THE PROLACTIN CELL OF A EURYHALINE FISH, THE TILAPIA, *OREOCHROMIS MOSSAMBICUS*: A MODEL FOR OSMORECEPTION

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DEDICATION

I dedicate this dissertation to my parents, Wayne and Denise Scale, for their constant support and encouragement.
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Prolactin (PRL) plays a central role in fresh water (FW) adaptation in teleost fish. Consistent with the freshwater-adapting actions of prolactin (PRL), the release of this hormone from the tilapia pituitary is stimulated as extracellular osmolality is reduced both \textit{in vitro} and \textit{in vivo}. The main objective of this research was to investigate the mechanisms involved in the mediation between an osmotic stimulus and PRL release.

The present studies also provided evidence of the unique osmosensitivity of the PRL cells when compared to the other pituitary cell types such as GH cells. Evidence now suggests that growth hormone (GH) acts in the seawater (SW) adaptation in some euryhaline fish. The release and content of PRL in response to long-term (days) changes in medium osmolality were markedly different than those of GH and corticotropin (ACTH) measured from the same pituitaries. Repeated blood withdrawal and transfer from SW to FW increased circulating PRL levels, whereas GH levels were unchanged. PRL release was not only more sensitive to a decrease in extracellular osmolality but long-lasting, when compared with the response of GH cells in dispersed cells and whole pituitaries.

The tilapia PRL cell provides an excellent model to investigate osmoreception. Working with this model, a technique was developed for examining changes in cell volume and PRL release from the same preparation of cells, as well as measurements of $[\text{Ca}^{2+}]_i$. These approaches were utilized to demonstrate that the rapid increase in PRL release in response to reductions in medium osmolality is largely dependent on extracellular calcium. Studying the relationships between cell volume, $[\text{Ca}^{2+}]_i$ and PRL
release in response to varying osmolalities, depolarizing conditions and ion channel blockers, provided evidence that stretch-activated calcium-permeant ion channels are responsible for the transduction of reduced extracellular osmolality into increased PRL release.

The present findings support the proposed signal transduction model for osmotic stimuli in PRL cells. A decrease in extracellular osmolality leads to an increase in cell volume. Cell swelling increases the open probability of stretch-activated ion channels, and results in the entrance of extracellular calcium into the cell and stimulation of PRL release.
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<td>[Ca(^{2+})]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>ACTH</td>
<td>Corticotropin</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol Bis-(B-aminoethyl ether)N,N,N',N' -Tetracetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh water</td>
</tr>
<tr>
<td>Gd(^{3+})</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GTH</td>
<td>Gonadotropin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol 1,4,5 - triphosphate</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>K(^+)</td>
<td>Potassium</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
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MEM................................................. Minimum essential medium
mOsmolal........................................... Milliosmolal
Na+ ................................................... Sodium
PAGE ................................................ Polyacrylamide gel electrophoresis
PBS ................................................... Phosphate buffered saline
PPD .................................................... Proximal pars distalis
PRL ..................................................... Prolactin
PrRP .................................................... Prolactin-releasing peptide
RIA ..................................................... Radioimmunoassay
RPD .................................................... Rostral pars distalis
RVD ..................................................... Regulatory volume decrease
SDS ..................................................... Sodium dodecyl sulfate
SERCA ................................................ Sarco-endoplasmic reticulum calcium activated ATPase
SGnRH ................................................ Salmon gonadotropin releasing hormone
SW ..................................................... Seawater
TMB-8 ................................................ 8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate
TRH ..................................................... Thyrotropin releasing hormone
VGCC .................................................. Voltage gated calcium channel
Xesto ................................................... Xestospongin-C
CHAPTER I

