Evidence that signal transduction for osmoreception is mediated by stretch-activated ion channels in tilapia


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The objectives of the present study were to determine whether stretch-activated Ca2+-permeant channels are involved in hyposmotically induced PRL release from dispersed tilapia PRL cells. The lanthanide ion, gadolinium (Gd3+), is widely utilized to block stretch-activated cation channels in general, including Ca2+ channels (14, 15), and its effect on hyposmotically induced PRL cell swelling, rise in intracellular Ca2+ (PRL release) can be measured simultaneously with other parameters involved in the osmoreceptive process, such as cell size, in a cell of known identity. Prolactin cells are easily isolated and dissociated and are remarkably sensitive to changes in extracellular osmolality, whether as dispersed cells or intact tissue (2, 3, 9, 10, 26, 27, 32). Thus small decreases in extracellular osmolality result in a robust elevation in PRL release (9, 10, 26, 27, 39). This direct sensitivity to changes in extracellular osmolality is observed both in vivo and in vitro (33, 34, 39). This response is physiologically relevant, because in the tilapia and other teleost fish PRL is a key osmoregulatory hormone that promotes freshwater adaptation (16, 22, 23). Thus the PRL cell can be defined as an osmoreceptor, not only by its sensitivity to changes in medium osmolality but also by its ability to signal osmotic balance adjustment by the whole organism. In a companion study (Ref. 32; see p. C1280 in this issue), we have shown the close relationship among hyposmotically induced cell swelling, rise in intracellular Ca2+, and PRL release. We thus have suggested that hyposmotically induced cell swelling activates extracellular Ca2+ entry through stretch-activated ion channels (8, 10, 29, 32).

Stretch-activated Ca2+ and K+ channels have been identified in several cell types (20). The activation of stretch-gated channels may be a link between mechanical stress and cell excitability, thus participating in cell volume regulation and in the transduction of mechanical and osmotic stimuli (13, 21, 24, 25, 31). Although mechanosensitive channels have been described in a wide range of cell types (for review, see Ref. 25), only in vasopressinergic and oxytocinergic neurons in the rat hypothalamus have stretch-inactivated ion channels been described that respond directly to physiologically relevant increases in osmolality (5).

The maintenance of a stable internal osmotic environment is fundamental to life, and deviations in such homeostasis lead to negative consequences, such as renal and cardiovascular failure. At the cellular level, volume regulation may represent an immediate and local response to minimize the effects of altering osmotic equilibrium (20). At an organismic level, animals have evolved several means for adapting to alterations in osmolality that include the development of neuroendocrine systems capable of detecting osmotic changes and initiating osmoregulatory action. The tilapia prolactin (PRL) cell is an excellent example of the integration between sensory and regulatory capabilities of the organism, within a single cell. The tilapia PRL cell represents a model system for osmoreception in which the osmoregulatory output...
concentration ([Ca^{2+}]), and PRL release were examined. Furthermore, hypotonic stimulation during exposure to nifedipine and high-[K^{+}] medium was employed to determine whether voltage-gated channels might mediate the response to reduced osmolality. The time course of changes in cell volume induced by the different treatments was observed together with the changes in PRL release. Changes in [Ca^{2+}], were determined from fura 2-AM-loaded cells.

MATERIALS AND METHODS

Fish. Tilapia (Oreochromis mossambicus) were obtained from a population maintained at the Hawaii Institute of Marine Biology. They were kept in 5,000-liter tanks in fresh-water under natural photoperiod. They were fed twice daily with Purina Trout Chow (−2% of body wt per 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 dispersions and perfusion. Pituitaries were removed from sexually mature tilapia (200–600 g) after decapitation. Each pituitary was placed individually in hypotonic (355 mosmol/kgH2O) Krebs bicarbonate-Ringer solution, containing glucose (500 mg/l), glutamine (290 mg/l), and Eagle’s minimal essential medium as described by Wigham et al. (38). The rostral pars distalis (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 hypotonic medium (355 mosmol/kgH2O). Medium osmolality was adjusted by varying the concentration of NaCl and checked using a vapor pressure osmometer (Wescor 5100C, Logan, UT). Tissues were preincubated overnight (18–20 h) at 26–28°C on a gyratory platform (80 rpm) under a humidified atmosphere composed of 95% O2-5% CO2. Cells were dispersed and plated as previously described (32) and perfused in a chamber that allowed for the measurement of cell size and collection of perfusate for quantification of PRL release. Media of different compositions were gravity fed through the chamber and changed using a manifold valve set upstream from the chamber. The dead time for new medium to reach the chamber has been corrected for in presenting the observations. Samples of the perfusate were collected every 5 min throughout the time course of the experiment.

