Effects of osmotic shrinkage on voltage-gated Ca\textsuperscript{2+} channel currents in rat anterior pituitary cells

Shlomo Ben-Tabou De-Leon, Galia Ben-Zeev, and Itzhak Nussinovitch

Department of Anatomy and Cell Biology, Hebrew University, Hadassah Medical School, Jerusalem, Israel

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Ben-Tabou De-Leon, Shlomo, Galia Ben-Zeev, and Itzhak Nussinovitch. Effects of osmotic shrinkage on voltage-gated Ca\textsuperscript{2+} channel currents in rat anterior pituitary cells. Am J Physiol Cell Physiol 290: C222–C232, 2006. First published September 7, 2005; doi:10.1152/ajpcell.00118.2005.—Increased extracellular osmolarity ([O\textsubscript{s}]\textsubscript{e}) suppresses stimulated hormone secretion from anterior pituitary cells. Ca\textsuperscript{2+} influx may mediate this effect. We show that increase in [O\textsubscript{s}]\textsubscript{e} (by 18–125%) differentially suppresses L-type and T-type Ca\textsuperscript{2+} channel currents (I\textsubscript{L} and I\textsubscript{T}, respectively); I\textsubscript{L} was more sensitive than I\textsubscript{T}. Hyperosmotic suppression of I\textsubscript{L} depended on the magnitude of increase in [O\textsubscript{s}]\textsubscript{e} and was correlated with the percent decrease in pituitary cell volume, suggesting that pituitary cell shrinkage can modulate L-type currents. The hyperosmotic suppression of I\textsubscript{L} and I\textsubscript{T} persisted after incubation of pituitary cells either with the actin-disrupter cytochalasin D or with the actin stabilizer phalloidin, suggesting that the actin cytoskeleton is not involved in this modulation. The hyperosmotic suppression of Ca\textsuperscript{2+} influx was not correlated with changes in reversal potential, membrane capacitance, and access resistance. Together, these results suggest that the hyperosmotic suppression of Ca\textsuperscript{2+} influx involves Ca\textsuperscript{2+} channel proteins. We therefore recorded the activity of L-type Ca\textsuperscript{2+} channels from cell-attached patches while exposing the cell outside the patch pipette to hyperosmotic media. Increased [O\textsubscript{s}]\textsubscript{e} reduced the activity of Ca\textsuperscript{2+} channels but did not change single-channel conductance. This hyperosmotic suppression of Ca\textsuperscript{2+} currents may therefore contribute to the previously reported hyperosmotic suppression of hormone secretion.

L-type Ca\textsuperscript{2+} channels; osmosensitivity; mechanosensitivity; osmolarity; hyperosmolarity

EARLY STUDIES demonstrated that hormone secretion from anterior pituitary cells (27), and other endocrine cells (4, 17), can be modulated by alterations in extracellular osmolarity [O\textsubscript{s}]\textsubscript{e}. This modulation was first attributed to direct effects of osmotic stress on the exocytosis of hormone containing granules (for reviews, see Refs. 13 and 20). However, later studies suggested that osmotic cell shrinkage, or swelling, may modulate Ca\textsuperscript{2+} influx and thereby hormone secretion. In these studies, it was demonstrated that hypertonicity inhibits stimulated increases in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and hormone secretion (50, 61), whereas hypotonicity increases [Ca\textsuperscript{2+}]\textsubscript{i} and hormone secretion (49, 51). Osmotic cell shrinkage, or cell swelling, might modulate voltage-sensitive Ca\textsuperscript{2+} influx either by a change in membrane potential or by direct effects on Ca\textsuperscript{2+} channels. Support for direct osmotic effects on Ca\textsuperscript{2+} channels comes from our previous studies, which demonstrated hyperosmotic suppression (35), and hyposmotic enhancements (3), of voltage-gated Ca\textsuperscript{2+} currents in anterior pituitary cells. Similar effects of increase, or decrease, in [O\textsubscript{s}]\textsubscript{e} on voltage-gated Ca\textsuperscript{2+} currents were observed in smooth muscle cells (30, 62), cardiac cells (34, 42), pancreatic β-cells (12), and hippocampal neurons (54). Therefore, it appears that voltage-gated Ca\textsuperscript{2+} channels in pituitary cells and other excitable cells are osmosensitive. This osmosensitivity of Ca\textsuperscript{2+} channels might reflect sensitivity to mechanical stress that is produced by cell shrinkage or swelling. Indeed, it was suggested that Ca\textsuperscript{2+} channels in pituitary cells (2, 44), in smooth muscle cells (30), and in cardiac myocytes (34), are mechanosensitive. In addition, it has been demonstrated that recombinant L-type (32) and N-type (6) Ca\textsuperscript{2+} channels are sensitive to membrane stretch, and that both native and recombinant glutamate receptors are mechanosensitive (8).

