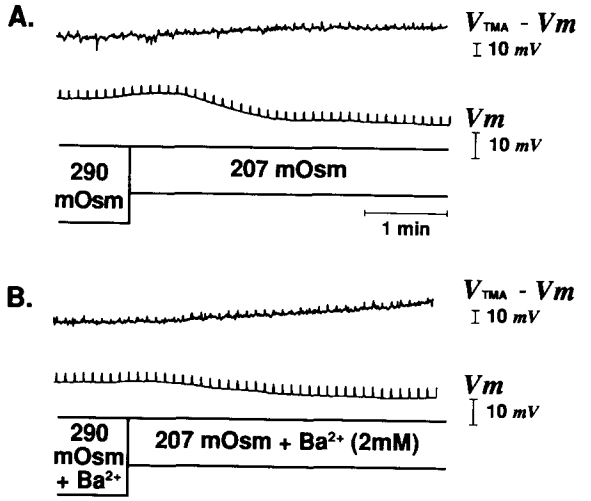

Fig. 9. Effects of added Ba^{2+} on intracellular TMA^+ activity and transmembrane potential (V_m) during osmotic stress of hepatocytes loaded with TMA^+. (A) Effects of hyposmotic stress under control conditions. V_m was −24 mV and increased during hyposmotic stress to −36 mV. The a^i_{TMA} was 22 mM and decreased within 3 min during hyposmotic stress to 19 mM. Control a^i_{TMA}/experimental a^i_{TMA} = 1.16. Relative osmolality: Experimental mosmolality/control mosmolality = 0.71. (B) Effect of hyposmotic stress with Ba^{2+} added at 2 mM. V_m was −31 mV and increased during hyposmotic stress to −34 mV. The a^i_{TMA} was 21 mM and decreased within 3 min during hyposmotic stress to 14 mM. Control a^i_{TMA}/experimental a^i_{TMA} = 1.50. Relative osmolality = 0.71. Time and voltage calibration applies to both traces in A and B. Downward is greater electronegativity for traces of V_m. Downward is more electropositive for traces of $V_{TMA}-V_m$. Intermittent upward voltage deflections throughout recordings result from 0.5 nA of constant current through the open-tip microelectrode to assess its input resistance.

a perfect osmometer (Fig. 8, dashed line). We assume here that TMA^+ distributes evenly throughout the total cell water and that changes in a^i_{TMA} reflect changes in cell water volume. These results indicate that either the hepatocyte membrane is not impermeant to sucrose or that the cells are capable of partially regulating their volume, in spite of the osmotic perturbations.

Howard and Wondergem [2] demonstrated that hepatocyte V_m behaves as an osmometer and varies with osmotic stress due to alterations in external NaCl concentration. The present V_m changes taken as paired measurements from the double-barreled TMA-electrode are shown in Fig. 8 (lower), and they demonstrate this characteristic of hepatocyte V_m also occurs with osmotic stress due to changes in external sucrose concentration.

Involvement of hepatocyte V_m in cell volume regulation

The physiologic significance of changes in hepatocyte V_m during osmotic stress is unknown. However, it may be linked to cell volume regulation, either as a transducer to signal volume change or as an electromotive force to redistribute osmotically active anions across the plasma membrane. As an initial step to explore these possibilities, we asked whether Ba^{2+} (2 mM), a known blocker of K^+ channels, affects both V_m and a^i_{TMA} during hyposmotic stress. Representative recordings taken from the same liver slice are shown in Fig. 9A and B. A decrease in external osmolality by about 30% increased V_m and decreased the differential voltage of $V_{TMA}-V_m$ (Fig. 9A). These voltage changes reversed when control conditions were restored (not shown). The measurements were repeated in the presence of Ba^{2+} (Fig. 9B). Now, during hyposmotic stress the increase in

TABLE II

Effect of $BaCl_2$ (2 mM) on hyposmotic stress-induced increases in hepatocyte transmembrane potential (V_m) and increases in hepatocyte water volume as measured by change in intracellular TMA^+ activity

Results are averages from four liver slices (n = 4 animals) representing 11 impalements (6 control and 5 experimental).

Hyposmotic stress (286 to 190 mosmol)	Relative increase in V_m (V_m: 4 min/V_m: 0)	Relative increase in cell water volume $((a^i_{TMA})_0/(a^i_{TMA})_{4min})$
Control	1.62±0.04	1.18±0.02
Added Ba^{2+}	1.24±0.09 *	1.44±0.02 **

* Differs from control, $P < 0.01$; ** Differs from control, $P < 0.001$.

