Effects of osmotic swelling on voltage-gated calcium channel currents in rat anterior pituitary cells

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Ben-Tabou De-Leon, Shlomo, Edna Blotnick, and Itzhak Nussinovitch. Effects of osmotic swelling on voltage-gated calcium channel currents in rat anterior pituitary cells. Am J Physiol Cell Physiol 285: C840–C852, 2003. First published May 28, 2003; 10.1152/ajpcell.00101.2003.—Decrease in extracellular osmolarity ([Os]e) results in stimulation of hormone secretion from pituitary cells. Different mechanisms can account for this stimulation of hormone secretion. In this study we examined the possibility that hyposmolality directly modulates voltage-gated calcium influx in pituitary cells. The effects of hyposmolality on L-type (I_L) and T-type (I_T) calcium currents in pituitary cells were investigated by using two hyposmotic stimuli, moderate (18–22% decrease in [Os]e) and strong (31–32% decrease in [Os]e). Exposure to moderate hyposmotic stimuli resulted in three response types in I_L (a decrease, a biphasic effect, and an increase in I_L) and in increase in I_T. Exposure to strong hyposmotic stimuli resulted only in increases in both I_L and I_T. Similarly, in intact pituitary cells (perforated patch method), exposure to either moderate or strong hyposmotic stimuli resulted only in increases in both I_L and I_T. Thus it appears that the main effect of decrease in [Os]e is increase in calcium channel currents. This increase was differential (I_L were more sensitive than I_T) and voltage independent. In addition, we show that these hyposmotic effects cannot be explained by activation of an anionic conductance or by an increase in cell membrane surface area. In conclusion, this study shows that osmotic swelling of pituitary cells can directly modulate voltage-gated calcium influx. This hyposmotic modulation of I_L and I_T may contribute to the previously reported hyposmotic stimulation of hormone secretion. The mechanisms underlying these hyposmotic effects and their possible physiological relevance are discussed.

L-type channels; mechanosensitivity; somatotrophs; lactotrophs

EARLY STUDIES SHOVED THAT hormone secretion from pituitary cells (28), pancreatic β-cells (5), and chromaffin cells (18) can be modulated by alterations in extracellular osmolarity ([Os]e). In pituitary cells it was demonstrated that hypertonicity inhibits stimulated increases in intracellular calcium concentration ([Ca]i) and hormone secretion (44, 52), whereas hypotonicity increases [Ca]i and hormone secretion (43, 46). The same group of researchers demonstrated that the hyposmotically induced increase in [Ca]i and hormone secretion can be mimicked by isosmotic media containing membrane-permeant molecules (43, 45), suggesting that cell swelling rather than osmotic stress per se underlies these effects of hypotonicity. These hyposmotically induced increases in [Ca]i and hormone secretion in pituitary cells were attributed largely to hyposmotically induced enhancement of calcium influx through L-type channels (43, 46).

Hyposmotic swelling may enhance calcium influx either by direct modulation of calcium channels, caused by increase in membrane tension, or by indirect modulation of calcium channels, caused by alterations in membrane potential. There are several examples for the latter possibility. Hyposmotically induced calcium influx in GH3 pituitary cells was attributed to osmotic activation of cationic stretch-activated channels that, in turn, caused membrane depolarization (10). Similarly, hyposmotically induced calcium influx in chromaffin cells (38) and in pancreatic β-cells (4, 14) was attributed to the activation of swell-dependent chloride channels that, in turn, caused membrane depolarization. However, there are also several examples that demonstrate direct hyposmotic enhancement of voltage-gated calcium currents, in smooth muscle cells (26, 31, 54), cardiac myocytes (36), pancreatic β-cells (14), and hippocampal neurons (49).

Because voltage-gated calcium channels play a key role in regulating the secretion of pituitary hormones (8, 27, 50), it was of interest to investigate whether or not hyposmotically induced cell swelling directly modulates voltage-gated calcium currents in pituitary cells. Both L-type and T-type calcium currents (I_L and I_T, respectively) were observed in anterior pituitary cells (2, 9, 13, 32, 40, 51). In a previous study we demonstrated that hypertonicity decreases calcium influx through L-type and T-type channels in anterior pituitary cells (37), presumably because of hyposmotic effects on the gating of L-type channels (3). In this study we show that hypotonicity directly enhances calcium influx through I_L and I_T, as previously suggested from secretion studies. A preliminary account of this work in abstract form has appeared elsewhere (1).

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Sensitivity of calcium channels to hyposmotic stress

Experimental procedures

Cell culture. Animals were killed and pituitary glands were obtained in accordance with the guidelines of the Authority for Animal Facilities-Ethics Committee, Hebrew University. Anterior pituitary glands were obtained after decapitation and dissection of three male rats (Sabra strain; 250–300 g) in a procedure following that previously described (2, 24). The anterior lobes were separated from the intermediate and posterior lobes and incubated for 25–35 min at 37°C in dissociation tissue culture medium (F-12; Biological Industries, Beth-Haemek, Israel). The dissociation medium contained the 0.15% protease (type XIV, 5.6 U/mg), 0.12% dispase (type II), 0.1% collagenase (type I), deoxyribonuclease I (type II, 40 U/ml), 0.25% bovine serum albumin (BSA, fraction V), and the antibiotic kanamycin sulfate (2.5 μM). All enzymes and chemicals were obtained from Sigma (St. Louis, MO) except dispase (Boehringer Mannheim, Mannheim, Germany). During the period of enzymatic dissociation, the cell suspension was separated gently with a 1-ml pipette every 5–7 min. After dissociation the cell suspension was washed twice to stop enzymatic activity. The cell suspension was then loaded on top of a Percoll discontinuous gradient to obtain enriched populations of pituitary growth hormone-secreting cells (somatotrophs) and prolactin-secreting cells (lactotrophs), as previously described (2). 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.