Because voltage-gated Ca\textsuperscript{2+} channels play a key role in regulating the secretion of pituitary hormones (26, 59) it was of interest to investigate whether or not hyperosmotic induced cell shrinkage modulates directly voltage-gated Ca\textsuperscript{2+} currents in pituitary cells. Both L-type and T-type Ca\textsuperscript{2+} currents (I\textsubscript{L} and I\textsubscript{T}, respectively) were observed in anterior pituitary cells (2, 9, 11, 40, 59). In a previous study (35), we have demonstrated that hypertonicity decreases Ca\textsuperscript{2+} influx through L-type and T-type channels in anterior pituitary cells. In this study, we further characterized the hyperosmotic effects on I\textsubscript{L} and I\textsubscript{T}. We show that the hyperosmotic suppression of I\textsubscript{L} and I\textsubscript{T} is differential (I\textsubscript{L} is more sensitive than I\textsubscript{T}), that the hyperosmotic suppression of I\textsubscript{L} is correlated with pituitary cell shrinkage and that the hyperosmotic suppression of I\textsubscript{L} and I\textsubscript{T} is not dependent on the integrity of the actin cytoskeleton. Furthermore, we show that the hyperosmotic suppression of I\textsubscript{L} stems from reduction in the activity of Ca\textsuperscript{2+} channels rather than from a decrease in single channel conductance. This hyperosmotic suppression of Ca\textsuperscript{2+} currents may contribute to the previously reported hyperosmotic suppression of hormone secretion from pituitary cells. The possible cellular mechanisms underlying these effects and their possible functional significance are discussed.

MATERIALS AND METHODS

Cell culture. The animals were euthanized in accordance with the guidelines of the Authority for Animal Facilities-Ethics Committee, Hebrew University. Anterior pituitary glands were dissected from three male rats (Sabra strain 250–300 g) in a procedure that follows a method previously described (2, 23). The anterior lobes were separated from the intermediate and posterior lobes and incubated for 25–35 min, at 37°C, in the dissociation tissue culture medium (F-12; Biological Industries, Beth-Haemek, Israel). The dissociation medium contained the following: 0.15% protease (type XIV, 5.6 U/mg), 0.12% dispase (type II), 0.1% collagenase (type I), DNA I (type II, 40 U/ml),

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0.25% BSA (Fraction V), and the antibiotic kanamycin sulfate (2.5 mM). All the enzymes and chemicals were obtained from Sigma (St. Louis, MO), except Dispase (Boehringer, Mannheim, Germany). During the period of enzymatic dissociation, the cell suspension was triturated gently with a 1-ml pipette every 5–7 min. After dissociation, the cell suspension was washed twice to stop enzymatic activity. The pituitary cell suspension was then loaded on top of a three-step discontinuous Percoll gradient (1.06/1.07/1.09 g/ml Percoll) to obtain an enriched populations of growth-hormone secreting cells (somatotrophs) and prolactin-secreting cells (lactotrophs), as previously described (11, 22, 40). By using the reverse hemolytic plaque assay (53), we found the 86% of the cells at the 1.07/1.09 boundary are somatotrophs and that 40% of the cells at the 1.06/1.07 g/ml boundary are lactotrophs (Nussinnovitch I, unpublished results). Cells were plated on round 16 mm glass coverslips, placed in 35 mm petri dishes, and kept in the incubator (37°C, 5% CO2) for 1–6 days before the experiment.

**Actin cytoskeleton imaging.** The actin cytoskeleton in pituitary cells was labeled with fluorescent isothiocyanate (FITC)-phalloidin as previously described (28). The staining procedure was performed at room temperature, in petri dishes containing pituitary cells attached to glass coverslips (see above). Shortly thereafter, pituitary cells were fixed with 4% paraformaldehyde (Fluka) in PBS for 40 min, and then permeabilized with 0.1% Triton X-100 (Baker) in PBS for 5 min. Nonspecific binding was then blocked by the addition of PBS containing 1% BSA (10 min) to the cells. Afterward, the pituitary cells were incubated with 13 μM FITC-phalloidin (Sigma) for 40 min, washed extensively (4×) with PBS, mounted with an antifade medium (Agar Scientific) to prevent rapid photobleaching, and then examined with an Olympus BX51 fluorescence microscope (×100 oil-immersion objective). Color images were captured using an Olympus DP70 digital camera. The involvement of the actin cytoskeleton in the hyperosmotic effects was examined with cytochalasin D (Cyto D; Sigma) and phalloidin (Sigma). Stacks were prepared in DMSO and kept at −20°C. Aliquots containing Cyto D or phalloidin were dissolved in the physiological solution before the start of experiments. In several control experiments, we found that final DMSO concentrations (0.001%-0.1%) were not affecting our results (see Fig. 6C).