There are technical considerations for using Gd3+ to identify stretch-activated channels. Gd3+ avidly binds to the phosphate and bicarbonate present in most incubation solutions, reducing the effective concentration of Gd3+ and these ions and possibly leading to false negative conclusions (6). To employ Gd3+ in our studies effectively, we validated the utility of our incubation system for studying the hypotomerically induced PRL release culture system in bicarbonate- and phosphate-free medium, containing HEPES (25 mM) to buffer the medium, and equilibrated with 100% O2. This medium preparation was utilized in all experiments involving Gd3+ and their respective controls.

Reagents. Stock solutions of nifedipine (Sigma) were dissolved in DMSO. An equivalent amount of DMSO (1:1,000) was added to the control medium. GdCl3 (Sigma) was first dissolved in distilled water (0.5 M) and added to media deprived of NaHCO3 and KH2PO4 and containing 25 mM HEPES (pH 7.4). Media containing depolarizing concentrations of K+ were prepared by adding 56 mM KCl and subtracting the osmotically equivalent amount of NaCl. Medium osmolality was measured and adjusted to either 355 mosmol/kgH2O (hypotonic) or 300 mosmol/kgH2O (hyposmotic). Substituting NaCl with KCl while keeping the same osmolality did not affect PRL cell volume.

Intracellular Ca^{2+} concentration and cell size. The methodology for measuring [Ca^{2+}], in tilapia PRL cells has been previously described (32). This procedure consists of monitoring [Ca^{2+}], by fluorescence imaging with the Ca^{2+}-sensitive dye fura 2. PRL cells, plated on poly-l-lysine-coated coverslips, were loaded with the membrane-permeant acetoxymethyl ester derivative of fura 2, fura 2-AM. Individual coverslips were mounted in a chamber that allows the cells to be perfused with media of varying compositions, which is in turn mounted on a microscope stage. Fura 2 ratio measurements were made on individual cells from images captured digitally through a charge-coupled device camera interfaced with an inverted microscope (Nikon). Images were acquired at 340 and 380 nm and were averaged and then background subtracted by use of images acquired at each wavelength from a field devoid of cells. All data were expressed as the ratio of fura 2 fluorescence excited at 340 nm (fura 2 bound to Ca^{2+}) to that excited at 380 nm (free fura 2). Increases of this ratio (340/380) result directly from increases of [Ca^{2+}]. (12). The ratio provides a measurement independent of dye concentration, cell thickness, and optical efficiency of the instrument.

The procedure for measuring cell size has been described previously (32). Briefly, the cross-sectional area of each cell was determined by tracing cells individually. All cell volume data were expressed as percent changes from baseline. The baseline was set at 100% and consisted of the mean of the first five values obtained at the beginning of each run.

Radioimmunoassays. The tilapia pituitary secretes two distinct PRL molecules that are encoded by separate genes. The release of the two PRLs, designated PRL177 and PRL188, was measured using homologous radioimmunoassays (1, 33, 39). Because the patterns of PRL188 and PRL177 were essentially identical in response to hypotonic medium and other treatments in all the experiments, only the release of PRL188 is presented in the present study (see Ref. 32, a companion article in this issue). Values obtained as nanograms per milliliter were converted to percent change from the baseline, set at 100%, which was determined from the average of the first five time points.

Statistical analysis. Because the absolute levels of PRL release varied among incubations, values for PRL release were obtained in nanograms per milliliter and normalized as percent change from baseline. Normalized values from each sampling period (5 min) were averaged for each treatment period (30 min). Values from each experimental replicate were log-transformed to conform to the assumptions of normality and equal variances. Comparisons between treatments were performed using a pairwise t-test. Significance level was set at 95% (P < 0.05). Calculations were performed using the Minitab statistical software package (State College, PA). Data are expressed as means ± SE.