Electrophysiological recording and analysis. Barium currents (I_{Ba}) through calcium channels were recorded with an Axopatch 1C amplifier (Axon Instruments, Union City, CA) by using the whole cell mode of the patch-clamp technique at room temperature (20–24°C). Patch electrodes were pulled from 1.6-mm (outer diameter) borosilicate glass (Hilgenberg, Malsfeld, Germany) on a two-stage puller (LM-3P-A; List Electronic, Darmstadt, Germany), and their resistance ranged from 3 to 6 MΩ when filled with the “intracellular solutions” (see Solutions). Membrane currents were sampled with an analog-to-digital converter (Digidata 1320A, Axon Instruments) at 5 kHz, filtered with a four-pole low-pass Bessel filter with a cutoff frequency (−3 dB) of 1 kHz, and stored in the hard disk of an IBM-based computer. Capacitive currents and access resistances (Rm) 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 test pulses (P) divided by 2 or 4 (P2 or P4 pulse protocols). The pCLAMP8 suite of programs (Axon Instruments) was used for on-line acquisition and off-line analysis of the membrane currents.

I_{Ba} 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 I_T (Vh = −30 mV) and I_L (Vh = 0 mV) simultaneously. To obtain instantaneous current-voltage (I-V) relationships of calcium channel currents, we used 500-ms voltage ramps ranging from −100 to +80 mV.

For simultaneous monitoring of I_{Ba}, cell membrane capacitance (Cm), and Rm, we used double-pulse protocols. The first subthreshold pulse, a 20-ms depolarization from −80 to −70 mV, activated capacitive currents. The second pulse, a 300-ms voltage ramp from −100 to +60 mV, activated Ib. In these experiments currents were sampled at a rate of 100 kHz (filter 10 kHz) and the capacitive currents were not compensated with the dials of the amplifier. The uncompensated capacitive currents were used to calculate the values of Cm and Rm by using the relation τ = Rm/Cm, where τ is the time constant of the decay of the capacitive currents (33). The value of τ was calculated by fitting a monoequivalent function to the decay of the capacitive currents. Cm was calculated from the relation $C = Q/V$, 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. $R_s$ was calculated from the relation $R_s = V/Cm$.

The among-group differences of tested parameters were examined either by paired or unpaired t-tests. Multiplecomparison tests were performed by one-way analysis of variance (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 α = 0.05 (see Fig. 4). Results are reported as means ± SE.

Solutions. Two types of hyposmotic stimuli were used to examine the effects of hyposmolality on calcium channel currents: moderate hyposmotic stimuli (18–22% decrease in [Os]e) and strong hyposmotic stimuli (31–32% decrease in [Os]e). To study hyposmotic effects while keeping ionic composition and strength constant we used as controls isosmotic media containing mannitol. Hyposmotic media were obtained by omitting mannitol from these isosmotic media. The compositions of the extracellular media used are given in Table 1. Barium was used as the charge carrier through calcium channels in all these experiments.

The pipette “intracellular” solution for whole cell recordings of I_{Ba}, contained in (mM) 138 CsCl, 11 EGTA, 10 HEPES, 2 Mg-ATP, 0.16 GTP, and 1.5 lidocaine N-ethyl chloride (QX-314) [adjusted to pH 7.2–7.3 with Cs(OH)], osmolarity 305 mosM. The pipette solution for perforated patch recordings contained in (mM) 75 CsCl, 75 Cs(OH), 75 l-aspartic acid, 0.1 EGTA, 10 HEPES, 1 MgCl2, and 200–300 μg/ml nystatin [adjusted to pH 7.2–7.3 with Cs(OH), osmolarity 300 mosM].

In some of the experiments we examined the hyposmotic effects on I_{Ba} in chloride-free media. In these experiments chloride was substituted with glutamate and acetate. The extracellular isosmotic media in these experiments contained (in mM) 95 l-glutamic acid, 10 Ba(acetate)2, 10 HEPES, 10 glucose, 115 mannitol [adjusted to pH 7.2–7.3 with tetraethylammonium (TEA(OH)), osmolarity 309 mosM]. The hyposmotic media (214 mosM) were obtained by omitting mannitol from this chloride-free isosmotic medium. The pipette intra-

Table 1. Composition of extracellular solutions

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<th>Isosmotic</th>
<th>Hyposmotic</th>
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<tr>
<td>TEA-Cl</td>
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<tr>
<td>Mannitol</td>
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<td>100/115</td>
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<tr>
<td>[Os]e, mosM</td>
<td>302/318</td>
<td>207/216</td>
<td>18.4/21.7</td>
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Concentrations are millimolar. These extracellular solutions were used for both whole cell and perforated-patch experiments (except for experiments in chloride-free media). pH of all solutions was adjusted to 7.2–7.3 with TEA(OH), TEA, tetraethylammonium; [Os]e, extracellular osmolarity.