INTRODUCTION

Few aspects of homeostasis are more fundamental to life in complex organisms than the maintenance of a stable intracellular environment. The structure and function of macromolecules is maintained by weak forces (hydrophobic interactions and hydrogen bonds), and thus is very sensitive to small changes in the ionic and osmotic environment. Two main strategies have been described by which organisms maintain an intracellular environment that supports proper cell functioning. Osmoregulation ensures a nearly constant intracellular osmotic milieu for the proper functioning of cellular biochemical reactions, whereas osmoconformity allows intracellular osmotic concentration to be dictated by the extracellular environment. Osmoconformers, however, are still able to maintain cellular homeostasis by regulating the composition of ions as well as other osmolytes that are essential for maintaining optimal enzyme function even in extreme environments (Yancey, 2001). For the animals that osmoconform, the intracellular composition of solutes may be made up largely by compatible osmolytes, since high concentrations of salts, such as Na⁺ and K⁺ can slow enzymatic activity (Yancey, 2001). In barnacle muscle fibers, for example, 70% of the intracellular osmotic concentration consists of amino acids, such as glycine (Clark and Hinke, 1981). Of particular importance in changing environments, osmoregulators may evolve enzyme systems that are optimized for specific conditions, rather than having to operate sub-optimally over a range of environmental osmotic concentrations. For this reason, many organisms invest
considerable energy in controlling precisely the composition of both intracellular and extracellular fluids. In fish, for example, osmoregulation can consume a high proportion of available energy, ranging from 25 to 50% of total metabolic output (Rao, 1968; Nordlie and Lefler, 1975; Nordlie et al., 1991; Bushnell and Brill, 1992; Toepfer and Barton, 1992). More recently, the calculated maximal O₂ consumption devoted to osmoregulation was estimated to account for 63% of the standard metabolic rate for a 100 g tilapia (Brill et al., 2001). Beyond its metabolic cost, osmotic equilibrium is a fragile balance that is maintained through the continuous interaction of a major portion of the (neuro) endocrine array. Even in humans, who have evolved complex homeostatic mechanisms for the maintenance of hydromineral balance, pathologic derangements in osmotic balance are common (McManus and Churchwell, 1994). Hypertonic states include renal failure, diabetes, diarrhea and dehydration, whereas clinical hypotonicity, usually associated with hyponatremia, is observed in malnutrition, congestive heart failure, nephrotic syndrome and hepatic cirrhosis (McManus and Churchwell, 1994). Indeed, one of the most difficult challenges in clinical medicine is the regulation of salt and water balance in seriously ill patients (Guyton, 1995).

The challenging search for mechanisms that mediate osmoreception

In view of the cost and importance of osmoregulation, it may seem at first surprising that so little is known about the physiological mechanisms which monitor, and in turn regulate, the maintenance of osmotic balance in vertebrates. Closer attention, however, reveals the impediment: the complex structure of osmoreceptive cells and tissues. Vasopressin cells of the mammalian hypothalamus illustrate this well. The cell
bodies of these neurosecretory cells are located mainly in the supraoptic nucleus with a smaller number in the paraventricular nucleus. In both sites, the vasopressin cells reside among a variety of cell types and receive input from these and a host of others. The axons of vasopressinergic neurons project a considerable distance to capillaries in the posterior pituitary, the site of vasopressin secretion. This kind of complexity in arrangement has virtually blocked attempts to clarify the mechanisms by which osmotic signals alter vasopressin secretion.

Most physiology texts reveal a wealth of detail about mechanoreceptors, thermoreceptors, nociceptors, electroreceptors, chemoreceptors, as well as receptors of neurotransmitters, hormones and other chemical mediators. The transduction mechanisms that serve these modalities are understood to a considerable extent. In contrast, the mechanism(s) by which the osmotic concentration of the extracellular fluid controls an osmoreceptive cell remains obscure. In the studies discussed herein, I explore the mechanisms by which the osmotic concentration of extracellular fluid regulates, via the directional movement of water, the release of the osmoregulatory hormone, prolactin (PRL), from the anterior pituitary of a euryhaline teleost, the tilapia, *Oreochromis mossambicus*.

