Cell volume increase and extracellular Ca\(^{2+}\) are needed for hyposmotically induced prolactin release in tilapia

A. P. Seale,\(^1,3\) N. H. Richman III,\(^1\) T. Hirano,\(^1\) I. Cooke,\(^2,3\) and E. G. Grau\(^1,3\)

\(^1\)Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe 96744; and \(^2\)Pacific Biomedical Research Center and \(^3\)Department of Zoology, University of Hawaii, Honolulu, Hawaii 96822

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Seale, A. P., N. H. Richman III, T. Hirano, I. Cooke, and E. G. Grau. Cell volume increase and extracellular Ca\(^{2+}\) are needed for hyposmotically induced prolactin release in tilapia. Am J Physiol Cell Physiol 284: C1280–C1289, 2003.—In the tilapia (Oreochromis mossambicus), as in many euryhaline teleost fish, prolactin (PRL) plays a central role in freshwater adaptation, acting on osmoregulatory surfaces to reduce ion and water permeability and increase solute retention. Consistent with these actions, PRL release is stimulated as extracellular osmolality is reduced both in vivo and in vitro. In the current experiments, a perfusion system utilizing dispersed PRL cells was developed for permitting the simultaneous measurement of cell volume and PRL release. Intracellular Ca\(^{2+}\) was monitored using fura 2-loaded cells under the same conditions. When PRL cells were exposed to hyposmotic medium, an increase in PRL cell volume preceded the increase in PRL release. Cell volume increased in proportion to decreases of 15 and 30% in osmolality. However, regulatory volume decrease was clearly seen only after a 30% reduction. The hyposmotically induced PRL release was sharply reduced in Ca\(^{2+}\)-deleted hyposmotic medium, although cell volume changes were identical to those observed in normal hyposmotic medium. In most cells, a rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\_i\)]) during hyposmotic stimulation was dependent on the availability of extracellular Ca\(^{2+}\), although small transient increases in [Ca\(^{2+}\)\(\_i\)] were sometimes observed upon introduction of Ca\(^{2+}\)-deleted media of the same or reduced osmolality. These results indicate that an increase in cell size is a critical step in the transduction of an osmotic signal into PRL release and that the hyposmotically induced increase in PRL release is greatly dependent on extracellular Ca\(^{2+}\).

osmoreception; signal transduction; regulatory volume decrease

IN MANY EURYHALINE FISH, including the tilapia (Oreochromis mossambicus), prolactin (PRL) plays a central role in freshwater osmoregulation. By acting on osmoregulatory surfaces, PRL stimulates ion retention and decreases water influx (3, 6, 13, 22). Consistent with its osmoregulatory activity, PRL release from the tilapia pituitary increases as extracellular osmolality is decreased both in vivo and in vitro (8, 25, 33, 34, 42). Blood osmolality in freshwater-acclimated tilapia (~310 mosmol/kgH\(_2\)O) is somewhat lower than that in sea-water-acclimated fish (350 mosmol/kgH\(_2\)O) (33, 42). The release of PRL increases in vitro in direct relation to reductions in osmotic pressure that fall within the physiological range observed in vivo (7, 8, 27). Thus PRL release is governed by extracellular osmolality, the factor that PRL regulates at the organismic level.

Under hyposmotic conditions, most cells swell and then undergo what has come to be called a regulatory volume decrease (RVD) (19). In perfusion studies, the rise in PRL release from intact RPDs reaches a peak within 30 min after the onset of hyposmotic stimulation before subsiding to an elevated plateau (7). Furthermore, this elevation in PRL release from whole pituitaries incubated in hyposmotic medium is maintained for up to 12 h compared with those maintained in hyperosmotic medium (33). These and other studies have employed either whole pituitaries or intact RPDs to investigate peak and sustained PRL release in response to hyposmotic medium. The time course followed by hyposmotically induced PRL release from dispersed PRL cells has not been described, although it is known that after overnight incubation, both intact RPDs and dispersed PRL cells show similar responses to hyposmotic medium (4). It is also not known whether the decrease in PRL release from peak levels after hyposmotic stimulation is due to a decrease in cell volume as a consequence of RVD.

Several studies using mammalian renal cells and cell lines have suggested that a rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(\_i\)]) is important for RVD and that this volume adjustment is dependent on extracellular Ca\(^{2+}\) (21, 37, 41). Increases in PRL release can be induced experimentally by increasing [Ca\(^{2+}\)\(\_i\)], with Ca\(^{2+}\) ionophores and, over a long term (18 h), are dependent on extracellular [Ca\(^{2+}\)] (8, 9, 11). Because hyposmotic medium alone is sufficient stimulus for a
rise in [Ca$^{2+}$], it is hypothesized that extracellular Ca$^{2+}$ entry is an important step in the activation of hyposmotically induced PRL release.