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cellular solution in these experiments contained (in mM) 130 l-glutamic acid, 5 TEA(acetate), 10 HEPES, 11 EGTA, 2 Mg-ATP, 0.16 GTP, 1.5 QX-314 [adjusted to pH = 72–7.3 with Cs(OH), osmolarity 310 mosM]. All chemicals for the extracellular and intracellular solution were purchased from Sigma except QX-314 (Alomone Labs, Jerusalem, Israel), which was used to block sodium 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 hyposmotic 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

Effects of moderate hyposmotic stimuli on calcium channel currents. Figure 1 demonstrates an experiment in which the pituitary cell was exposed to a moderate hyposmotic stimulus. $I_{Ba}$ were activated by a double-pulse protocol. The first test pulse, to $-30$ mV, activated a transient $I_T$ and the second pulse, to 0 mV, activated a slowly inactivating $I_L$ (Fig. 1A). Decrease in $[Os]_e$ by 17% resulted in an increase in the amplitude of both $I_T$ and $I_L$. The full time course of this experiment is illustrated in Fig. 1B. Exposure to the hyposmotic medium (249 mosM) resulted in a reversible increase in the amplitude of $I_L$ (by 56%) and $I_T$ (by 25%). These hyposmotic increases in $I_{Ba}$ were not associated with changes in holding currents, as illustrated in Fig. 1B. Similar effects were observed in additional experiments. $I_L$ were increased by $32 \pm 4\%$ ($n = 18$), and $I_T$ were increased by $22 \pm 5\%$ ($n = 11$) ($P < 0.05$, unpaired $t$-test).

However, the response of $I_L$ to moderate hyposmotic stimuli was more complex, as illustrated in Fig. 2. Three different response types were actually observed (Fig. 2, A–C). Figure 2A illustrates a decrease (by 42%) in the amplitude of $I_L$ (type I response). Figure 2B illustrates a biphasic effect (17% decrease followed by 29% increase) on the amplitude of $I_L$ (type II response). Figure 2C illustrates an increase (by 33%) in the amplitude of $I_L$ (type III response). Responses were defined as “biphasic” when the initial decrease in the amplitude of $I_L$ recovered back toward control values by >50% during exposure to the hyposmotic medium. Three similar response types were observed in 40 experiments (first exposures in 40 cells). The distribution of these response types ($I_L$) is depicted in Fig. 2D. Type I response was observed in 25% of the experiments, type II response in 50% of the experiments, and type III response in 25% of the experiments. Similar responses were not observed in $I_T$. Moderate hyposmotic stimuli increased $I_T$ in 11 cells. These increases in $I_T$ were accompanied by three response types in $I_L$: decrease ($n = 2$), biphasic response ($n = 7$), and increase ($n = 2$). Hence, moderate hyposmotic stimuli elicited both differential and independent effects on $I_T$ and $I_L$.

In some of these experiments cells were repeatedly exposed to the same moderate hyposmotic stimulus (while $I_L$ were recorded). Figure 3A illustrates a pituitary cell that was exposed three consecutive times to the same moderate hyposmotic stimulus. The first exposure resulted in a biphasic response, a decrease in $I_L$ (by 30%) that was followed by a recovery to control levels. The second exposure (5 min later) resulted also in a biphasic response. However, at this time, the decrease (by 23%) was followed by a substantial increase (by 30%) in $I_L$. In the third exposure only an increase (by 40%) in $I_L$ was observed. Similar results were obtained in 10 cells that were exposed three consecutive times to the same moderate hyposmotic stimulus, as illustrated in Fig. 3B. The first 10 exposures resulted mostly in biphasic responses (9 of 10); both the first and second phases exhibited decrease in $I_L$. The second 10 exposures also resulted mostly in biphasic responses (8 of 10 cells). However, at this time, the second phase exhibited a clear increase in $I_L$. The third 10 exposures resulted in biphasic responses (with a substantial increase in $I_L$ in the second phase; 6 of 10 cells) and in type III responses, increase in $I_L$ (3 of 10 cells). A similar trend of enhancement was observed in 20 cells that were exposed two consecutive times to the same moderate hyposmotic stimulus (not shown). Thus it appears that repetitive exposures to
the same moderate hyposmotic stimulus result in enhanced manifestation of type III responses, increase in $I_L$.

One way to explain this shift toward type III responses is by assuming that the biphasic responses (type II) are actually composed of two superimposed responses, decreases (type I) and increases (type III) in $I_L$. Repeated exposures to moderate hyposmotic stimuli cause a use-dependent inactivation of type I responses, thus leading to better manifestation of type III responses, increase in $I_L$. Support for this hypothesis comes from the nature of the biphasic response. The decrease in $I_{Ba}$ (first phase) always preceded the increase in $I_{Ba}$ (second phase). In addition, the distribution of delay times to the onset of these two phases in type II response is similar to the distribution of delay times to the onset of type I and III responses, respectively, as illustrated in Fig. 4.