**Electrophysiological recording and analysis.** Whole cell barium currents (Iba) and single-channel barium currents, through voltage-gated Ca2+ channels, were recorded with an Axopatch 1C amplifier (Axon Instruments, Union City, CA) using the whole cell and cell-attached modes of the patch-clamp technique. Patch-electrodes were pulled from 1.6 mm (OD) borosilicate glass (Hilgenberg, Malsfeld, Germany) on a two-stage puller (L/M-3P-A, List-Electronic, Darmstadt, Germany) and their resistance ranged from 3 to 6 MΩ, when filled with the “patch pipette solutions” (see below). Membrane currents were recorded at room temperature (20–24°C), sampled with an analog-to-digital converter (TL-1 DMA interface or Digidata 1320A, Axon Instruments) at 5 kHz, filtered with a four-pole low-pass Bessel filter with a cut-off frequency (~3 dB) of 1 kHz and stored in the hard drive of an IBM-based computer. Capacitive currents and access resistances (Ra) were electronically compensated with the potentiometers provided with the amplifier. Final access resistance was usually ~15 MΩ. Linear leak currents (and residual capacitive currents) were digitally subtracted after extrapolation of averaged leak currents that were obtained in response to P/2 or P/4 pulse protocols. The pCLAMP6 or pCLAMP8 programs (Axon Instruments) were used for on-line acquisition and for off-line analysis of the membrane currents. Iba through L-type and T-type channels were usually activated with 200-ms voltage steps (interval 10–15 s) from a holding potential (Vh) of ~80 mV to various test potentials (VT). In some of the experiments double-pulse protocols were used to activate simultaneously T-type currents (VT = −30 mV) and L-type currents (VT = 0 mV). To obtain instantaneous V/I relationships of Ca2+ channel currents we have used 600-ms voltage ramps ranging from ~100 to +80 mV.

For simultaneous monitoring of Iba, Cm, and Rm we used double-pulse protocols. The first subthreshold pulse, a 10-ms depolarization from ~80 to ~70 mV, activated capacitive currents. The second pulse, a 20-ms depolarization from ~80 to 0 mV, activated Iba. In these experiments, currents were sampled at a rate of 50 or 100 kHz (filter 10 kHz and cell membrane capacitive currents were not compensated electronically). The uncompensated membrane capacitive currents were used to calculate the values of Cm and Rm by using the relation \( Rm = Rm' × Cm \), where \( Rm' \) is the time constant of the decay of the capacitive currents (31). The \( Rm' \) was calculated by fitting a monoeponential function to the decay of the capacitive currents. Cm was calculated from the relation \( Cm = QV / V \), where Q is the amount of charge needed to discharge the cell membrane (obtained by integrating the capacitive current) and V is the amplitude of the subthreshold pulse. Ra was calculated from the relation \( Ra = Rm' × Cm \). Calculated Cm values may be affected by alterations in electrode capacitance due to changes in bath level. However, electrode capacitive currents were consistently compensated in all the experiments. In addition, Cm values stayed constant throughout long perfusion experiments, suggesting that changes in electrode capacitance were not a major factor affecting Cm values.

Single channel Ca2+ currents were recorded with the cell-attached mode of the patch-clamp technique. Single channel currents were activated by voltage steps from a Vh of ~80 mV to various test potentials (pulse duration 200 or 340 ms, interval 15 s), sampled at a rate of 5 kHz, and filtered at 0.5–1 kHz. To clamp the membrane patch at ~80 mV in cell-attached recording, the patch pipette was held at ~80 mV. Recordings were always obtained from multi-Ca2+ channel patches. To examine the hyperosmotic effects on these multichannel patches single traces were leak subtracted and analyzed (pCLAMP 6 or 8) as follows: 1) individual current traces in control and during hyperosmotic stimuli were averaged (ensemble currents) and compared. 2) NP, was used as a measure of Ca2+ channel activity (where N is the number of channels in the patch and P, is the probability of Ca2+ channels to be in the open state). The value of NP, for each current trace in the experiment was calculated from the ratio \( f_i \) (where \( f_i \) is the average current of this trace and \( i \) is the corresponding single channel current amplitude). 3) All point amplitude histograms (APAH). These histograms were constructed from data points in control, hyperosmotic stimulus, and fitted with a Gaussian fit. From the peaks of these histograms, representing closed times and the number of channels in the patch, we usually monitored changes in \( i \) and changes in closed probability (\( P_i \)).

Statistical differences among groups of tested parameters were examined either by paired or by unpaired t-tests. Multiple-comparison tests were performed by one-way ANOVA. When significant differences were indicated in the F-test (\( P < 0.001 \)), the significance of differences between the means of any of these groups was determined by the Tukey method for multiple comparisons with \( \alpha = 0.05 \) (see Fig. 4). Results are always reported as means ± SE.