In the present experiments, a perfusion system utilizing dispersed PRL cells permitted switches to media of different osmotic concentrations and ionic compositions with minimal mixing. By video imaging the cells during perfusion, the time course of the hyposmotically induced increase in cell volume could be observed together with the increase in PRL release into the perfusate. A similar chamber, containing PRL cells exposed to the same conditions, allowed the determination of the changes in [Ca$^{2+}$], from fura 2-AM-loaded cells. Thus we are able to show for the first time that a cell volume increase, occurring rapidly in response to hyposmotic medium, precedes the rise in PRL release in a model in which osmotically sensitive cells secrete a hormone responsible for maintaining osmotic homeostasis at the organismic level. Furthermore, the involvement of extracellular Ca$^{2+}$ in changes in [Ca$^{2+}$], cell volume, and PRL release after hyposmotic stimulation has been characterized.

**MATERIALS AND METHODS**

**Fish.** Mature tilapia (*Oreochromis mossambicus*) of both sexes, weighing 200–600 g, were obtained from a population maintained at the Hawaii Institute of Marine Biology. They were kept outdoors in 5,000-liter tanks in fresh water and fed twice daily with Purina Trout Chow (~2% of body wt, twice a day). Water temperature was 22–26°C. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

**Cell dispersion and perfusion.** Pituitaries were removed after decapitation. The RPD, composed of nearly 100% PRL cells, was dissected and placed in groups of 3 in a 24-well culture plate with 500 μl of hyperosmotic (355 mosmol/kgH$_2$O) Krebs bicarbonate-Ringer solution, containing glucose (500 mg/l), glutamine (290 mg/l), and Eagle's minimal medium, preceded the rise in PRL release in a model in which osmotically sensitive cells secrete a hormone responsible for maintaining osmotic homeostasis at the organismic level. Furthermore, the involvement of extracellular Ca$^{2+}$ in changes in [Ca$^{2+}$], cell volume, and PRL release after hyposmotic stimulation has been characterized.

**Intracellular Ca$^{2+}$ concentration.** The procedure for fura 2 loading in dispersed PRL cells has been described previously (Hyde GN, Seale AP, Grau EG, and Borski RJ, unpublished observations). Briefly, PRL cells were dispersed as described in Cell dispersion and perfusion but were placed on round coverslips (22-mm diameter) previously coated with poly-L-lysine (0.1 mg/ml) and preincubated overnight in 355 mosmol/kgH$_2$O medium. Cells were then loaded with 5 μM fura 2-AM (Molecular Probes, Eugene, OR), freshly diluted from a stock solution of 5 mM in anhydrous DMSO, for 90 min at 28°C. After loading, the coverslips with cells were rinsed and placed in baseline medium (355 mosmol/kgH$_2$O) for 30 min prior to data recording. Individual coverslips were mounted in a metal chamber that allows the cells to be perfused with media of different compositions (4). The chamber was mounted on the stage of an inverted microscope (Nikon).

**Cell volume.** Cell images were captured every 5 min with a video camera and stored in a computer (Macintosh Hex). The microscope was equipped with a ×100 oil-immersion objective lens (Nikon, Japan) and the total magnification of the image, as seen on the screen, was ×1,200. The cross-sectional areas of cells were estimated by tracing each cell from digitally captured images. Images were processed with the NSC Imaging software. Areas (A) were obtained in pixels and then transformed into square micrometers as determined by viewing a stage micrometer. Cell volume (V) was estimated from the area as follows

$$r = \sqrt{\frac{A}{\pi}} \quad \text{and} \quad V = 4/3\pi(r^3)$$

Cell volume was expressed as a percent change from the baseline (taken as 100%). The baseline value was taken as the mean of the volume calculated for the first five time points in pretreatment medium.