Fig. 2. Moderate hyposmotic stimuli evoked 3 response types in L-type current. Exposure of pituitary cells to moderate hyposmotic stimuli resulted in the following 3 effects on L-type currents: decrease in L-type currents (type I response; A); biphasic effect on L-type currents [a decrease (1st phase, H1) followed by an increase (2nd phase, H2) in $I_{Ba}$] (type II response; B); and an increase in L-type currents (type III response; C). Left: individual $I_{Ba}$ waveforms. Right: the full time course of the experiments. Gray line, hyposmotic medium. Note that the 3 effects on $I_{Ba}$ were not associated with significant changes in $I_{H}$. D: left, summary of the 3 response types (I, II, and III). Right: the distribution of the 3 response types: 25%, 50%, and 25% for response types I, II, and III, respectively. Only the first exposures to hyposmotic media are included in these experiments; $n$, no. of cells.
Effects of strong hyposmotic stimuli on calcium channel currents. Because repeated exposures to the same moderate hyposmotic stimulus resulted in better manifestation of type III response (increase in $I_L$), we thought that it was of interest to examine the effects of stronger hyposmotic stimuli on calcium channel currents. One of these experiments is illustrated in Fig. 5. $I_T$ and $I_L$ were activated by a double-pulse protocol, as shown in Fig. 1. Decrease in [O$s$] by 32% resulted in a substantial increase in the amplitude of both $I_T$ and $I_L$ (Fig. 5A). The full time course of this experiment shows that the hyposmotic enhancement in the amplitude of $I_T$ (by 43%) and $I_L$ (by 63%) was reversible and that it was not associated with significant changes in holding currents (Fig. 5B). Similar results were observed in additional experiments. $I_L$ were increased by 44 ± 5% ($n = 20$), whereas $I_T$ were increased by 28 ± 4% ($n = 7$) ($P < 0.04$, unpaired $t$-test). Hence, only increases in calcium channel currents were observed in response to strong hyposmotic stimuli. This difference between strong and moderate hyposmotic stimuli in their effects on $I_L$ may stem from differences in the extent of pituitary cell swelling. Indeed, Table 2 shows that swelling in response to strong hyposmotic stimuli was larger than swelling in response to moderate hyposmotic stimuli.

Hyposmotic effects on $I_L$: voltage independence. The hyposmotic effects on $I_L$ may have resulted from hyposmotically induced changes in the voltage dependence of $I_L$. We therefore examined the effects of hypomolarity on $I-V$ relationships of $I_{Ba}$ that were activated with voltage ramps from $-100$ to $80$ mV (ramp duration $0.5$ s, interval $15$ s).

Figure 6A shows $I-V$ curves that were activated by voltage ramps before and during exposure to a strong hyposmotic stimulus. The small hump on the rising phase of the $I-V$ curve (at about $-30$ mV) may represent the activation of T-type barium currents, whereas the maximal peak of the $I-V$ curve (defined as $I_{max}$, at $-5$ mV) represents mainly the activation of L-type barium currents. Exposure to the strong hyposmotic stimulus resulted in a substantial increase in $I_{max}$ (by 30%) that was not associated with a significant voltage shift in the $I-V$ relationship. This voltage independence of the hyposmotic effects is further illustrated in the normalized $I-V$ curves (Fig. 6B) that were obtained by normalizing the $I-V$ curves in Fig. 6A to $I_{max}$. It is clear from these normalized curves that the hyposmotic effect was not associated with any significant changes in the voltage at which $I_{Ba}/I_{max}$ reached 50% ($V_{0.5}$) or in the voltage at which $I_{Ba}/I_{max}$ reached 100% ($V_{peak}$). The full time course of the experiment (Fig. 6C) shows that the hyposmotic effects on the $I-V$ relationships (on $I_{max}$) are fully reversible. Similar effects were observed in eight cells that were exposed to strong hyposmotic stimuli and in six cells that were exposed to moderate hyposmotic stimuli.
To intact pituitary cells: perforated patch recordings. Exposure to a strong hyposmotic stimulus (207 mosM) resulted in a reversible increase in both $I_T$ (by 17%) and $I_L$ (by 29%). Similar effects were observed in additional cells. $I_L$ were increased by 25 ± 3% ($n = 11$), and $I_T$ were increased by 17 ± 4% ($n = 5$) ($P < 0.05$, unpaired t-test). Figure 8B shows the effects of a strong hyposmotic stimulus on $I-V$ curves that were elicited by voltage ramps as described above (see Fig. 6). Exposure to the strong hyposmotic stimulus (207 mosM) resulted in an increase in the peak current of the $I-V$ curve (by 12%) without any significant shift in $V_{0.5}$ or $V_{peak}$ (as judged from the normalized $I-V$ curves seen below).

Similar effects were observed in four cells that were exposed to strong hyposmotic stimuli and in four cells that were exposed to moderate hyposmotic stimuli. Table 3 shows that the voltage shifts in $V_{0.5}$, $V_{peak}$, and $E_r$ in these experiments were insignificant ($<2$ mV). Hence, the hyposmotic effects on calcium channel currents in intact pituitary cells cannot be attributed to hyposmotic stimuli. Table 3 shows that the voltage shifts in $V_{0.5}$, $V_{peak}$, and apparent reversal potential ($E_r$) in these experiments were insignificant ($<2$ mV). Therefore, it is reasonable to suppose that the effects of osmotic swelling on $I_{Ba}$ result from hyposmotic effects on the activity of calcium channels rather than voltage-dependent shifts in the activation of calcium channels.