*The tilapia PRL cell: an osmoreceptive cell with an osmoregulatory output*

The PRL cell of the tilapia is not encumbered by the impediments that hinder the investigation of most osmoreceptive cells. The anatomical arrangement of the tilapia pituitary gland has been described in detail (Dharmamba and Nishioka, 1968; Bern et al., 1975; Nishioka et al., 1988) and illustrates the advantage of utilizing the PRL cell as a
model to study osmoreception. There are two features of the pituitary gland morphology common to most teleost fishes that differ from the mammalian pattern. The fish pituitary lacks a median eminence and a hypothalamo-hypophysial portal system. Hormone-producing cell types are segregated in teleosts into distinct areas of the pituitary (Norris, 1997). In the tilapia, as in many other teleost fishes, PRL cells are approximately spherical and, unlike those of mammals, are isolated in a nearly homogeneous tissue; they comprise nearly 100% of the rostral pars distalis (RPD) of the anterior pituitary (Nishioka et al., 1988; Nishioka et al., 1993). They are easily isolated and dissociated, and respond identically as an intact tissue or as dispersed cells (Borski, 1992). These cells are remarkably sensitive to changes in extracellular osmolality (cf. Bern, 1980; Nishioka et al., 1988; Grau et al., 1994). A decrease in extracellular osmolality of less than 5% (325 to 310 mOsmolal), a change well within the physiological range experienced by the tilapia in nature, results in a marked elevation in PRL release (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1994). This direct sensitivity to changes in extracellular osmolality is observed both in vivo and in vitro (Shepherd et al., 1999).

Prolactin has long been known to be essential to fresh water (FW) osmoregulation in tilapia and other euryhaline teleosts. Three lines of evidence indicate that the response of the tilapia PRL cell to extracellular osmolality has real physiological significance and is not merely a phenomenon associated with cell volume regulation. First, the response of the PRL cell (i.e., the release of PRL) to extracellular osmolality is entirely appropriate to a cell that releases hormones which maintain salt and water balance in FW fish. That is, as extracellular osmolality decreases, PRL release increases, and as extracellular
osmolality increases, PRL release decreases. Second, the range of extracellular osmolality to which the PRL cell responds is well within the normal range of blood osmolalities observed in the organism. Third, the response of the PRL cell to an osmotic stimulus is mediated through intracellular mechanisms that are well known for their control of cell secretory activity. These include second messenger systems and related intracellular signaling mechanisms that mediate both acute and sustained PRL release.

The supporting evidence for these three points follows.

The most striking feature of the tilapia PRL cell is the close association between secretory output and extracellular osmolality. The tilapia pituitary releases two distinct PRL molecules that are encoded by separate genes (Specker et al., 1985; Yamaguchi et al., 1988; Rentier-Delrue et al., 1989). The amount of the two tilapia PRLs released increases in direct proportion to the degree that osmolality is reduced (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981; Grau et al., 1987; Nishioka et al., 1988). These two hormones, designated PRL$_{177}$ and PRL$_{188}$, possess similar activity in an osmoregulation-based PRL bioassay (Specker et al., 1985). The only activity which has been found in tilapia to distinguish these PRLs to date is an apparently unique somatotropic action of PRL$_{177}$ (Shepherd et al., 1997).

The range of extracellular osmolality to which the tilapia PRL cell responds in vitro is well within the range of blood osmolality observed in vivo, which ranges between 325 and 335 mOsmolal for both FW- and seawater (SW)-acclimated fish (Yada et al., 1994). The movement of a FW-acclimated tilapia to SW produces a rapid, and sometimes large, increase in blood osmolality. Conversely, the transfer of a SW-acclimated fish to FW elicits a rapid drop in blood osmolality. The degree of these
changes depends to a considerable extent on the past experience of each animal with
different salinities, but deviations in plasma osmolality between 290 and 450 mOsmolal
are common in tilapia that adapt successfully when faced with acute osmoregulatory
challenges. Within this range of normal plasma osmotic variations, Grau et al (1981)
have shown that shifts in extracellular osmolality as small as 15 mOsmolal, less than 5%
of normal serum osmolality, are sufficient to markedly alter the secretory activity of the
tilapia PRL cell.

Is the osmoreceptive ability of PRL cells shared by other cell types?

Both PRL and growth hormone (GH) cells in the tilapia have the ability to
respond to changes in extracellular solute concentration in vitro. While PRL cells
respond to reductions in medium osmolality, GH cells respond to elevations in osmolality
over an 18 h incubation (Helms et al., 1987; Grau et al., 1994). In order to verify the
unique osmoreceptive characteristics of the PRL cell, I compared week-long in vitro PRL
release in response to changes in medium osmolality with GH and corticotropin (ACTH)
release from whole pituitaries (Chapter II). In this study I also investigated the effects of
transferring tilapia from FW to SW or from SW to FW on plasma osmolality and
circulating levels of PRL and GH. In a separate study, I compared early (minutes)
release of PRL and GH in response to changes in extracellular osmolality in dispersed
cells (Chapter III). Growth hormone promotes SW adaptation in several teleosts,
including the tilapia (Sakamoto et al., 1997; McCormick, 2002). Based on the differing
osmoregulatory roles that GH and PRL play in the tilapia, I hypothesized that GH release
would not be affected in the same manner as PRL release when whole pituitaries or dispersed cells were exposed to changes in extracellular osmolality.