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goldfish gonadotropes and somatotropes incubated at 28°C (17). The calibration procedure described by Kao (18) was utilized for obtaining minimum and maximum ratios, R_{min} and R_{max}. Briefly, low and high [Ca^{2+}]_{i} values were obtained by employing EGTA-buffered media and normal media containing 20 μM digitonin, respectively, to flush 2-AM-loaded PRL cells. The calibration equation was then plotted with the Image-1/FL software, and [Ca^{2+}]_{i} determinations were extrapolated from the curve. The calibration estimates indicate that resting PRL cells, with ratios of 0.4–0.6, have a [Ca^{2+}]_{i} of 3–10 × 10^{-8} M, and PRL cells depolarized with high [K^{+}] show a ratio of 2–3, corresponding to a [Ca^{2+}]_{i} of 1–2.5 × 10^{-6} M. Perfusion experiments for [Ca^{2+}]_{i} determinations were replicated at least three times.

Radioimmunoassays. The tilapia pituitary secretes two distinct PRL molecules, PRL_{188} and PRL_{177}, that are encoded by separate genes. The quantity of both PRLs in the perfusate was measured by homologous radioimmunoassays (1, 33, 42). In the present experiment, PRL_{188} and PRL_{177} release showed highly similar patterns in response to a reduction in osmolality (Fig. 1). Thus, for clarity, only the PRL_{188} response is presented. Values obtained as nanograms per milliliter were converted to a percent change from the baseline set at 100%, which was determined from the average of the first five time points under pretreatment conditions (355 mosmol/kgH_{2}O).

Statistical analysis. Slope comparisons of cell volume changes were carried out by multiple regression and simple regression analyses. For the comparison of PRL release in Ca^{2+}-deleted medium and normal hyposmotic medium, five values for each treatment period were added to generate a net response over 25 min. The net response over 25 min during the pretreatment was subtracted from the net response over 25 min during treatment for each replicate and then averaged. A pairwise t-test was employed to compare the average percent pretreatment change between cells in hyposmotic medium and Ca^{2+}-deleted hyposmotic medium. For comparisons of cell volume in Ca^{2+}-deleted medium and normal hyposmotic medium, the net change in cell volume under both conditions was computed by subtracting the average cell volume change in pretreatment medium from that in treatment medium. Comparisons between treatments were performed using a pairwise t-test. Calculations were performed using the Minitab statistical software package (State College, PA).

RESULTS

Effects of a reduction in medium osmolality on cell volume and PRL release. The effects of a hyposmotic medium on PRL release and cell volume were examined over a time course of 80 min (Fig. 2A). The increase in cell volume was proportional to the degree that medium osmolality was reduced. As the osmolality was reduced by 15%, the peak volume increase amounted to ~15% above baseline. Cell volume was consistently increased at the first measurement (5 min) after hyposmotic medium reached the cells and was maximum at the second measurement (10 min). Volume then decreased gradually, following a trajectory that could be well fitted by a linear regression (see below). The second PRL determination (but not the first) following change to the hyposmotic medium consistently showed a sevenfold increase in PRL, on average 700% for a 15% decrease in medium osmolality. A peak in PRL release occurred at the third determination (15 min), reaching as much as 11-fold above baseline release. The rate of PRL release then declined sharply to reach a plateau of two- to threefold above the baseline after 25 min. This plateau was sustained throughout the exposure to hyposmotic medium. The baseline rate of PRL release ranged between 0.8 and 1.6 ng/min, whereas the peak release following hyposmotic stimulation reached 12.5 ng/min. After the return to hyperosmotic (355 mosmol/kgH_{2}O) medium, cell volume declined to the original size within a single measurement, whereas PRL release declined more slowly.

Though a 15% reduction in osmolality falls well within the physiological range tolerated by this species, a 30% reduction is extreme (Fig. 2B). Cell volume increased rapidly after medium osmolality was reduced from 355 to 248 mosmol/kgH_{2}O, increasing to 30% above the baseline at the first determination. Cell volume then declined to 15% above baseline, following a slope than can be fitted to a linear regression (see below). The increase in PRL release observed after exposure to 248 mosmol/kgH_{2}O medium reached an average of 600% at the second determination (10 min) and a peak of 10-fold at the third determination (15 min). The time course of the peak response and the magnitude of PRL release are similar to those observed after a 15% decrease in medium osmolality. As at 15%, the PRL release declined to an elevated plateau of approximately twofold over baseline, which was sustained throughout the remaining exposure to 248 mosmol/kgH_{2}O medium. Both cell volume and PRL release returned to baseline levels after medium osmolality was switched back to 355 mosmol/kgH_{2}O.