Hyposmotic effects on $I_L$: chloride-free media. To rule out the possibility that activation of a swell-dependent chloride conductance contributes to the hyposmotic effects on calcium channels, we examined hyposmotic effects on $I_{Ba}$ in chloride-free media. Figure 7A shows that exposure of a pituitary cell to a strong hyposmotic stimulus in a chloride-free medium resulted in a reversible increase in the amplitude of $I_L$. The full time course of this experiment is illustrated in Fig. 7B. The hyposmotic stimulus (214 mosM) caused a reversible increase in $I_L$ (by 35%) without changing the holding currents. Similar results were observed in additional experiments that were performed in chloride-free media with strong hyposmotic stimuli. $I_L$ were increased by 26 ± 5% ($n = 5$), and $I_T$ were increased by 19 ± 3% ($n = 4$).

Hyposmotic effects on calcium channel currents in intact pituitary cells: perforated patch recordings. To examine hyposmotic effects on $I_{Ba}$ under conditions that are more similar to the normal physiological conditions of the cell, we used the perforated patch method (21). With this method the cell remains intact and retains most of its intracellular components. Figure 8 summarizes experiments that were performed with the perforated patch method. Figure 8A shows the effects of a strong hyposmotic stimulus on $I_T$ and $I_L$ that were activated by a double-pulse protocol. Exposure to the strong hyposmotic stimulus (207 mosM) resulted with a reversible increase in both $I_T$ (by 17%) and $I_L$ (by 29%). Similar effects were observed in additional cells. $I_L$ were increased by 25 ± 3% ($n = 11$), and $I_T$ were increased by 17 ± 4% ($n = 5$) ($P < 0.05$, unpaired t-test). Figure 8B shows the effects of a strong hyposmotic stimulus on $I-V$ curves that were elicited by voltage ramps as described above (see Fig. 6). Exposure to the strong hyposmotic stimulus (207 mosM) resulted in an increase in the peak current of the $I-V$ curve (by 12%) without any significant shift in $V_{0.5}$ or $V_{peak}$ (as judged from the normalized $I-V$ curves seen below).

Similar effects were observed in four cells that were exposed to strong hyposmotic stimuli and in four cells that were exposed to moderate hyposmotic stimuli. Table 3 shows that the voltage shifts in $V_{0.5}$, $V_{peak}$, and $E_r$ in these experiments were insignificant ($<2$ mV). Hence, the hyposmotic effects on calcium channel currents in intact pituitary cells cannot be attributed to

Fig. 4. Distribution of latencies to response types I, II, and III in L-type currents. The histogram illustrates the mean latencies ($L_{II}$) to response types I, II (phase I and phase II), and III. Analysis of variance shows significant differences between the mean values of $L_{II}$ ($F = 16.60, P < 0.0001$). $L_T$ to type I response is statistically different from $L_T$ to type III response. Similarly, $L_T$ to phase I and phase II (in type II response) are statistically different. In addition, the post hoc test shows there is no statistical difference between $L_T$ to type I and III responses and $L_T$ to phases I and II (type II response), respectively (Tukey’s method for multiple comparisons, $\alpha = 0.005$).

This statistical analysis suggests that phases I and II of type II response correspond to type I and III responses, respectively. Latencies were measured as the time difference between the onset of perfusion (with the hyposmotic medium) and the peak of the hyposmotic response.

Fig. 5. Effects of strong hyposmotic stimuli on T-type and L-type $I_{Ba}$. A: T-type and L-type $I_{Ba}$ are activated by a double-pulse protocol to −30 and 0 mV, respectively. Decrease in $[Os]_o$, by 32% (strong hyposmotic stimulus) resulted in an increase in both L-type and T-type currents. B: the full time course of the experiment shows that the hyposmotic effect is reversible and that $I_{Ba}$ throughout the hyposmotic effect stays constant. Hypo (214), $[Os]_o = 214$ mosM.
Table 2. Percent increase in pituitary cell volume in response to strong and moderate hyposmotic stimuli

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<tr>
<th>Strong Stimuli (All Response Types)</th>
<th>Moderate Stimuli (Different Response Types)</th>
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<td>80 ± 7 (17)</td>
<td>80 ± 7 (17)</td>
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Values are means ± SE for no. of cells in parentheses. Changes in cell diameter were used to calculate changes in cell volume. Swelling in response to strong stimuli was larger than swelling in response to moderate stimuli. The insignificant P value (<0.08) may result from the low-resolution method used to measure changes in cell diameter (see RESULTS). *P < 0.08, unpaired t-test, compared with strong stimuli; †P < 0.03, unpaired t-test, compared with strong stimuli; ‡P < 0.3, unpaired t-test, compared with type II and type III responses.

Hyposmotic-induced changes in the voltage dependence of the calcium channels. Figure 8C summarizes the hyposmotic effects on $I_L$ and $I_T$ that were obtained with perforated patch recording. Interestingly, the histogram in Fig. 8C shows only increases in $I_L$ in response to moderate hyposmotic stimuli, in contrast to the three response types that were observed with whole cell recordings (Fig. 2). Thus it appears that in intact pituitary cells exposure to either moderate or strong hyposmotic stimuli results only in increases in calcium channel currents.