V_m diminished, but the decrease in the differential voltage was much greater than under the control condition. This indicated a greater increase in cell water volume. Results obtained from four liver slices are summarized in Table II. Hyposmotic stress of 0.66-times the control osmolality to hepatocytes in liver slices hyperpolarized V_m by a factor of 1.62. In the same cells, cell water volume increased by a factor of 1.18, which indicated a 18% increase in cell water volume. If Ba^{2+} was present at 2 mM in both control and hyposmotic solutions, V_m hyperpolarized by only 1.24, which was significantly less than that under control conditions. Furthermore, addition of Ba^{2+} resulted in an increase in cell water volume by a factor of 1.44, which indicated a 44% increase in cell water volume. This was significantly greater than that measured in cells under control conditions, $P < 0.001$.

Discussion

Volume regulation in hepatocytes has been linked to increases in membrane K^+ permeability and K^+ efflux [2,3,5,19,20]. Ba^{2+} blocks conductance of various K^+-channels, depolarizes hepatocytes [10], and disrupts their volume regulation during hypotonicity [4,5,20]. Our results showing difference in changes of V_m and a^i_{TMA} in control compared with Ba^{2+}-treated cells further demonstrate that changes in V_m are linked to osmotically induced changes in membrane K^+ permeability. To the best of our knowledge, these findings also are the first to implicate that changes in V_m due to osmotic stress play a direct role in cell volume regulation.

We postulate that changes in V_m with osmotic stress provide an electromotive force that redistributes osmotically active ions, such as Cl^-. Cl^- distributes passively with V_m in hepatocytes as determined by Cl^--sensitive microelectrodes [17,21,22]. Graf et al. [17] used current-clamp techniques in hepatocyte cultures to demonstrate that changes in intracellular Cl^- activity result directly from changes in V_m. Correspondingly, change in hepatocyte V_m with osmotic stress quickly alters the intracellular Cl^- activity [3]. Depolarization of V_m, which accompanies hyperosmotic stress, increases intracellular Cl^-; whereas hyperpolarization of V_m which accompanies hyposmotic stress, decreases intracellular Cl^-. Transmembrane fluxes of cations, either Na^+ or K^+, can follow by separate channels to maintain bulk electroneutrality within the cells. This exchangeable Cl^- pool provides a means for rapid volume regulation by hepatocytes, which may be physiologically important during metabolic variation in the concentrations of intracellular organic solutes, such as amino acids, glucose, or bile salts.

Our results also show that hepatocytes in mouse liver slices regulate their volume during osmotic perturbations of the extracellular media. The data in Fig. 8 do not fit the plot predicted for a perfect osmometer, which indicates that net transmembrane water movement in hepatocytes is less than predicted by the change in osmotic pressure. Alpini et al. [23] showed that sucrose does not permeate the hepatocyte plasma membrane. Thus, we infer a reflection coefficient of unity for sucrose and the hepatocyte membrane. A sucrose-impermeant hepatocyte plasma membrane alone is inconsistent with data not fitting the predicted curve in fig. 8.

Other studies demonstrate that hepatocytes regulate their volume, particularly during cell swelling [4,5,19]. Hepatocyte swelling can occur after water intake [24] or during changes in intracellular concentrations of organic solutes, such as amino acids [6,19,25]. Changes in a^i_{TMA} with time during osmotic stress show that volume regulation is concurrent with the osmotic imbalance instead of following initial increases or decreases in cell volume. Thus, volume regulatory decreases or increases, which are evident in many cells in suspension including rat hepatocytes [19,20] do not occur in mouse liver slices. This could reflect different regulatory capabilities of cells in situ compared with hepatocytes in suspension. Here, extracellular matrix in concert with the cell cytoskeleton may dampen sudden changes in cell volume. Also, 4-min time limits on the present measurements may not be long enough to record significant volume regulatory events. Finally, TMA^+ may alter the time-course for cell volume regulation, as might mixing and diffusion delays for media changes in liver slices. We have yet to explore these possibilities.