**Solutions.** For whole cell recordings of voltage-gated Iba the extracellular (bath) solution contained (in mM) 150 TEA-Cl, 10 BaCl2, 10 glucose, and 10 HEPES [adjusted to pH 7.3 with TEA (OH), 300 mosmol/L]. The pipette “intracellular” solution contained (in mM) 138 CsCl, 11 EGTA, 10 HEPES, 2 Mg-ATP, 0.16 guanosine triphosphate, and 1.5 lidocaine N-ethyl chloride (QX-314) [adjusted to pH 7.3 with Cs(OH), osmolarity 305 mosmol/L]. [Os], was increased by the addition of 50, 100, 200, or 400 mM mannitol to the extracellular solution (corresponding to 18%, 30%, 63%, and 125% increase in [Os]). For cell-attached recordings of single channel currents the extracellular solution contained (in mM) 140 K-aspartate, 2 MgCl2, 5 EGTA, 10 glucose, and 10 HEPES [adjusted to pH 7.3 with KOH(OH)]. The patch-pipette solution contained (in mM) 100 BaCl2, 15 TEA-Cl, and 10 HEPES. BAY K 8644 or FPL 64176 (3–10 μM) were added to the bath and patch pipette solutions in all the single channel experiments. [Os], in these single channel experiments was increased.
as described above, by adding mannitol to the bath solution. All chemicals for these extracellular and intracellular solutions were purchased from Sigma, except QX-314 (Alomone Labs, Jerusalem, Israel), which was used to block Na⁺ currents.

Before each experiment coverslips containing pituitary cells were placed in a perfusion chamber (RC-16, Warner Instruments). Cells were exposed to control (isosmotic) or hyperosmotic solutions by perfusing the chamber at a rate of ~1 ml/min. The volume of solution in the perfusion chamber was ~0.4 ml and the cells were exposed to the different experimental solutions for 2–4 min.

RESULTS

Hyperosmotic suppression of I_L and I_T: differential sensitivity. In control experiments using isosmotic solutions, I_L usually increased in size (run up) during the first 1–5 min of recordings. After this run-up period, I_L either remained constant or was diminished by <20%, during the next 20 min of recordings. Hyperosmotic effects on I_L were usually examined after the run-up period. The maximal hyperosmotic suppression of I_L was usually observed within 1–2 min; therefore run down was not a major factor in the hyperosmotic effects that we observed. Figure 1, A and B, illustrates L-type and T-type Ca²⁺ current waveforms that were activated by a double pulse protocol to −30 and 0 mV, respectively. A 63% increase in [Os]ₑ reversibly suppressed I_L and I_T by ~40% and 20%, respectively. Similar results were obtained in additional double pulse experiments. In these experiments, 63% increase in [Os]ₑ suppressed I_L by 49 ± 3% and I_T by 30 ± 5% (n = 4) (P < 0.02, paired t-test). This differential sensitivity was also observed when we compared I_L and I_T that were recorded from different cells, in response to different hyperosmotic stimuli, as illustrated in Fig. 1C.

Hyperosmotic suppression of I_L: dependence on [Os]ₑ and on cell shrinkage. Figure 2A shows that the hyperosmotic suppression of I_L depends on the magnitude of hyperosmotic stimuli. Exposure of pituitary cells to five different hyperosmotic stimuli (for 40 to 70 s) resulted with a significant dose-dependent suppression of L-type currents (one-way ANOVA, P < 0.05). The dependence on the magnitude of osmotic-stress may stem from dependence on hyperosmotic induced pituitary cell shrinkage. Indeed, the hyperosmotic suppression of I_L was consistently accompanied with a decrease in pituitary cell diameter (measured by using a scale that was inserted into the ocular of the microscope). We therefore correlated between the percent decrease in I_L and the percent decrease in pituitary cell volume at the end of 200 s of hyperosmotic stimuli (estimated from decrease in cell diameter), in response to four different hyperosmotic stimuli. Figure 2B illustrates high correlation (r² = 0.9) between the percent decrease in cell volume and percent decrease in I_L, in these experiments, suggesting that pituitary cell shrinkage may underlie the hyperosmotic suppression of Ca²⁺ currents.

Hyperosmotic suppression of I_L: voltage dependence. The hyperosmotic suppression of I_L may have resulted from hyperosmotic-induced voltage shifts in the activation of I_L. We therefore examined hyperosmotic effects on current-voltage (I-V) relationships of I_Ba that were activated either by discrete voltage steps (from −50 to 40 mV) or by voltage ramps (from −100 to 80 mV, duration 0.6 s, interval 15 s). Figure 3A shows I-V curves of L-type currents that were activated by voltage steps, before and during 30% increase in [Os]ₑ. This increase in [Os]ₑ resulted with a decrease in the maximal current (Iₘₐₓ) (by 37%), and with a small negative voltage shift, in the I-V curve. The magnitude of the shift was estimated from the normalized I-V curves (I/Iₘₐₓ), shown in Fig. 3B. V₀.5 (voltage at which Iₗ/Iₘₐₓ = 0.5) and V_peak (voltage at which Iₗ/Iₘₐₓ = 1) shifted by −2.3 and −4.5 mV, respectively. Similar small negative shifts were observed in ramp experiments, as shown in Fig. 3, C and D. A 30% increase in [Os]ₑ reduced Iₘₐₓ by 36% and...
shifted $V_{0.5}$ and $V_{\text{peak}}$ by $-4$ and $-3$ mV, respectively. In some of these ramp experiments whole cell currents reversed from inward $I_{Ba}$ to outward currents. These outward currents may reflect efflux of cesium through the Ca$^{2+}$ channels (Ca$^{2+}$ channels are permeable to monovalent ions when divalent ion concentration is reduced below the micromolar level). We therefore compared reversal potential ($E_r$) values under isosmotic and hyperosmotic conditions. Figure 3C shows a reversal potential of $\sim 60$ mV that was not changed during the hyperosmotic stimulus. Similar results were obtained in addi-
tional experiments, in response to 30% increase in [Os]e. 
V_{0.5} negatively shifted by 1.6 ± 0.3 mV (P < 0.0001, paired t-test, n = 17) and V_{peak} negatively shifted by 2.2 ± 0.4 mV (P < 0.0001, paired t-test, n = 17). However, E_L in control (67 ± 2 mV) was not different from E_L during hyperosmotic challenges (66 ± 5 mV), (P = 0.64, paired t-test, n = 8). Thus our results demonstrate a small, but significant, negative voltage shift during hyperosmotic challenges. Thus it cannot be argued that the hyperosmotic suppression of I_L results from a depolarizing shift in the activation of the I_L, or from changes in the reversal potential of the currents.