The extent to which PRL cells undergo RVD in response to different reductions in medium osmolality was analyzed in light of the role that these cells play in osmoreception. The slope (between 40 and 110 min) of
cell volume decrease following the peak increase after a 30% reduction in osmolality was significantly steeper (P < 0.001) than the slope after a 15% reduction, reflecting a higher degree of cell volume decrease (Fig. 2C). Simple linear regressions of the changes in cell volume were also utilized as an indication of RVD. When the whole period of hyposmotic exposure (80 min) was accounted for, a significant decrease in cell volume was observed following the initial cell swelling for both 15 and 30% reductions in osmolality (P < 0.05 and P < 0.001, respectively). A significant linear regression (P < 0.01) for cell volume after a 30% drop in osmolality, but not after a 15% drop, was seen as early as 35 min after exposure to hyposmotic medium.

Intracellular Ca\(^{2+}\) oscillations. The baseline [Ca\(^{2+}\)]\(_i\) of PRL cells perfused with 355 mosmol/kg\(\text{H}_2\text{O}\) medium was expressed as 340/380 ratios and classified into three main patterns (Fig. 3). Quiescent or silent cells, exhibiting low spontaneous variation from baseline (Fig. 3A), accounted for 60% of the cells studied (111 of 186 cells). The remainder showed spontaneous activity.

Fig. 2. Effects of 15 (A) and 30% (B) reduction in medium osmolality (from 355 to 300 mosmol/kg\(\text{H}_2\text{O}\) and from 355 to 248 mosmol/kg\(\text{H}_2\text{O}\), respectively) on cell volume change and PRL release measured from the same preparation of dispersed PRL cells. PRL release and cell volume are expressed as percent change from the baseline. Horizontal bars represent the estimated time in which the solution reached the cells and are corrected for the temporal lag between cell volume and PRL release measurements. A: after exposure to 300 mosmol/kg\(\text{H}_2\text{O}\) medium, cell volume increased 15% above baseline (○), closely followed by a 10- to 11-fold increase in PRL release (●). B: exposure to 248 mosmol/kg\(\text{H}_2\text{O}\) medium increased cell volume 30% above baseline (●), also followed by a 10-fold increase in PRL release (○). Values are means ± SE (n = 4 replicate experiments for PRL release and 16 cells, taken from all experiments, for volume measurements). C: the slope of cell volume decline after the peak induced by a 30% reduction in osmolality (248 mosmol/kg\(\text{H}_2\text{O}\)) is significantly (P < 0.001) steeper than that of a 15% reduction (300 mosmol/kg\(\text{H}_2\text{O}\)). The slopes were compared by multiple regression analysis and are shown as solid lines.

Fig. 3. Patterns of basal intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in tilapia PRL cells. Traces represent the 340/380 ratio of single cells under control (355 mosmol/kg\(\text{H}_2\text{O}\)) medium and directly reflect [Ca\(^{2+}\)]\(_i\). Measurements under control medium were taken every 15 s for 15 min. A: trace of a single quiescent PRL cell displaying stable basal [Ca\(^{2+}\)]\(_i\), (observed in 111 of 186 cells). B: typical pattern of [Ca\(^{2+}\)]\(_i\), in a spontaneously active high-frequency oscillator (observed in 60 of 186 cells). C: a spontaneously active low-frequency oscillator (observed in 15 of 186 cells). These and all subsequent 340/380 ratios shown are from fura 2-AM-loaded, dispersed PRL cells. Traces shown are representative of those obtained in 186 PRL cells studied in 26 independent experiments.
that could be further classified into two patterns: spontaneous high-frequency oscillators (Fig. 3B) and low-frequency oscillators (Fig. 3C). The cells exhibiting these two patterns accounted for 32 and 8% respectively, of the total cells studied. Sporadic and transient peaks that show a range of amplitudes characterize the high-frequency spontaneous oscillators. Low-frequency oscillators are characterized by periods of slow increase and recovery to resting $[\text{Ca}^{2+}]_i$.

Effects of reduction in medium osmolality on $[\text{Ca}^{2+}]_i$. When medium osmolality was reduced from 355 to 300 mosmol/kgH$_2$O, $[\text{Ca}^{2+}]_i$ quickly increased (within 1–2 min) as indicated by the increase in 340/380 ratios (Fig. 4). Both oscillating and silent cells responded to the hyposmotic stimulus, and the traces presented in Fig. 4, A and B, are representative of 23 and 35 cells, respectively. The magnitude of $[\text{Ca}^{2+}]_i$ responses to hyposmotic medium varied among cells, reaching ratios of up to 6 and as low as 0.5 (Fig. 4, C and D, respectively). Of the 69 cells analyzed for $[\text{Ca}^{2+}]_i$ responses to hyposmotic medium, 55% responded with ratios up to 1, 25% responded with ratios up to 2, and the remaining 20% had peaks with ratios higher than 2. Most cells responded with a peak in $[\text{Ca}^{2+}]_i$ that typically lasted from 5 to 10 min and subsided either to an elevated plateau (45% of cells) or baseline levels (39% of cells). The remaining 16% of cells did not increase the ratio above baseline after exposure to hyposmotic medium but did respond to high-[K$^+$] medium at the end of the experiment by increasing $[\text{Ca}^{2+}]_i$.