*Hyposmotic stimulation of tilapia PRLs is dependent on an increase in intracellular free Ca\(^{2+}\).*

The examination of the second messenger metabolism of the tilapia PRL cell also supports the notion that it is a physiologically important osmoreceptor (cf. Grau and Helms, 1989; 1990). Briefly, it has been shown that PRL release is Ca\(^{2+}\)-dependent and can be induced experimentally by increasing intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) or cAMP (Grau et al., 1982; Grau et al., 1986). It has also shown that cAMP turnover and [Ca\(^{2+}\)]\(_i\) change inversely with extracellular osmolality over a time-course which is compatible with their involvement in the stimulation of PRL release (Richman et al., 1990; Helms et al., 1991; Richman et al., 1991).

More recently, investigations of Ca\(^{2+}\) function have been extended to the measurement of [Ca\(^{2+}\)]\(_i\) (Borski, 1992). Through the use of the Ca\(^{2+}\)-sensitive fluorescent dye, fura-2, Borski (1992) observed that [Ca\(^{2+}\)]\(_i\) rises within 20 seconds after a reduction in the osmolality (300 mOsmolal) of the incubation medium. This rise is reversed when medium osmolality is restored to its original level (355 mOsmolal). Measurements of [Ca\(^{2+}\)]\(_i\) show two distinct types of PRL cells. The first type, which is described as silent, constitutes approximately 75% of the cells measured while a second, spontaneously active cell comprises the remainder. In silent PRL cells, [Ca\(^{2+}\)]\(_i\) remains stable and relatively low during incubation in slightly hyperosmotic medium (355 mOsmolal). Spontaneously active PRL cells, by contrast, display oscillations in [Ca\(^{2+}\)]\(_i\) with an
amplitude approximately 2-fold above baseline and periods of 45-60 seconds, when maintained in 355 mOsmolal medium. Both types of PRL-secreting cells have been reported in mammals, which show patterns of $[\text{Ca}^{2+}]_i$ that are remarkably similar to those which I have observed in the tilapia PRL cell (Stojilkovic and Catt, 1992; Tomic et al., 1994).

Both PRL cell types respond with equal rapidity upon exposure to hyposmotic medium (300 mOsmolal). Silent cells show a biphasic rise in $[\text{Ca}^{2+}]_i$ that peaks at about twice the basal $[\text{Ca}^{2+}]_i$ level before tailing to an intermediate level between the peak and baseline. In spontaneously active cells, the height of the peaks of $[\text{Ca}^{2+}]_i$ remains unchanged during hyposmotic stimulation, while the amplitude of the oscillations in $[\text{Ca}^{2+}]_i$ is reduced, due to an elevation of baseline $\text{Ca}^{2+}$ levels. The overall effect is a rise in $[\text{Ca}^{2+}]_i$. The oscillations of $[\text{Ca}^{2+}]_o$, though reduced, are not eliminated completely. It is clear that osmotic signals induce changes in the metabolism of $\text{Ca}^{2+}$ in the tilapia PRL cell that are of the same variety that one sees with hormonally-induced stimulation in a wide variety of endocrine cells (Stojilkovic and Catt, 1992). In the present studies, I further characterized the $[\text{Ca}^{2+}]_i$ oscillatory patterns in PRL cells, in steady state and in response to reductions in medium osmolality (Chapter IV). Furthermore, I employed $[\text{Ca}^{2+}]_i$ measurements in conjunction with all experiments designed to investigate the mechanisms involved in the signal transduction of the osmotic signal in PRL cells.