Hyperosmotic suppression of I_L: independence on cell membrane surface area and on access resistance. The hyperosmotic suppression of I_{Ba} may have resulted from decrease in C_m (pituitary cell shrinkage) or from substantial increases in R_a. We therefore monitored simultaneously hyperosmotic effects on I_{Ba}, C_m, and R_a by using double-pulse protocols; the first subthreshold pulse activated capacitive currents and the second pulse activated L-type Ca^{2+} currents. The capacitive currents were used to calculate changes in C_m and R_a (see MATERIALS AND METHODS). Figure 4A illustrates one of these experiments. 63% increase in [Os]e suppressed I_L by ~40%, without any significant effects on the capacitive current, suggesting that changes in R_a and C_m are not responsible for the decrease in I_L. Similar experiments were performed in additional cells. Figure 4, B and C, summarizes 14 experiments in which pituitary cells were challenged with 30% increase [Os]e for 150–225 s. The hyperosmotic suppression of I_L in these experiments (by 35 ± 3%, n = 14) was not accompanied with significant changes in C_m (Fig. 4B), but was associated with ~30% increases in R_a (Fig. 4C), from 14 ± 2 to 20 ± 4 MΩ (P < 0.004, paired t-test, n = 14). This raises the possibility that increase in R_a underlies (or contributes) to the hyperosmotic suppression of I_{Ba}. However, closer inspection of Fig. 4C, right panel, reveals that this is not the case. First, there is no correlation between the hyperosmotic suppressions of I_L and their corresponding increases in R_a. Second, in at least 4 out of these 14 experiments the hyperosmotic suppression of I_L was not accompanied with a significant increase in R_a.

Hyperosmotic suppression of I_L and I_{Ba} independence on the actin cytoskeleton. The hyperosmotic suppression of I_{Ba} may have resulted from decrease in membrane tension that is transferred to the channel proteins via the cytoskeleton. The involvement of the actin cytoskeleton in the hyperosmotic

![Fig. 4. Simultaneous hyperosmotic effects on I_L, membrane capacitance (C_m), and access resistances (R_a). A: a double-pulse protocol was used to activate capacitive currents and L-type currents. The capacitive currents were used to calculate C_m and R_a (see MATERIALS AND METHODS). The arrow at left points to the capacitive currents that are illustrated on a faster time scale at right. Note that the hyperosmotic challenge (63% increase in [Os]e) resulted with a decrease in I_L (left), but not with any significant changes in the capacitive currents (right). Hence, the hyperosmotic induced decrease in I_L was not accompanied with changes in R_a and C_m. Similar experiments were performed in pituitary cell that were exposed to 30% increase in [Os]e, summarized in B and C. B: the hyperosmotic induced decrease in I_L was not accompanied with changes in C_m. C: hyperosmotic induced decrease in I_L was accompanied with a 40% increase in R_a (left). However, there was no correlation between the %decrease in I_L and the %increase in R_a (right), suggesting that the hyperosmotic suppression of I_L was independent on these increases in R_a (each filled circle in these plots represents one experiment).](http://ajpcell.physiology.org/)

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Hyperosmotic suppression of $I_L$ at the single channel level. The hyperosmotic suppression of $I_L$ may have resulted either from a reduction in the number of openings (or “activity”) of $Ca^{2+}$ channels or from a reduction in single channel conductance ($\gamma$). These possibilities were investigated by recording the activity of L-type channels in the cell-attached mode, while exposing pituitary cells, outside the patch pipette, to hyperosmotic media.