Prolactin cells respond strongly to depolarizing conditions by increasing $[\text{Ca}^{2+}]_i$ and PRL release (Hyde GN, Seale AP, Grau EG, and Borksi RJ, unpublished observations, and Ref. 30), and this property, or a second exposure to hyposmotic medium, was used as an indicator of cell integrity at the end of $[\text{Ca}^{2+}]_i$ measurements. The mean of ratios from each observed time point was taken from cells ($n = 10$) recorded during the same experimental run (Fig. 4E). In this example, after exposure to hyposmotic medium, the mean ratio increased from 0.7 to a peak of 1, after which it declined to baseline levels within 12 min. After the switch back to hyperosmotic medium, the mean ratio gradually declined to 0.6. A second exposure to hyposmotic medium increased the mean ratio to 0.9, and before the peak in $[\text{Ca}^{2+}]_i$ returned to

![Fig. 4. Effects of hyposmotic medium (300 mosmol/kgH$_2$O) on $[\text{Ca}^{2+}]_i$ in single PRL cells. Arrows indicate the estimated time in which the medium reached the cells. A: patterns of $[\text{Ca}^{2+}]_i$, from a single spontaneously active PRL cell in response to hyposmotic medium. B: patterns of $[\text{Ca}^{2+}]_i$, from a single quiescent PRL cell in response to hyposmotic medium. The magnitude of $[\text{Ca}^{2+}]_i$ changes in response to hyposmotic medium varied among single PRL cells, from very strong (C) to slight increases in 340/380 ratio (D). E: mean ratio of cells from a single representative experiment in which medium osmolality was changed from 355 to 300 mosmol/kgH$_2$O, switched back to 355 mosmol/kgH$_2$O, and reexposed to 300 mosmol/kgH$_2$O, followed by high-[K$^+$] hyperosmotic medium (355 mosmol/kgH$_2$O). Values are means ± SE ($n = 10$ distinct cells from a single experiment) at 1-min intervals. An increase in 340/380 ratio was observed after both hyposmotic stimulations, followed by a strong and rapid increase in ratios after exposure to high-[K$^+$] hyperosmotic medium. Responsiveness to high-[K$^+$] or hyposmotic medium at the end of all experimental runs was used as an indicator of cell viability.](image-url)
baseline, high-[K+] medium (355 mosmol/kgH2O) was introduced and elicited a sharp and strong increase in the mean ratio.

Effects of Ca2+ deletion on PRL release and cell volume. To examine the involvement of extracellular Ca2+ in hyposmotically induced PRL release, we exposed PRL cells to hyposmotic medium devoid of CaCl2 (Ca2+-deleted). Cells were initially perfused with normal hyperosmotic medium (355 mosmol/kgH2O, with 2 mM CaCl2), and after the switch to normal hyposmotic medium, PRL release was increased over threefold above the baseline. A significant (P < 0.05) decrease in net PRL release over a period of 25 min was observed in hyposmotic Ca2+-deleted medium compared with the normal hyposmotic control (Fig. 5A). Experiments were also conducted with EGTA (2 mM) in the incubation medium to ensure a low extracellular Ca2+ concentration. These experiments produced results similar to those when Ca2+ was deleted from the incubation medium (data not shown). Prolactin cell volumes increased in proportion to the reduction in osmolality regardless of the presence or absence of Ca2+ (Fig. 5B).

In both normal hyposmotic (i.e., containing Ca2+) and Ca2+-deleted hyposmotic media, RVD was not observed during the 30 min following a 15% drop in osmolality, as confirmed by regression analysis. As mentioned previously, RVD becomes measurable after 35 min in hyposmotic medium. In all treatments, cell volume increased ~10–15% above the baseline established in hyperosmotic medium, thus clearly indicating that deletion of Ca2+ from the incubation medium reduced hyposmotically induced PRL release but not cell swelling.