One of the specific aims of this investigation was to determine whether the rise in PRL release in response to reduced extracellular osmolality is triggered by an increase in cell size that leads to an increase in $[\text{Ca}^{2+}]_i$. I hypothesized that a fall in extracellular osmolality leads to a passive influx of water into the PRL cell that increases cell volume;
this initiates an influx of extracellular Ca\(^{2+}\) and a rise in \([Ca^{2+}]_{i}\), which mediates the increase in PRL release. In order to clarify these processes, experiments were proposed to determine: 1) whether an increase in cell size leads to an increase in \([Ca^{2+}]_{i}\); 2) whether an increase in \([Ca^{2+}]_{i}\) or cell size initiates an increase in PRL release; and 3) whether an increase in cell size and PRL release following exposure to hyposmotic medium is dependent on the entry of extracellular Ca\(^{2+}\) (Chapter IV).

_Transection of an osmotic signal into changes in PRL cell activity_

Stretch-activated Ca\(^{2+}\) and K\(^{+}\) channels were identified in several cell types (Lang et al., 1998). These channels show the common property that their activity varies directly with membrane tension; that is, the probability that these channels will open, and remain open longer, increases as pressure is applied to the cell membrane. A second class of channels, stretch-inactivated, is inhibited by increased membrane tension (cf. Morris and Sigurdson, 1989). It has been proposed that the activation of stretch-gated channels may be a link between mechanical stress and cell excitability, and that the channels participate in cell volume regulation and in the transduction of mechanical and osmotic stimuli (cf. Guharay and Sachs, 1984; Lansman et al., 1987; Sackin, 1987; Morris and Sigurdson, 1989). The proposal that stretch-sensitive ion channels may act in the transduction of osmotic information is particularly attractive in light of the fact that a 1 mOsmolal decrease in medium osmolality increases membrane stretch to a degree equivalent to 18 mm Hg, well within the range that stretch-sensitive ion channels are responsive (Morris and Sigurdson, 1989).
Cell volume changes following an osmotic stimulus could account for the osmosensitivity of certain endocrine and neuroendocrine pathways. Cell volume changes would lead to the activation or inactivation of stretch-sensitive ion channels that are linked to secretory mechanisms. An alternative explanation for the osmosensitivity, which does not invoke stretch-operated channels, would involve channels whose gating element is directly sensitive to external osmolality, presumably through modifications in channel protein structure. Until now, however, critical investigations have not been conducted on an isolated cell that has a clearly demonstrated osmoregulatory output.

To clarify the transduction mechanism that underlies the osmoreceptive ability of the tilapia PRL cell, RPDs were incubated in media whose osmolality was raised either by adding NaCl (55 mOsmolal) to the medium or by adding either the membrane-permeant molecule, urea, or the cell membrane-impermeant molecule, mannitol (Weber et al., 2002). In these experiments, perifusion of PRL cells was employed to clarify the temporal relationship between changes in PRL release and cell size. When medium osmolality was reduced from 355 mOsmolal to 300 mOsmolal, PRL cell size increased rapidly and was followed closely by a rise in PRL release. The close relation between cell size and PRL release was further underlined in another perifusion study. After establishing a low level of PRL release in 355 mOsmolal (hyperosmotic) medium, a portion of the NaCl (55 mOsmolal) was replaced with an equivalent amount of urea. This substitution resulted in an increase in cell size and in a rise in PRL release. The rate of increase in cell size and in medium PRL was slower, however, than that observed after the introduction of hyposmotic medium. While urea moves freely across cell membranes, its penetration rate is considerably lower than that of water. Thus, water
movement, and consequently cell size, is determined by the rate at which urea moves
down its concentration gradient into the cell. Once urea has equilibrated across the cell,
cell size and PRL release reach the same levels as those observed following exposure to
hyposmotic medium, but the time-course is prolonged. Neither cell size nor PRL release
changed following the substitution of 55 mOsmolal of NaCl with an osmotically
equivalent amount of mannitol. Thus, the pattern of PRL release coincided with changes
in osmotic gradient and not extracellular osmolality. These studies, then, provided a
direct demonstration that the osmotic regulation of the PRL cell is tied to changes in cell
volume that result from the osmotically induced movement of water across the cell rather
than as a direct response to extracellular osmolality per se. In one of the experiments
presented in Chapter III, medium osmolality was slowly reduced from 355 to 250
mOsmolal to mimic the slow change in the osmotic gradient that is induced when urea is
introduced as a substitute for NaCl. In this case, cell size and PRL release also increased
gradually in close coordination. Conversely, when osmolality was gradually increased
from 300 to 450 mOsmolal, cell size and PRL release decreased concordantly.