Changes in intracellular activity of membrane impermeant foreign ions such as TMA^+ have been used to measure volume change in the epithelial cells of gallbladder [7,8]. In applying the TMA^+ technique to measure change in liver cell volume, we established and met specific criteria (see Methods and Results) regarding: (1) effectiveness of nystatin for loading hepatocytes with TMA^+; (2) stability and integrity of cells loaded with TMA^+; and (3) finite duration for constant a^i_{TMA} with respect to possible degredation or transport from the cells.

Some quirks encountered in attempting to meet these criteria were unexpected. Nystatin, for example, has no immediate effect on hepatocyte V_m. In contrast, nystatin caused a large V_m depolarization on the luminal side of epithelial cells in *Necturus* gallbladder [7,26], and similar effects occurred in urinary bladder epithelium [27], and in fungal cells [28]. Nonetheless, nystatin depolarized hepatocytes when added to cells whose Na^+-K^+ pump had been inhibited by ouabain (1 mM). We conclude that an electrogenic Na^+-K^+ pump in hepatocytes compensates the depolarizing effect of the non-selective cation leak by nystatin, which perhaps is activated by nystatin- or amphotericin-induced rise in intracellular Na^+ activity. Activation of Na^+-K^+ pump by polyene antibiotics also occurs in frog skin [29]. This

may be by primary activation resulting from conformational changes that occur in the presence of polyenes in the cell membrane, or it may be a secondary activation by increasing Na^+ influx. Direct measurements of intracellular Na^+ activity (a^i_{Na}) or substituting external Na^+ with lithium during exposure to nystatin will be required to distinguish between these possibilities.

Reuss [7] found that a^i_{TMA} in epithelial cells of *Necturus* gallbladder loaded with TMA^+ was constant and did not change appreciably over a 3 h period. Results of our measurements in hepatocytes showed that a^i_{TMA} decreased by 60% over 1 h. The liver is an important organ for detoxification of many compounds, and it may degrade TMA^+. Previous studies showed that TMA^+ does not permeate epithelial cells of gallbladder [7] or human and sheep erythrocytes [30]. However, hepatic transport and biliary excretion of organic cations like quaternary ammonium compounds are well known [31]. The time-course for decrease in hepatocyte a^i_{TMA} is consistent with a membrane carrier for quaternary ammonium. However, our results show a^i_{TMA} remains relatively constant for 10 min and does not decrease significantly within 4 min. This poses a 4 min time limit within which this technique can be used to measure changes in hepatocyte volume.

An increase in hepatocyte V_m during hyposmotic stress results in part from an increase in membrane K^+ conductance [2,3]. Inhibition by nystatin of this increase in V_m during hyposmotic stress due to reduction of external NaCl is consistent with the prediction that nystatin forms a membrane pore that acts as a non-selective cation conductance [18]. There are two possible explanations here. First, the non-selective cation leak may have diminished the effective decrease in osmolality as measured by reduction of NaCl. The presence of nystatin in the membrane increases the transmembrane Na^+ leak, and some transmembrane ion equilibration may have already occurred prior to osmotic stress. Second, by similar reasoning nystatin alone effectively increases membrane cation permeability, which results in cell swelling [32]. Thus, further swelling during subsequent hyposmotic stress is limited and corresponding increases in V_m are less, Fig. 4. In contrast, sucrose does not permeate the nystatin pore, and its presence reduces cell swelling due to nystatin alone [32]. Subsequent hyposmotic stress results in complete swelling and corresponding increases in V_m. We also show reversibility of nystatin's inhibition of swelling-induced increases in V_m. This shows that there is no long-term effect of nystatin that might interfere with volume measurements, and it demonstrates that nystatin can be removed from the hepatocyte cell membrane. This is consistent with previous observations of Holz [33].

In conclusion, the onset of osmotic stress to hepatocytes triggers adaptive changes in the V_m. We have demonstrated, by inhibiting change in V_m with the K^+ channel blocker, Ba^{2+}, that variation in V_m is important for the control of hepatocyte volume. Here, we postulate that the change in V_m alters the transmembrane Cl^- gradient, since hepatocyte Cl^- distributes passively according to V_m. Thus, the intracellular Cl^- constitutes part of an exchangeable pool of osmolytes participating in cell volume regulation. Moreover, we have demonstrated the utility of an electrophysiological method to measure change in cell water volume. This depended on the effective loading of hepatocytes with TMA^+ using nystatin, a non-selective, membrane cation pore.

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