Effects of Ca2+ deletion on [Ca2+]i. The changes in [Ca2+]i in response to Ca2+-deleted hyposmotic medium clearly indicate the role of extracellular Ca2+ triggering hyposmotically induced PRL release. Exposure to Ca2+-deleted hyposmotic medium (300 mosmol/kgH2O) immediately after normal hyperosmotic medium (355 mosmol/kgH2O, with 2 mM CaCl2) did not alter the 340/380 ratio in 9 of 11 cells. This pattern, followed by an increase in [Ca2+]i in normal (i.e., Ca2+-containing) hyposmotic medium, is exemplified by the single cell recording in Fig. 6A. The other 2 of 11 cells responded to Ca2+-deleted hyposmotic medium with a transient increase in [Ca2+]i. A switch from normal hyperosmotic medium to Ca2+-deleted hyposmotic medium also produced either a transient increase
that transiently and strongly increased \([\text{Ca}^{2+}]_i\) at the end of the experiment.

Strongly to high \([\text{K}^+]\) in osmotic medium, is shown in Fig. 6B. This cell also responded to normal \([\text{Ca}^{2+}]_i\) following exposure to \([\text{Ca}^{2+}]_i\)-deleted hyperosmotic medium. This response was seen in 12 of 52 cells. After recovery, responsiveness to normal hyperosmotic medium or high \([\text{K}^+]\) was observed in 75 and 87% of cells, respectively, although to different magnitudes.

Of all cells subjected to \([\text{Ca}^{2+}]_i\)-deleted medium, 75% exhibited a recovery in their ability to respond to normal hyposmotic medium by increasing \([\text{Ca}^{2+}]_i\), and 87% responded to high \([\text{K}^+]\) in normal hyposmotic media at the end of the experiments by increasing \([\text{Ca}^{2+}]_i\). Of the 39 cells exposed to \([\text{Ca}^{2+}]_i\)-deleted hyperosmotic medium (devoid of CaCl2 and containing 2 mM EGTA) after pretreatment in normal hyperosmotic medium, 48% responded with a transient increase in \([\text{Ca}^{2+}]_i\), but none showed an increase in \([\text{Ca}^{2+}]_i\) after subsequent exposure to \([\text{Ca}^{2+}]_i\)-deleted hyperosmotic medium. Only 53% of these cells recovered by responding to normal hyposmotic medium after removal of EGTA, but 18 of 19 cells that were exposed to high \([\text{K}^+]\), under normal hyperosmotic medium, increased \([\text{Ca}^{2+}]_i\).

**DISCUSSION**

This is the first report to correlate changes in cell size and PRL release in an osmoreceptive model system, based on observations on the same population of PRL cells. The use of the tilapia PRL cell offers distinct advantages that are unavailable to researchers addressing similar questions in other osmoregulatory systems, such as the hypothalamo-neurohypophysial magnocellular systems that secrete vasopressin and oxytocin in mammals (5). Specifically, the tilapia PRL cell represents a model system in which the osmoregulatory output (PRL release) can be measured simultaneously with other parameters, such as cell size, that are involved in the osmoreceptive process.

The present findings indicate the importance of extracellular \([\text{Ca}^{2+}]_i\) in hyposmotically-induced PRL release. In the absence of extracellular \([\text{Ca}^{2+}]_i\), a reduction in osmolality and the subsequent increase in cell volume do not significantly increase PRL release. In the presence of extracellular \([\text{Ca}^{2+}]_i\), however, an increase in cell volume seems to be the trigger for a rise in \([\text{Ca}^{2+}]_i\) and subsequent PRL release. Thus, in the activation of the osmoreceptive transduction pathway, the extent to which an increase in cell volume triggers a rise in \([\text{Ca}^{2+}]_i\) and subsequent PRL release. This is the tendency of extracellular \([\text{Ca}^{2+}]_i\) to be essential for the full response of PRL secretion in hyposmic environments, although the participation of intracellular \([\text{Ca}^{2+}]_i\) stores in this process remains to be examined.

In the present perfusion incubations, dispersed PRL cells responded within minutes to decreases in medium osmolality. Pro lactin cells were preincubated in hyperosmotic medium (355 mosmol/kgH2O) to show the effects of a physiologically relevant reduction in medium osmolality. The movement of a freshwater-acclimated tilapia to seawater produces a rapid increase in blood osmolality. Conversely, the transfer of a seawater-acclimated fish to freshwater elicits a rapid drop in blood osmolality. The degree of these changes depends to a considerable extent on the past experience of the animal with different salinities, and deviations between 290 and 450 mosmol/kgH2O in the blood osmolality are commonly observed in tilapia subjected to transfer between freshwater and seawater (10, 33).