Hyperosmotic effects on activity of L-type $Ca^{2+}$ channels. Figure 7 shows that 63% increase in $[Os]$ results with reversible suppression of $Ca^{2+}$ channel activity in a multichannel patch. This reduction in L-type $Ca^{2+}$ channel activity is also manifested as a reduction in the amplitude of ensemble $Ca^{2+}$ channel currents.
channel currents, as illustrated in Fig. 7B. These effects of hyperosmolarity on Ca\(^{2+}\) channel activity were quantitatively estimated by using two additional methods of analysis; by calculating values of NP\(_o\), as a measure of L-type Ca\(^{2+}\) channel activity, and by constructing APAH (see MATERIALS AND METHODS). The results of this analysis, for the experiment shown in Fig. 7, are illustrated in Fig. 8. Figure 8A illustrates that 63% increase in [Os], reversibly decreased NP\(_o\), values by ∼50%. This decrease in the activity of L-type channels was also manifested as an increase in the proportion of P\(_c\) (by ∼50%) in the APAH of the same experiment, as illustrated in Fig. 8B. Similar results (63% increase in [Os]e), were obtained in additional cells. NP\(_o\) values were reduced by ∼45% from 0.82 ± 0.10 to 0.44 ± 0.09 (P < 0.001, paired t-test, n = 9), and P\(_c\) values were increased by ∼60% from 0.43 ± 0.06 to 0.69 ± 0.07 (P < 0.001, paired t-test, n = 9). Hence, these experiments demonstrate that exposing pituitary cells to hyperosmotic media reduced the activity of L-type Ca\(^{2+}\) channels inside the cell-attached membrane patch.

**Hyperosmotic effects on single channel conductance.** The hyperosmotic suppression of L-type Ca\(^{2+}\) channel activity was not accompanied with changes in single channel conductance (γ), as shown in Fig. 9A. γ in control (23.8 pS) was not different from γ during hyperosmotic challenges (63% increase in [Os]e, 25 pS). The small increase in the amplitude of single channel currents at each one of the voltage steps, observed during exposure to the hyperosmotic challenge, may stem from a 4 mV hyperpolarizing shift across the membrane patch. This hyperpolarization by itself may reduce the activity of L-type Ca\(^{2+}\) channels. However, from the activity curve (NP\(_o\) voltage relationship) of this experiment (Fig. 9B), it is clear that a hyperpolarization of 4 mV cannot explain the strong hyperosmotic suppression of Ca\(^{2+}\) channel activity. Similar results were obtained from averaged I-V curves of single channel currents (n = 16; Fig. 10A); γ in control (27.3 pS) was not different from γ during hyperosmotic challenges (26.8 pS). A small positive voltage shift was also observed in these averaged I-V (∼2 mV). However, from the activity curve that was constructed from the same experiments (Fig. 10B), it is clear that this hyperpolarizing shift cannot explain the hyperosmotic suppression of L-type channels. The reason for this small voltage shift is not clear, however, it may stem from hyperosmotic effects on membrane potential outside the membrane.
Thus it appears that the hyperosmotic suppression of whole cell Ca\(^{2+}\) channel currents results from reduction in \(N_{P_o}\) and not from reduction in single channel conductance.

**DISCUSSION**

**Overview:** This study demonstrates that increase in [Os]e (by 18–125%) results with suppression of Ca\(^{2+}\) channel currents in pituitary cells. This hyperosmotic suppression was observed in every pituitary cell studied, either from the somatotroph or from the lactotroph enriched cell populations. Therefore, it is reasonable that sensitivity to hyperosmotic stress is a common feature of Ca\(^{2+}\) channels in both somatotrophs and lactotrophs, and possibly also in other pituitary cell types. In addition, our study shows that this hyperosmotic suppression was differential; \(I_L\) was more sensitive than \(I_T\) to hyperosmotic stimuli (Fig. 1), similar to the previously described differential sensitivity of \(I_L\) and \(I_T\) to hyposmotic stimuli (2), and similar to the differential sensitivity of recombinant \(I_L\) and \(I_T\) to membrane stretch (6). This differential sensitivity may stem either from different sensitivities of the channel proteins to osmotic stimuli or from different locations of the channel proteins in different membrane microdomains (41, 58). The present study shows also that the hyperosmotic suppression of \(I_L\) depends on the magnitude of increase in [Os]e and is correlated with decrease in pituitary cell volume (Fig. 2), suggesting that pituitary cell shrinkage can modulate Ca\(^{2+}\) influx. The hyperosmotic suppression of Ca\(^{2+}\) channel currents was associated with a small negative voltage shift (2–4 mV) in the \(I-V\) relationship of Ca\(^{2+}\) channels.

**Fig. 9.** Hyperosmotic effects on single channel conductance of L-type Ca\(^{2+}\) channels. A: \(I-V\) relationship of single channel currents. 63% increase in [Os], had no effect on single channel conductance (γ). γ in control (23.8 pS, filled circles) was not different from γ during the hyperosmotic stimulus (25 pS, empty circles). The amplitude of single channel current (i) was obtained from APAHs (calculated from 30 current traces, at each voltage). B: \(N_{P_o}-V\) relationship in the same experiment. 63% increase in [Os], substantially decreased the activity of Ca\(^{2+}\) channels at all voltages. \(N_{P_o}\) values were calculated and averaged from 30 current traces, at each voltage. The ensemble currents, in response to a constant 80-mV voltage step, were averaged from at least 60 current traces each. Control recordings were carried out before Control \(I-V\). “Hyper” recordings were carried out after exposure to the hyperosmotic medium before the “hyper \(I-V\).” “Wash” recordings were carried out after the hyper \(I-V\) (during exposure to isosmotic medium). Representative current traces, to demonstrate single channel activity, are illustrated above each ensemble current. Note that the hyperosmotic effect on ensemble currents was reversible.