Hyposmotic swelling is not accompanied by increase in cell surface area. The hyposmotic effects on calcium channel currents were consistently accompanied by an increase in pituitary cell diameter (measured at the end of exposure to hyposmotic stimuli by using a scale that was inserted into the ocular of the microscope). Pituitary cell diameter increased from 11.9 ± 0.3 μm in control to 14.5 ± 0.4 μm (n = 37) during exposure to hyposmotic media ($P < 0.001$, paired t-test) and returned back to control levels (11.8 ± 0.4 μm, n = 26) after washout of hyposmotic media (see also Table 2). Because pituitary cells are spherical cells, this increase in cell diameter corresponds to ~80% increase in pituitary cell volume and ~48% increase in pituitary cell surface area. These changes in cell volume and cell membrane surface area can affect our experimental results. We therefore monitored in several experiments simultaneous changes in $I_{Ba}$, $C_m$, and $R_a$. In these experiments we used a double-pulse protocol. The first subthreshold pulse activated capacitive currents (uncompensated) to measure changes in $C_m$ and $R_a$, and the second pulse, a voltage ramp, activated $I_{Ba}$ (see EXPERIMENTAL PROCEDURES).

Fig. 9 shows that a strong hyposmotic stimulus elicited a 26% increase in peak $I_{Ba}$ (Fig. 9, A and C) without significant changes in $C_m$ and $R_a$ (Fig. 9B). $C_m$ in control conditions (8.0 pF) was not much different from $C_m$ measured during exposure to the hyposmotic stimulus (7.4 pF). Similarly, $R_a$ in control conditions (9.1 MΩ) was not much different from $R_a$ measured during exposure to the hyposmotic stimulus (9.6 MΩ). Similar results were obtained in seven experiments. Exposure to strong hyposmotic stimuli resulted in significant increases in peak $I_{Ba}$ (by 48 ± 6%) but not in significant changes in $C_m$ and $R_a$. $C_m$ values in control conditions (8.5 ± 0.4 pF) were not significantly different from $C_m$ values during the hyposmotic stimulus (8.4 ± 0.4 pF; $P < 0.709$, paired t-test). Similarly, $R_a$ values in control conditions (18.1 ± 2.9 MΩ) were not significantly different from $R_a$ values during the hyposmotic stimuli (16.3 ± 2.7 MΩ; $P < 0.089$, paired t-test). Thus the hyposmotically induced changes in $I_{Ba}$ observed in this study cannot be attributed to changes in either $C_m$ or $R_a$. This result goes along with previous studies that demonstrated that hyposmotic cell swelling (11, 15, 83) is not accompanied by significant increases in $C_m$ (or cell membrane surface area).
Table 3. *Hyposmotic effects on normalized I-V relationships*

<table>
<thead>
<tr>
<th></th>
<th>%Response</th>
<th>(\Delta V_{0.5}, \text{mV} )</th>
<th>(V_{\text{peak}}, \text{mV} )</th>
<th>(E^* ), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Hypo</td>
</tr>
<tr>
<td>Whole cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong stimulus</td>
<td>46 (\pm) 10(8)</td>
<td>0.8 (\pm) 0.4(8)</td>
<td>3.1 (\pm) 1.1(8)</td>
<td>3.1 (\pm) 1.3(8)</td>
</tr>
<tr>
<td>Moderate stimulus†</td>
<td>5 (\pm) 3(6)</td>
<td>-0.1 (\pm) 1.1(6)</td>
<td>7.5 (\pm) 1.7(6)</td>
<td>7.3 (\pm) 2.1(6)</td>
</tr>
<tr>
<td>Perforated patch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong stimulus</td>
<td>21 (\pm) 3(4)</td>
<td>-1.4 (\pm) 0.4(4)</td>
<td>8.5 (\pm) 0.4(4)</td>
<td>6.9 (\pm) 0.7(4)</td>
</tr>
<tr>
<td>Moderate stimulus</td>
<td>9 (\pm) 2(4)</td>
<td>-0.9 (\pm) 0.4(4)</td>
<td>4.8 (\pm) 2.0(4)</td>
<td>3.2 (\pm) 2.6(4)</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE for no. of experiments in parenthesis. \(\Delta V_{0.5}\), voltage shift in voltage at which barium current \(I_{\text{Ba}}\) normalized to maximal peak of current-voltage voltage \((I-V)\) curve \(I_{\text{max}}\) reached 50%; \(V_{\text{peak}}\), voltage at which \(I_{\text{Ba}}/I_{\text{max}}\) reached 100%; \(E^*\), reversal potential; Hypo, hyposmotic. The effects of osmotic swelling on \(V_{0.5}\), \(V_{\text{peak}}\), and \(E^*\), were very small (<2 mV) and insignificant statistically (paired t-test). †\(E^*\) measured only when \(I_{\text{Ba}}\) reversed from inward to outward currents. ‡Only biphasic responses were observed in these experiments. The parameters introduced are for phase III (increase in \(I_{\text{Ba}}\)), similar results were obtained for phase I (decrease in \(I_{\text{Ba}}\)).