RESULTS

Effects of Gd3+ on PRL release, cell volume change, and [Ca^{2+}]. The lack of NaHCO3 and KH2PO4 in the media did not prevent an increase in either cell volume or PRL release after hypotonic stimulation (Fig. 1A). In the HEPES-buffered media, PRL release rose rapidly when medium osmolality was reduced to 300 from 355 mosmol/kgH2O (P < 0.01 for the first stimulation and P < 0.05 for the second stimulation). Gd3+ at 100
μM substantially reduced the response to hyposmotic medium (Fig. 1B). Cell volume increased about 15% after exposure to both normal and Gd³⁺-containing hyposmotic media. Addition of 1 mM GdCl₃ but not by 0.1 mM Gd³⁺. Cell volume increased as medium osmolality was reduced and was not affected by Gd³⁺. Data are expressed as means ± SE (n = 4 replicate experiments for PRL release; n = 16–20 for cell volume determinations). *P < 0.05, **P < 0.01: significantly different from baseline in 355 mosmol/kgH₂O medium.

To determine whether suppression by Gd³⁺ of the hyposmotically induced increase in PRL release is attributable to a blockage of extracellular Ca²⁺ entry, we measured [Ca²⁺]ᵢ in PRL cells treated with Gd³⁺. The absence of NaHCO₃ and Na₂PO₄ did not alter the hyposmotically induced increase in [Ca²⁺]ᵢ (Fig. 2A). Exposure to 1 mM GdCl₃ in hyposmotic medium blocked the rise in [Ca²⁺]ᵢ but did not affect subsequent [Ca²⁺]ᵢ responses to hyposmotic medium after its removal in 10 of 12 cells (Fig. 2B). Thus Gd³⁺ uncoupled PRL release from changes in cell volume, apparently by blocking the rise in [Ca²⁺]ᵢ. This finding supports the notion that extracellular Ca²⁺ entry through stretch-activated channels is a critical step for hyposmotically induced increase in PRL release.

Effects of nifedipine, high [K⁺], and hyposmotic medium on PRL release, cell volume, and [Ca²⁺]ᵢ. The following experiments were designed to further investigate the nature of the ion channels that participate in hyposmotically induced PRL release. Nifedipine (10 μM), an L-type Ca²⁺ channel blocker, significantly (P < 0.05) reduced high [K⁺]-induced PRL release after 10 min, compared with a parallel high-[K⁺] control (Fig. 3A). However, nifedipine did not prevent an increase in PRL release in hyposmotic medium, suggesting that this type of voltage-gated Ca²⁺ channel is unlikely to be involved in hyposmotically induced PRL release (Fig. 3B).

Depolarizing concentrations of KCl produce a strong but brief stimulation of PRL release in tilapia (18, 30). When 56 mM KCl was added to hyperosmotic medium (355 mosmol/kgH₂O), PRL release increased within 5 min and returned to baseline levels after 15 min (Fig. 4A). We found previously that PRL cells remain unre-
responsive to further stimulation by high $[K^+]$ until they are allowed to repolarize in normal medium (30). Extended preexposure to high $[K^+]$, however, did not block the rise in PRL release produced by exposure to hyposmotic medium. In preparations where preexposure to high $[K^+]$ lasted 1 h (in hyperosmotic medium), the rise in PRL release reached 900% above baseline when cells were exposed to hyposmotic medium with high $[K^+]$ (Fig. 4B). Similarly, the increase in cell volume evoked by hyposmotic medium was unaffected by depolarizing $[K^+]$. Interestingly, after transfer to hyperosmotic medium with normal $[K^+]$, cell volumes decreased to below the original baseline. Nevertheless, cell volume increased once more, when hyposmotic medium was perfused. These results suggest that channels other than voltage-gated channels may mediate the PRL cell response to reduced osmolality.

$[Ca^{2+}]$ rose briefly after exposure to high $[K^+]$ in a manner that was similar to the pattern observed with PRL release (Fig. 5). Under depolarizing conditions, the introduction of hyposmotic medium produced a second high and sustained rise in $[Ca^{2+}]$, gradually declined to pretreatment levels after the reintroduction of normal hyperosmotic medium. A final exposure to hyposmotic medium with normal nondepolarizing $[K^+]$ again produced a rise in $[Ca^{2+}]$. Although varying in magnitude, these overall patterns of response were observed in all of the 24 cells examined. These results suggest that the rise in $[Ca^{2+}]$ after hyposmotic stimulation does not involve the entry of $Ca^{2+}$ through voltage-gated channels.