**Fig. 10.** Summary of hyperosmotic effects on "single channel conductance" of L-type channels. Left: averaged \(I-V\) relationships of single channel currents. Each point represents averaged currents that were recorded from at least 8 cells (SE values are smaller than symbol size). γ in control (27.3 pS, filled circles) was not different from γ during hyperosmotic stimuli (26.8 pS, open circles, 63% and 125% increase in [Os],). Right: averaged \(N_{P_o}-V\) relationships that calculated from the same experiments. Each point represents \(N_{P_o}\) values that were obtained from at least 8 cells. Increase in [Os], substantially decreased \(N_{P_o}\) values. This suggests that the hyperosmotic suppression of Ca\(^{2+}\) currents results from a decrease in \(N_{P_o}\) rather than a decrease in γ.

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currents but with no change in the reversal potential (Fig. 3). Hence, it is reasonable that the suppression of Ca\(^{2+}\) channel currents was not caused by the voltage shift or by the activation of a different ionic conductance. It is possible that the small negative voltage shift in the I-V relationship results from an increase in \(R_s\) (33) observed in some of these experiments (Fig. 4). To rule out the possibility that the hyperosmotic suppression of Ca\(^{2+}\) currents simply results from a decrease in pituitary cell membrane surface area, or from increase in access resistance, we simultaneously monitored hyperosmotic induced changes in \(I_L\), \(C_m\), and \(R_s\) (Fig. 4). Our results demonstrate that the hyperosmotic suppression of Ca\(^{2+}\) currents is not correlated with changes in \(C_m\) and \(R_s\), although an increase in \(R_s\) was observed in some of these experiments. Together these results suggest that the hyperosmotic suppression of voltage gated Ca\(^{2+}\) currents stems from a genuine effect of osmotic cell shrinkage on the Ca\(^{2+}\) channel proteins.

To assess the biophysical basis for these hyperosmotic effects on whole cell Ca\(^{2+}\) channel currents we recorded the activity of L-type Ca\(^{2+}\) channels from cell-attached patches, while exposing the cell, outside the patch pipette, to hyperosmotic media. Our results show that increase in [Os]e suppressed \(N P_o\) and increased \(P_c\) (Figs. 7 and 8) without having any significant changes in single channel conductance (Figs. 9 and 10). The suppression in \(N P_o\) was similar in magnitude to the suppression of whole cell currents; 63% increase in [Os]e suppressed both \(I_L\) and \(N P_o\) by 40–50%. This similarity suggests that the mechanisms underlying hyperosmotic suppression of whole cell and cell-attached currents are similar, despite the different configurations of recordings and despite the different modes of exposure to hyperosmotic media. Moreover, the similarity in responses may suggest that our cell-attached patches were similar in morphology to the whole cell membranes, and that our cell-attached membrane patches were not lipid blebs devoid of cortical cytoskeleton (15, 36, 64). It has been shown that uncoupling of cortical actin from the plasma membrane, and the formation of lipid blebs during tight seal cell-attached recordings, underlies the discrepancy between the activities of mechan gated ion channels under cell-attached and whole cell recordings (16, 37, 60, 65).

The results of this study show that decrease in Ca\(^{2+}\) channel activity underlies the hyperosmotic suppression of whole cell Ca\(^{2+}\) currents. This is the first study, to the best of our knowledge, which compares hyperosmotic effects both at the whole cell and single channel level. The decrease in Ca\(^{2+}\) channel activity may result either from a decrease in \(N\) or in \(P_o\). The prominent suppression of maximal \(I_{Ba}\) in contrast to the lack of prominent effects on \(V_{0.5}\) (Fig. 3) might suggest that \(N\) rather than \(P_o\) is affected by hyperosmotic stress, as proposed for the effects of membrane stretch on N-type channels (6). On the other hand, the clear increase in \(P_c\) (Fig. 6) might suggest that \(P_c\) rather than \(N\) is affected by hyperosmotic stress. This increase in \(P_c\) cannot be attributed to changes in the efficacy of BAY K 8644, due to osmotic alterations in intracellular ionic strength. BAY K 8644 was added extracellularly and it was shown that dihydropyridine (DHP) agonists gain access to their receptor site from the outer surface of the cell membrane (18, 57). Additional experiments are needed to resolve this question as to whether \(N\) or \(P_o\) is affected by osmotic stress.