**DISCUSSION**

**Overview.** This study demonstrates that calcium channel currents in pituitary cells can be modulated by decrease in \([\text{Os}]_e\). Exposure of pituitary cells to moderate hyposmotic stimuli (18–22% decrease in \([\text{Os}]_e\)) resulted in three different response types in \(I_L\), type I, II, and III responses, and in increases in \(I_T\). Exposure of pituitary cells to strong hyposmotic stimuli (31–32% decrease in \([\text{Os}]_e\)) resulted only in increases in both \(I_L\) and \(I_T\). Similarly, when the perforated patch method was used, exposure to either moderate or strong hyposmotic stimuli resulted only in increases in both \(I_L\) and \(I_T\). Thus it appears that the main effect of decrease in \([\text{Os}]_e\) (and consequently pituitary cell swelling) is increase in calcium channel currents. This increase was differential: \(I_L\) were more sensitive than \(I_T\) to the hyposmotic stimuli (see Figs. 1, 5, and 8). This differential sensitivity may stem either from different sensitivities of the channel proteins to osmotic swelling or from different arrangements of the channel proteins in the plasma membrane. The latter possibility may also explain the independent effects of moderate hyposmotic stimuli on \(I_L\) and \(I_T\).

The three response types in \(I_L\) that were observed in response to moderate hyposmotic stimuli (Fig. 2) may be explained by two opposing hyposmotic effects on \(I_L\): decrease (type I response) and increase (type III response) in \(I_L\). Simultaneous activation (or superposition) of these two responses may result in the appearance of the biphasic response (type II response). Indeed, type I response was observed only while pituitary cells were exposed to moderate hyposmotic stimuli. It was attenuated or disappeared upon repetitive exposures to the same moderate hyposmotic stimulus (Fig. 3). Its onset was always faster than the onset of type III response (Fig. 4), and, in addition, it was associated with smaller increases in pituitary cell volume (Table 2). Thus it appears that the mechanism underlying type I response is different from that underlying type III response and that type I response is possibly overrun (or inactivated) by strong hyposmotic stimuli. Interestingly, the use-dependent inactivation of type I response resembles the use-dependent adaptation of mechanosensitive ion channels in *Xenopus laevis* oocytes (17) and in snail neurons (48).

Are calcium channels in pituitary cells mechanosensitive? The sensitivity of calcium channels in the membrane of pituitary cells to hyposmotic swelling implies that they are mechanosensitive. This sensitivity to hyposmotic swelling corroborates previous studies that demonstrated that calcium channels in pituitary cells are sensitive to hyperosmotic shrinkage (37), to a stream of physiological solution (2), and to membrane...
tension produced during seal formation (41). Interestingly, the use of hyposmotic swelling as a tool to study mechanosensitivity of ion channels gained support from recent studies that demonstrated, by using laser tweezers, that hyposmotic swelling is associated with increase in mechanical membrane tension (11, 12). Indeed, the hyposmotic enhancements of $I_L$ in smooth muscle cells (26, 31, 54) and in cardiac myocytes (36) were mimicked by increase in mechanical membrane tension, suggesting that L-type channels in these cells are mechanosensitive. Furthermore, it was demonstrated recently that recombinant L-type (35) and N-type (6) channels are mechanosensitive, suggesting that the mechanosensitivity of voltage-gated calcium channels might be a general phenomenon. Therefore, it is reasonable to suppose that the hyposmotic enhancements

![Diagrams showing the effects of hyposmotic stress on calcium channel currents](http://ajpcell.physiology.org)
of $I_L$ and $I_T$ observed in this study reflect their sensitivity to mechanical membrane tension.

Possible mechanisms for effects of hyposmolarity on calcium channels. Our study shows that the hyposmotic enhancements of $I_{Ba}$ cannot be attributed to a voltage shift in the activation of calcium channel currents (Figs. 6 and 8), to activation of an anionic conductance (Fig. 7), or to an increase in membrane surface area (Fig. 9). In addition, because $[Ca]_{i}$ was strongly buffered (see Experimental Procedures), the hyposmotic enhancements of $I_{Ba}$ cannot be attributed to release of calcium from internal stores.

The biophysical basis for the hyposmotic effects is unknown at this stage. However, it is reasonable to suppose that osmotic swelling alters the activity of calcium channels ($NP_{o}$, where $N$ is the number of channels contributing to the calcium current and $P_{o}$ is the open probability of single calcium channels). The voltage independence of the hyposmotic effects on $I_L$ implies that $N$ might be the parameter affected. However, a detailed kinetic study will be needed to distinguish between the role of parameters $N$ and $P_{o}$ in the hyposmotic effects. Interestingly, it was recently reported that membrane stretch increased the activity of recombinant L-type (35) and N-type (6) calcium channels. In the case of L-type channels this increase in activity was thought by the authors to reflect a change in $P_{o}$ (35). In the case of N-type channels, neither $I$-$V$ shifts nor activation rates of whole cell currents accompanied this increase in activity, implying that $N$ might be the parameter affected (6).

The hyposmotic enhancements of $I_L$ and $I_T$ may stem from direct mechanical effects of membrane expansion on the calcium channel protein or, alternatively, from various indirect effects. It has been shown that the effect of membrane stretch on recombinant L- and N-type channels is not dependent on interactions between the $\alpha_1$-subunit and its accessory subunits (6, 35). Thus it appears that membrane tension may be transferred to the $\alpha_1$-subunit either directly or indirectly via other proteins that interact with the $\alpha_1$-subunit. The cytoskeleton of the cell is a natural candidate for this transfer of membrane tension (16, 42). Indeed, it has been shown that hyposmotic enhancement of $I_L$ in smooth muscle cells is reduced by the actin-cytoskeleton disrupter cytochalasin D (Cyt-D) and potentiated by the actin cytoskeleton stabilizer phalloidin (55). Interestingly, several studies in recent years pointed to a functional link between calcium channels and the cytoskeleton (22, 23). In two recent studies it was demonstrated that actin cytoskeleton disruptors and stabilizers can modulate the amplitude of $I_{Ba}$ in cardiac cells (29) and in vascular smooth muscle cells (39).