Several studies have investigated whether other endocrine cells respond to
changes in extracellular osmolality. These cells include mammalian anterior pituitary
cells, median eminence cells, rat pituitary tumor-derived GH\textsubscript{4}C\textsubscript{1} cells and pancreatic β
cells (Blackard \textit{et al.}, 1975; Sato \textit{et al.}, 1991a; Inukai \textit{et al.}, 1993; Srbak and Greer,
2000). It is unlikely, however, that these cells function as true osmoreceptors. First,
none of the hormones measured in these investigations have been demonstrated to have a
clear osmoregulatory function and the range of osmolality required to elicit a response
was by and large well outside the physiological range. Moreover, the responses were not
sustained. Typically, there was a burst of release followed by a return to baseline levels within 10 min of exposure to reduced osmolality. The character of the response contrasts with that of the tilapia PRL cell which is evoked by physiologically appropriate changes in osmolality and is sustained for hours. Rather, available information indicates that a wide range of cells that cannot be characterized as osmoreceptors can respond to osmotic shock with a nonspecific release of hormone (Strbak and Greer, 2000).

In rats, neurosecretory cells with osmoreceptor response properties occur in the supraoptic and paraventricular nuclei and in the organum vasculosum of the lamina terminalis (Honda et al., 1990; Bourque and Oliet, 1997; Bourque, 1998). In this case, increases in extracellular solute concentration enhance the release of both oxytocin and vasopressin in association with an increase the frequency of action potentials in magnocellular neurons (Negoro et al., 1988; Bourque, 1998). There is evidence that this signal transduction occurs via stretch-inactivated cation channels (Oliet and Bourque, 1993; Bourque, 1998). The osmotic regulation of oxytocin and vasopressin release undoubtedly represents an important physiological response in mammals, since these hormones are involved in sodium excretion and water retention (Verbalis et al., 1986; Honda et al., 1990; Verbalis et al., 1991; Bourque, 1998). Nevertheless, as mentioned previously, the complex anatomical arrangement of these hypothalamic osmoreceptors, has prevented direct experimental approaches. Specifically, one cannot simultaneously measure changes in cell size and the hormonal osmoregulatory output in a cell that can be distinguished specifically as either a vasopressin or oxytocin neuron (Oliet and Bourque, 1992).
As previously mentioned, I hypothesized that stretch-activated ion channels play a central role in the transduction of osmotic stimuli in the tilapia PRL cell. Thus, I examined the link between an increase in PRL cell volume, the rise in $[\mathrm{Ca^{2+}}]$ and PRL release by employing pharmacological approaches suited to test the involvement of stretch-activated $\mathrm{Ca^{2+}}$ channels in this process (Chapter V).

**The induction of changes in gene expression by changes in medium osmolality**

It is now clear that the administration of an osmotic stimulus *in vivo* can alter the expression of at least two homologous osmoregulatory peptides of the vertebrate neurohypophyses, vasotocin and vasopressin (Hyodo and Urano, 1991; Chaturvedi *et al.*, 1997; Jaccoby *et al.*, 1997; Murphy *et al.*, 1998; Saito and Grossmann, 1998; Hyodo, 1999; Chaturvedi *et al.*, 2000). In FW fish, vasotocin stimulates a rise in glomerular filtration rate and in urine flow. This diuretic action is not observed in SW fish in which the excessive loss of water would be maladaptive (Perks, 1987). In the flounder and rainbow trout, plasma levels of vasotocin are higher when fish are in FW compared with fish in SW (Perrott *et al.*, 1991). Likewise, Hyodo and Urano (1991) have shown that mRNA levels of vasotocin increase during FW adaptation in the rainbow trout.

Vasopressin is antidiuretic in mammals. Levels of vasopressin mRNA rise under osmotic salt challenge *in vivo* (McCabe *et al.*, 1990). This and other evidence make it increasingly clear that the expression of vasopressin and vasotocin genes, as well as the secretion of the two hormones, is governed by an osmoreceptor (Schrier *et al.*, 1979; Leng *et al.*, 1982; Leng *et al.*, 1999). However, because the cells which produce vasopressin and vasotocin cannot be isolated, it remains to be determined whether the
effects of extracellular osmolality are produced directly at the vasopressin and vasotocin cells or whether they are mediated through one or more other cell types that are themselves osmosensitive.