Thus the changes of hyperosmotic medium (355 mosmol/kgH2O) to hyposmotic medium (300 mosmol/kgH2O) employed in this study are well within the range of blood osmolalities observed in vivo.

In the present study, a 15% reduction in osmolality elicited an increase in cell volume of roughly 15%, which returned gradually toward the baseline over the next 80 min. However, this cell volume decrease was slow, and it took 35 min of hyposmotic stimulation before a significant volume reduction could be observed. In many cells placed in hyposmotic media, the
initial swelling due to water influx is followed rapidly by losses of ions and/or organic solutes that lead to RVD (19, 20). In many cell types, RVD is observed within 20 min of hyposmotic stimulation (2, 20, 21, 32, 37, 39). The absence of a rapid RVD in the PRL cell may reflect the smaller shift in osmolality used in this study (15% reduction in osmolality) compared with those typically used by others, i.e., 25–50% (2, 20, 21, 31, 32, 39). Therefore, in addition to testing a physiologically relevant 15% change, we subjected the cells to a 30% decrease in osmolality to examine PRL cell volume changes and PRL release. Indeed, after a 30% increase in cell volume, the RVD following a 30% reduction in osmolality was more accentuated than that observed after a 15% reduction, indicating that the degree of osmotic deviation may dictate the extent of cellular RVD.

No single mechanism for RVD is known to operate in all cells. A wide array of different ions, organic solutes, and pathways has been implicated in different cell types, and different transduction mechanisms linking the initial swelling and subsequent activation of transport pathways have been postulated (19). Many of these studies, however, are based on in vitro experiments that often extrapolate the conditions observed in vivo. In normal and tumor-derived GH4C1 and MMQ rat pituitary cells, for example, RVD was observed within 10 min after exposure to 27% hyposmotic medium (36). On the other hand, pancreatic β-cells may exhibit RVD after a reduction in osmolality as low as 10% (23). In the latter case, however, the physiological significance of RVD is related to the regulation of insulin by blood glucose, rather than to osmoreception. If PRL cells are responding as osmoreceptors to physiological decreases in extracellular osmolality, changes in cell volume would need to be compatible with the time course of hyposmotically induced PRL response. The present study indicates that regulatory volume adjustments in the PRL cells occur only after extended periods (after 35 min when exposed to a 15% reduction in osmolality) or an extreme deviation (30%) in osmolality. The delayed or slow decrease in tilapia PRL cells exposed to small, physiological changes (15%) in extracellular osmolality may be of adaptive significance to the animal. Continued stimulation (hyposmotic extracellular environment) of the PRL cell maintains elevated rates of PRL secretion for up to 12 h (33). The absence of rapid volume regulation within the physiological range of osmolality indicates that volume regulatory changes in intracellular osmolyte pools do not occur during the time frame necessary for the peak in PRL release. Thus we believe that these findings provide significant evidence that PRL release in response to changes in osmolality is a physiologically important process and not a nonspecific outcome of cell volume regulatory mechanisms.

Evidence from our laboratory suggests that the effect of reduced osmolality on PRL release in the tilapia is mediated through a signal transduction system linked to changes in [Ca2+]. By measuring the uptake and loss of 45Ca2+ in the RPD, Richman et al. (29, 30) have shown that intracellular Ca2+ metabolism is modified in response to extracellular osmolality and is directly linked to the stimulation of PRL release. Through the use of the Ca2+-sensitive fluorescent dye fura 2, we observed that [Ca2+]i rises rapidly after a reduction in medium osmolality from 355 to 300 mosmol/kg H2O. It was previously reported that 25% of tilapia PRL cells show spontaneous oscillatory activity, whereas the remainder are silent (10). The baseline of the oscillatory cells was elevated, whereas the amplitude of oscillations was decreased at the onset of hyposmotic stimulation. This report is consistent with our current findings, in which the majority of cells in hypertonic medium were silent (60%). Regardless of the oscillation pattern, PRL cells responded to hyposmotic medium by elevating [Ca2+]i. This rise in [Ca2+]i, varied in magnitude among experiments. Although this variation may represent natural fluctuation in PRL cell responsiveness, it may be a reflection of variations in cell preparation. As an indication of cell responsiveness, high-[K+] medium was introduced at the end of the experiments, and >90% of the cells studied responded with a sharp increase in [Ca2+]i. In addition to silent and spontaneously active cells, low-frequency oscillation patterns are described for the first time in the tilapia PRL cell.