**DISCUSSION**

This is the first study to provide evidence that stretch-activated $Ca^{2+}$-permeant channels are involved in mediating the signal transduction for osmoreception in tilapia PRL cells. Our results show that the stretch-activated channel blocker Gd$^{3+}$ blocked hyposmotically induced increase in $[Ca^{2+}]$, and the rise in PRL release but did not prevent PRL cells from swell-

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**Fig. 2.** Effects of Gd$^{3+}$ on the hyposmotically induced rise in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]$) of single PRL cells. Arrows indicate the estimated time at which the indicated medium reached the cells. All recordings were taken from cells incubated in bicarbonate- and phosphate-free medium containing HEPES (25 mM). A: $[Ca^{2+}]$, trace of a single PRL cell that increased the 340/380 ratio after the switch from hyperosmotic (355 mosmol/kgH$_2$O) to hyposmotic medium (300 mosmol/kgH$_2$O) and that maintained the elevated $[Ca^{2+}]$, throughout the remainder of hyposmotic exposure. B: $[Ca^{2+}]$, trace of a single PRL cell exposed to hyperosmotic and hyposmotic medium containing 1 mM Gd$^{3+}$ followed by recovery in normal hyperosmotic and hyposmotic medium. Gd$^{3+}$ blocked the hyposmotically induced rise in $[Ca^{2+}]$, but did not prevent the rise in $[Ca^{2+}]$, induced by normal hyposmotic medium after recovery. The trace shown is representative of 10 cells from 3 replicate experiments.

**Fig. 3.** Effects of nifedipine on high $[K^+]$-induced and hyposmotically induced PRL release. PRL release is expressed as percent change from the baseline. Horizontal bars indicate the estimated time at which media reached the cells. A: exposure of dispersed PRL cells to high-$[K^+]$ medium containing 10 $\mu$M nifedipine (○) significantly reduced PRL release compared with high-$[K^+]$ controls (●). B: exposure of dispersed PRL cells to hyposmotic medium (300 mosmol/kgH$_2$O) containing 10 $\mu$M nifedipine (○) did not prevent hypsomotically induced PRL release. This response was indistinguishable from that of cells exposed to normal hyperosmotic medium (●). Values represent means ± SE ($n$ = 4 replicate experiments).
Firing rate (4). The osmotic control of oxytocin and vasopressin likely represents an important osmoregulatory response in mammals, because vasopressin at least is involved in sodium excretion and water retention (4, 17, 36, 37). We have hypothesized that a similar mechanism operates in the tilapia PRL cell. Specifically, we propose that changes in cell volume lead to the activation or inactivation of stretch-sensitive ion channels that are linked to secretory mechanisms. In tilapia PRL cells, osmotic influx of water during hyposmotic stimulation leads to an increase in cell volume, which in turn would increase the open probability of stretch-activated Ca2+-permeant channels. In the present study we used osmotic, ionic, and pharmacological manipulations to investigate this hypothesis while observing their effects on PRL release and [Ca2+].

Our finding that Gd3+ uncouples hyposmotically driven cell swelling from the increase in PRL release is particularly interesting in light of the fact that removing extracellular Ca2+ from the medium also reduces PRL release, without preventing cell swelling (see Ref. 32, a companion article in this issue). Although Gd3+ has also been found to block other types of Ca2+ channels, it currently provides one of the best available pharmacological tools to address the involvement of stretch-activated channels in signal transduction processes and has been widely used to determine the presence of stretch-activated ion channels in a variety of cell types (14, 15). Gd3+ has also been used effectively to block stretch-inactivated channels that are believed to be involved in mammalian osmoreception (28). In the present study, Gd3+ blocked the hyposmotically induced rise in [Ca2+]i and PRL release, without preventing the osmometric increase in cell volume. This result indicates that an increase in cell size can occur independently of a Ca2+ signal, thus suggesting

![Diagram](http://ajpcell.physiology.org/)

**Fig. 4.** Effects of depolarizing conditions on hyposmotically induced PRL release and cell volume from dispersed PRL cell preparations. Horizontal bars indicate the estimated time at which media reached the cells. PRL release (●) and cell volumes (○) are expressed as percent change from baseline, and the former is plotted in log scale for clarity. A: dispersed PRL cells were exposed to hyposmotic medium containing 56 mM KCl for 30 min before being switched to hyposmotic medium with 56 mM KCl. PRL release increased transiently in response to high [K+] and was more sustained when hyposmotic medium was introduced. Cell volumes increased only with reductions in medium osmolality. B: dispersed PRL cells were exposed to hyperosmotic medium containing 56 mM KCl for 1 h before being switched to hyposmotic medium in the same conditions. PRL release increased transiently in response to high [K+] and again during perfusion with high-[K+] hyposmotic medium. Cell volumes increased only with reductions in medium osmolality. Values represent means ± SE (n = 4 replicate experiments for PRL release; n = 17–19 for cell volumes).
that the entry of extracellular Ca\(^{2+}\), but not osmotically driven water movement into the cell, is a critical factor in initiating PRL release.