Continued exposure to an osmotic stimulus evokes changes in the tilapia PRL cell in vitro that extend beyond the rapid effects on PRL release and on second messenger metabolism. Sustained exposure to reduced extracellular osmolality has been known to alter the rate of synthesis of the two tilapia PRLs and the levels of their respective mRNAs (Yoshikawa, 1992, Uchida, unpublished data). Exposure to reduced extracellular osmolality augments the incorporation of $^3$H-leucine into each of the two PRLs after 4-6 hours, but not before (Yoshikawa, 1992). The full time course of this response, however, has yet to be fully characterized. It is possible that osmotically induced changes in Ca$^{2+}$ or cAMP metabolism may secondarily induce changes in the mRNA and synthesis of the two PRLs. Although gene expression was outside the scope of this dissertation, I examined the effects of long-term (1 week) exposure to changes in extracellular osmolality on PRL release and content from whole pituitaries (Chapter II). This study provided additional information on the role of increased PRL synthesis in maintaining sustained release.

Research objectives

The main objective of my research was to investigate the mechanisms involved in transducing an osmotic stimulus into an increase in PRL release. In this process I studied the relationships between cell volume, [Ca$^{2+}$], and PRL release in response to varying...
osmolalities, depolarizing K⁺ and ion channel blockers. In the following five chapters of the dissertation, I will describe:

1) The release and content of PRL, GH and ACTH from whole pituitaries in response to a long-term (days) reduction in medium osmolality and the effects of blood withdrawal and salinity change on plasma osmolality and circulating levels of PRL and GH (Chapter II).

2) The release of PRL and GH from dispersed cells and whole pituitaries in response to reductions and elevations in medium osmolality. In this study I also characterized the relationship between gradual changes in osmolality, cell volume and PRL and GH release (Chapter III).

3) The patterns of \([\text{Ca}^2+]_{i}\) in PRL cells and the role of extracellular \(\text{Ca}^2+\) in hyposmotically-induced PRL release (Chapter IV).

4) The involvement of stretch-activated ion channels in the transduction of reduced extracellular osmolality into PRL release (Chapter V).

5) In Chapter VI, I make some final remarks and conclusions.

Chapter II has been published in General and Comparative Endocrinology (Vol. 128, pp. 91-101) and Chapter III is in preparation for publication. Chapters IV and V have been submitted for publication in the American Journal of Physiology.
CHAPTER II

Effects of environmental osmolality on release of prolactin, growth hormone and ACTH from the tilapia pituitary

INTRODUCTION

Prolactin (PRL) is centrally important to the maintenance of hydromineral balance of fish in fresh water (FW), and cortisol is a key factor in seawater (SW) (Utida et al., 1971; Assem and Hanke, 1981; Hirano, 1986; Brown and Brown, 1987; McCormick, 2002). Recently, growth hormone (GH) and its mediator, insulin-like growth factor I (IGF-I), have also been implicated in the control of SW adaptation in several species of salmonids (McCormick et al., 1991; Sakamoto et al., 1991) as well as in the euryhaline tilapia, Oreochromis mossambicus (Sakamoto et al., 1997; Fruchtman et al., 2000) and the killifish, Fundulus heteroclitus (Mancera and McCormick, 1998).

Consistent with its role in FW osmoregulation, PRL cell activity is higher in FW fish than in SW fish (Nagahama et al., 1975; Nishioka et al., 1988). In some euryhaline species, such as tilapias and mollies, the in vitro release of PRL is stimulated by reducing the osmolality of the incubation medium (Nagahama et al., 1975; Nishioka et al., 1988; Grau and Helms, 1990; Grau et al., 1994). An increase in circulating levels of PRL was observed when tilapia were transferred from SW to FW, whereas there was a reduction in PRL levels when they were transferred from FW to SW or brackish water (Nicoll et al., 1981; Ayson et al., 1993; Auperin et al., 1994; Yada et al., 1994).
In tilapia, a transient but significant increase in plasma GH levels occurred when fish were transferred from FW to 70% SW, which corroborates the role of GH in SW adaptation in this species (Yada et al., 1994; Sakamoto et al., 1997). Furthermore, pituitary GH content and GH cell activity were found to be higher in SW compared with FW tilapia (Borski et al., 1994). On the other hand, there seems to be no report on the effect of environmental salinity on adrenocorticotropic hormone (ACTH) release, although plasma cortisol, which is stimulated by ACTH, is elevated in SW (Assem and Hanke, 1981).