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**Fig. 9.** Hyposmotic effects on $I_{Ba}$ are not associated with increase in cell membrane surface area. Cell membrane capacitance ($C_m$) was taken as a measure of cell membrane surface area. A: to monitor simultaneously hyposmotic effects on $C_m$ and on $I_{Ba}$, we used a double-pulse protocol. The first subthreshold pulse activated capacitive currents, and the second pulse, a voltage ramp, activated $I_{Ba}$. Exposure to a strong hyposmotic stimulus resulted in increase in $I_{Ba}$ without significant changes in capacitive currents. This is further illustrated in B and C: B: capacitive currents before (black line) and after (gray line) exposure to the hyposmotic medium (expanded from A). The hyposmotic stimulus had no significant effect on the shape of the capacitive currents. $C_m$ in control conditions was 8.0 pF, and $C_m$ during the hyposmotic stimulus was 7.4 pF. C: $I$-$V$ relationship before (black line) and after (gray line) exposure to the hyposmotic medium (expanded from A). A significant hyposmotic increase in $I_{Ba}$ was observed.
Cyt-D produced a decrease in $I_{Ba}$, whereas phallolidin increased $I_{Ba}$.

Increased tension in the cytoskeleton may affect the calcium channel directly, leading to changes in the conformational state of the channel protein and thus altering its activity. Alternatively, increased tension in the cytoskeleton may affect the activity of phosphorylating enzymes known as regulators of L-type calcium channels. In a recent study it was shown that inhibitors of protein tyrosine kinase (PTK) modulate hyposmotically enhanced $I_{Ba}$ in smooth muscle cells (26). On the basis of these results these authors suggest that c-SRC, a cytoplasmic PTK that may be associated with the cytoskeleton, is involved in the hyposmotic modulation of L-type calcium currents in these smooth muscle cells.

Membrane tension may also be conferred to the calcium channel protein via the phospholipid bilayer (16, 42). In fact, it has been suggested that altering membrane stiffness by using lipophilic compounds can modulate influx through N-type channels in neuroblastoma cells (34). However, cholesterol, which was used to mimic membrane stretch in that study, had no effect on the extent of activation of N-type currents, in contrast to the effect of osmotic swelling on calcium currents described here (and to the effects of membrane stretch on recombinant channels). Interestingly, it has been suggested that membrane phospholipids, rather than the cytoskeleton, mediate osmomechanical effects on glutamate receptors (7).

Possible physiological significance. The idea that hyposmotically induced membrane expansion directly modulates calcium influx through L-type channels in pituitary cells and thereby [Ca]i and hormone secretion was first proposed by Greer and colleagues (43). Previous studies suggested that hyposmotic swelling may affect calcium influx indirectly through L-type channels by altering membrane potential (4, 10, 38). The present study demonstrates direct effects of hyposmotic swelling on calcium influx through L-type channels. Calcium influx through L-type channels plays a key role in regulating both basal and stimulated hormone secretion from pituitary somatotrophs and lactotrophs (8, 27, 50). Therefore, it is expected that hypotonic swelling would enhance hormone secretion from these pituitary cells. However, the physiological significance of this sensitivity to membrane tension is not clear. Although the hyposmotic stimuli used in this study were not excessively high (18–32% decrease in [Os]e), it is not feasible that pituitary cells would be challenged with similar hyposmotic stimuli during normal physiological life. Pituitary cells, like most mammalian cells (except kidney cells and intestinal cells), experience very small alterations in [Os]e during normal physiological conditions (30), and decrease in [Os]e by >10% is considered to be pathological. Nevertheless, alteration in membrane tension may occur also under normal physiological conditions. One interesting possibility is that changes in the metabolic state of pituitary cells may be associated with changes in intracellular osmolarity, and as a result with changes in pituitary cell volume, under normal physiological conditions (19, 30). According to this hypothesis, volume regulatory mechanisms are continuously activated during normal physiological life and calcium channels may participate in these volume regulatory mechanisms (43). Another interesting possibility is that calcium channels sense alteration in membrane tension during the process of exocytosis (12, 25) and that these alterations in membrane tension participate in regulating calcium influx during the exocytotic event, thus providing a novel autoregulatory mechanism. This latter hypothesis is based on the recently reported physical and functional links between calcium channels and proteins of the exocytotic machinery. Most of this evidence was obtained for neuronal calcium channels (47), and similar evidence exists also for L-type calcium channels (53).

Interestingly, it was proposed recently that mechanosensitive L-type calcium channels in human intestinal smooth muscle cells participate in a feedback mechanism that controls intestinal muscle contraction during normal or pathological digestive activity (20). In addition, it has been proposed that mechanosensitivity of L-type channels in cardiac myocytes may affect the contraction of cardiac muscle under normal and pathophysiological conditions such as ischemic heart disease (36).

DISCLOSURES

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REFERENCES