Depolarizing concentrations of KCl have been shown to directly stimulate a transient rise in [Ca\(^{2+}\)]\(_i\) in PRL cells (Hyde GN, Seak AP, Grau EG, and Borski RJ, unpublished observations) as well as PRL release from the cultured RPD (11, 30). The L-type Ca\(^{2+}\) channel, the most widely determined type of voltage-gated Ca\(^{2+}\) channel in endocrine cells, can be activated by depolarization of the membrane (35). Nifedipine is a selective blocker of L-type voltage-gated Ca\(^{2+}\) channels and has been shown to block the high [K\(^+\)]- and BAY K-induced PRL release in tilapia PRL cells after 4 h (Hyde GN, Seak AP, Grau EG, and Borski RJ, unpublished observations). In the present study, 10 \(\mu\)M nifedipine significantly reduced the PRL response to high [K\(^+\)] after 10 min. This finding is consistent with recent electrophysiological studies showing that \(-30\%\) of the voltage-gated Ca\(^{2+}\) current under voltage clamp in the tilapia PRL cell was blocked by \(\geq 1\) \(\mu\)M nifedipine and, thus, was attributable to L-type Ca\(^{2+}\) channels (Cooke I and Xu S, unpublished observations). It was predicted that if nifedipine were able to block hypsomotically induced PRL release, then voltage-gated channels could play a role in the signal transduction of the osmotic stimulus. However, nifedipine failed to block the release of PRL during exposure to hypsomotic medium, suggesting that L-type channels are not involved in hypsomotically induced PRL release.

Many voltage-gated Ca\(^{2+}\) channels, with the exception of the L-type, become inactivated after depolarization (7); thus depolarizing the membrane with high [K\(^+\)] would not be expected to lead to any sustained rise of [Ca\(^{2+}\)]\(_i\), via these channels. The transduction mechanisms that respond to depolarization do not support sustained PRL release from the tilapia PRL cell. Rather, after a transient rise in release, PRL levels return to the original baseline, and cells only become responsive to further stimulation by depolarization if they are allowed to repolarize (30). If hypsomotically induced PRL release were dependent on voltage-gated Ca\(^{2+}\) channels, then preexisting depolarizing conditions should prevent subsequent responses to hypsomotic medium. However, in the present experiments, the stimulation of PRL release by hypsomotic medium was unaffected by previous and sustained exposure to depolarizing [K\(^+\)]. Thus the observed hypsomotically induced PRL release in depolarizing conditions suggests the presence of Ca\(^{2+}\)-permeant channels that are not voltage gated. In these experiments, cell volume did not respond to depolarizing [K\(^+\)]. An increase in cell volume, however, always preceded the hypsomotically induced rise in PRL release in either depolarizing or normal media. The reason cell volumes decreased below baseline after extracellular conditions were changed from high [K\(^+\)] to normal remains to be investigated, but one might speculate that it may be linked to a possible efflux of K\(^+\), which would result in the hyperpolarization of the cell. A similar approach has been employed to investigate the nature of Ca\(^{2+}\) signals mediating gonadotropin-releasing hormone (GnRH)-stimulated growth hormone (GH) release in goldfish GH cells (19). It was found that depolarizing conditions did not block GnRH-induced GH release, and the authors concluded that the response operates through pathways that are independent of voltage-gated Ca\(^{2+}\) channels. In a similar manner, the current data provide evidence that the signal transduction for the hypsomotic signal in the tilapia PRL cell is not mediated by voltage-gated Ca\(^{2+}\) channels but, rather, through stretch-activated Ca\(^{2+}\)-permeant channels, a conclusion that is compatible with the data from the present Gd\(^{3+}\) and nifedipine experiments. This study provided several lines of evidence that support the involvement of stretch-activated Ca\(^{2+}\) channels in mediating the hypsomotically induced rise in PRL release. Whereas the osmotic control of vasopressin release in rats is believed to be mediated by stretch-inactivated ion channels present in cellular osmoreceptors responsive to a hyperosmotic stimulus (4, 5), this is the first study to provide evidence for transduction by stretch-activated ion channels of a hypsomotic stimulus serving a key role in the endocrine response of the whole organism’s osmotic homeostasis. Although the participation of intracellular Ca\(^{2+}\) stores in this process remains to be investigated, the initial influx of extracellular Ca\(^{2+}\) through channels that are sensitive to cell swelling appears to be a critical step in this signal transduction.

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