Sensitivity of Ca\(^{2+}\) channels to osmotic cell shrinkage: possible cellular mechanisms. The present study shows that the hyperosmotic suppression of \(I_L\) depends on the percent increase in [Os]e (Fig. 2A) and is highly correlated with the percent decrease in pituitary cell volume (Fig. 2B). Hence voltage-gated Ca\(^{2+}\) influx in pituitary cells can be suppressed by pituitary cell shrinkage. This osmosensitivity may result either from alterations in mechanical membrane tension or from alterations in ionic strength underneath the plasma membrane. Changes in membrane tension may be conveyed to the channel protein either through the cytoskeleton of the cell or through the phospholipid bilayer (14, 46). An increasing number of studies point to a functional link between the actin cytoskeleton and L-type Ca\(^{2+}\) channels (28, 38, 45, 47, 52). Moreover, it was shown that hyposmotic-induced increase in \(I_L\) in smooth muscle cells was abolished by the actin microfilament disrupter Cyto D, and augmented by the actin microfilament stabilizer phalloidin (63). Similar effects were reported for T-type Ca\(^{2+}\) currents in cardiac myocytes (42). However, in contrast to these findings, we demonstrate here that the hyperosmotic suppression of \(I_L\) and \(I_T\) persisted after disruption of the actin cytoskeleton with Cyto D or stabilizing it with phalloidin (Figs. 5 and 6). Hence, our results suggest that the actin cytoskeleton does not play a significant role in the hyperosmotic suppression of Ca\(^{2+}\) currents in pituitary cells. This conclusion is based on the assumption that cortical actin is not washed away under conditions of whole cell recordings (60, 65). In this context, it is interesting that shear-stress effects on L-type Ca\(^{2+}\) currents (but not on sodium currents) persisted after disruption of the actin cytoskeleton in smooth muscle cells (56). In addition, shear-stress effects on recombinant L-type channels persisted after truncation of the \(\alpha_{1c}\) COOH terminus, a putative site for interaction of Ca\(^{2+}\) channels with the cytoskeleton (32). These findings are in support to the notion that cortical actin cytoskeleton is not the key player in mechanical modulation of L-type channels. It is therefore tempting to speculate that decrease in membrane tension is conveyed directly to the Ca\(^{2+}\) channel proteins through the phospholipid bilayer. It is well established that increased tension in the lipid bilayer activates mechano-gated ion channels in both prokaryotic and eukaryotic cells (14). Previous studies (8) have shown by using amphiphilic compounds that tension in the phospholipid bilayer underlies the mechanosensitivity of glutamate receptor in mouse central neurons. Similarly, it has been shown that tension in the phospholipid bilayer underlies the mechanosensitivity of two-pore domain K channels, TREK and TRAAK (25, 43).

It is also possible that the osmosensitivity of whole cell Ca\(^{2+}\) currents results from local alterations in ionic strength underneath the plasma membrane. Hyperosmotic shrinkage may be associated with efflux of water molecules and, as a result, with local increases in ion concentration underneath the plasma membrane, despite the constant ionic composition of the intracellular solution. It is possible that these local increases in ionic strength underneath the plasma membrane suppress in some way the activity of Ca\(^{2+}\) channels and as a result reduce whole cell Ca\(^{2+}\) influx. This ionic-strength hypothesis is also supported by our single channel results. Hyperosmotic induced cell shrinkage, during cell-attached recordings, is expected to increase ionic strength all over the cytoplasm of the intact cell, including the cytoplasm underneath the cell-attached mem-
brane patch. Some indirect support for this ionic-strength hypothesis comes also from studies that demonstrated that decrease in extracellular ionic strength facilitates Ca\(^{2+}\) influx in hippocampal neurons (5, 54). In addition, it was shown that decrease in intracellular ionic strength triggers the activation of volume regulated anion channels (7, 39), and it was proposed that deprivation of the open-channel volume underlies the hyperosmotic suppression of potassium channels in squid giant axons (66).

Sensitivity of Ca\(^{2+}\) channels to osmotic cell shrinkage: functional significance. The findings of this study demonstrating hyperosmotic suppression of \(I_h\) and \(I_f\) are in agreement with previous studies demonstrating hyperosmotic suppression of stimulated hormone secretion from pituitary cells (49, 61). The relevance of these findings to hormone secretion is not clear because pituitary cells, like most mammalian cells, experience very small alterations in [Os]\(_e\), due to changes in the metabolic state of pituitary cells. However, alterations in intracellular osmolarity ([Os]i) due to changes in the metabolic state of pituitary cells, may occur under normal physiological conditions (29). It is plausible that such putative alterations in [Os]i will cause continuous alterations in pituitary cell volume under normal physiological conditions, thereby affecting Ca\(^{2+}\) influx and hormone secretion.

Another interesting possibility is that Ca\(^{2+}\) channels sense alterations in membrane tension during the process of exocytosis. A substantial decrease in membrane tension during the process of exocytosis by anterior pituitary cells identified by a plaque assay. Am J Physiol 425: 29 – 42, 1990.


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