Intracellular Ca2+ oscillations have been described in many cell types, including mammalian PRL cells (35, 38) and goldfish GH cells (43). Oscillations have been implicated in modulating specific signal transduction pathways (15, 28, 35), although there is little evidence describing how this modulation occurs. In goldfish somatotropes, 88% of cells were quiescent, and low-frequency oscillations were not reported (43). In mammalian PRL cells, four [Ca2+]i oscillation patterns have been described (38). Some cells (~25%) showed no spontaneous [Ca2+]i oscillations, whereas the remainder were divided into low-amplitude, high-frequency oscillators (42%), high-amplitude, high-frequency oscillators (25%), and low-frequency oscillators (8%).

This study also examined whether the short-term rise in [Ca2+]i is dependent on an increase in cell volume. Alternatively, an increase in cell volume may directly lead to an increase in PRL release, independent of extracellular [Ca2+]i. Indeed, the osmotically induced release of hormones from rat anterior pituitary cells seems to occur independently of extracellular Ca2+ availability (36). In such cases, however, large deviations in osmolality are required to induce a short-lived burst of the hormone release, and cell swelling may induce a universal secretion of exocytotic material that may represent a pathological response (36). The entry of extracellular Ca2+ following cell swelling has been described in several cases (21, 37, 41). Increases in [Ca2+]i are often attributed to activation of a RVD mechanism that does not appear to be operating in the tilapia PRL cell. For example, in Xenopus renal A6 cell lines, removal of extracellular Ca2+ from the medium prevented a full RVD (37). In the present study, cell volume was maintained at an elevated level during hyposmotic stimulation in both Ca2+-deleted and nor-
mal media, suggesting that extracellular Ca\textsuperscript{2+} does not affect cell volume.

The deletion of Ca\textsuperscript{2+} from hyposmotic medium significantly reduced the hyposmotically induced PRL release, indicating that extracellular Ca\textsuperscript{2+} is a crucial component for the initiation of this signal transduction. The near absence of release in response to hyposmotic Ca\textsuperscript{2+}-deleted medium has been previously described from intact RPDs perfused under similar conditions (29). The small (<2-fold) response in PRL release observed in Ca\textsuperscript{2+}-deleted hyposmotic media suggests the participation of other second messenger systems and intracellular Ca\textsuperscript{2+} stores in the transduction pathway. Alternatively, this transient response may be a reflection of compensatory release of Ca\textsuperscript{2+} from intracellular stores, because it occurred after extracellular Ca\textsuperscript{2+} was depleted with either EGTA or with nominally Ca\textsuperscript{2+}-free media. In some cells, a transient peak in [Ca\textsuperscript{2+}]\textsubscript{i} was observed after exposure to hyposmotic or hyposmotic Ca\textsuperscript{2+}- and Ca\textsuperscript{2+}-deleted media, followed by a decline or return to the baseline ratio. Because these transient responses occurred in both hyper- and hyposmotic media deprived of Ca\textsuperscript{2+}, they may represent compensatory Ca\textsuperscript{2+} release from intracellular stores. This compensatory release has also been suggested in goldfish somatotropes (16), but the mechanism underlying such a response remains to be clarified.

The notion that stretch-sensitive ion channels may act in the transduction of changes in transmembrane osmotic pressure is particularly attractive in light of the fact that a 1 mosmol/kgH\textsubscript{2}O decrease in medium osmotic pressure increases membrane tension to a degree that is well within the range to which stretch-sensitive ion channels are responsive (24). Changes in cell volume in response to an osmotic stimulus could account for the osmosensitivity of certain endocrine and neuroendocrine pathways. Changes in cell volume would lead to the activation or inactivation of stretch-sensitive ion channels that are linked to secretory mechanisms. In the case of tilapia PRL cells, this transduction process would link a reduction in extracellular osmolality to an increase in PRL, consistent with the osmoregulatory role of this hormone. Although the importance of cell volume and extracellular Ca\textsuperscript{2+} to this process has been identified, further study of the nature and control of this transduction pathway is necessary. We are currently testing the hypothesis that the increase in cell volume following hyposmotic stimulation will increase the open probability of putative stretch-activated channels that allow extracellular Ca\textsuperscript{2+} entry and a subsequent increase in PRL release (Ref. 32a; see p. C1290 in this issue).

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