The tilapia pituitary produces two distinct PRL molecules, PRL$_{177}$ and PRL$_{188}$ that are encoded by separate genes (Specker et al., 1985; Yamaguchi et al., 1988; Rentier-Delrue et al., 1989). This study describes the short- and long-term effects of osmolality on simultaneous PRL$_{177}$, PRL$_{188}$, GH and ACTH release from the pituitary of euryhaline tilapia (*Oreochromis mossambicus*) in vitro. Changes in plasma levels of the two PRLs and GH as well as plasma osmolality were examined simultaneously after transfer from FW to 80% SW and also from SW to FW. Since preliminary experiments indicated that repeated blood withdrawal brings about a significant reduction in blood osmolality, changes in PRLs and GH were simultaneously determined after repeated blood withdrawal from fish acclimated to FW.
MATERIALS AND METHODS

Fish
Euryhaline tilapia (*Oreochromis mossambicus*) were obtained from a population maintained at the Hawaii Institute of Marine Biology. They were kept in 5000-liter tanks in FW or SW (32 ppt) under natural photoperiod. They were fed twice daily with Purina Trout Chow (approximately 2% of body weight per day). Water temperature was 22-26°C.

Transfer experiment
Mature male and female tilapia (80-100 g) were acclimated for at least 2 weeks in 600-liter oval fiberglass tanks containing either FW or SW. One group of fish was transferred from SW to FW, while the other group was transferred from FW to 80% SW (26 ppt). Fish were sacrificed 6 h after transfer. Prior to blood collection, fish were anesthetized in tricaine methanesulfonate (MS222, Sigma, St. Louis, MO, 0.5 g/l) neutralized with NaHCO₃ (0.5 g/l). Blood was taken by caudal puncture using a syringe coated with ammonium heparin (200 units/ml). After centrifugation at 10,000 rpm for 5 min, plasma was removed and stored at -20°C until the hormones were assayed. Plasma osmolality was measured with a vapor pressure osmometer (Wescor 5100C, Logan, UT).

Repeated blood withdrawal
Mature male and female tilapia, weighing 300-500 g, were kept in 5000-liter
oval fiberglass tanks containing FW. Blood samples were taken at 0, 0.5, 1, 4, 8, 12 h and 1, 2, 3, 5 and 7 days. Each sample consisted of 2-3% of total blood volume as estimated by Okimoto et al. (1994). Prior to blood collection, fish were anesthetized in MS222 (0.5 g/l) neutralized with NaHCO₃ (0.5 g/l). Blood was taken by caudal puncture and plasma was removed as described above.

Pituitary incubation

For the long-term incubation, pituitaries were removed from sexually mature tilapia (80-150 g). Each pituitary was placed individually in hyperosmotic (365 mOsmolal) 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. (1977) together with the antibiotic, gentamycin sulfate (Sigma, 50 μg/ml). Whole pituitaries were pre-incubated for 2 h, at 26-28 °C on a gyratory platform (80 rpm) under a humidified atmosphere of 95% O₂/5% CO₂ and then rinsed once more with hyperosmotic medium. Eight pituitaries (4 from males and 4 from females) were incubated separately in either hyperosmotic medium or hyposmotic medium (290 mOsmolal). The medium was changed after 6 and 12 h, and 1, 2, 3, 4, 5, 6 and 7 days. On day 7, the pituitaries were sonicated in 200 μl of RIA buffer (0.01 M phosphate buffer, pH 7.3, containing 0.14 M NaCl, 1% BSA, 0.01% NaN₃, and 0.1% Triton X-100) and kept frozen at -20°C until hormone quantification.

For comparison with the long-term incubation of pituitaries, hormone content was determined in single pituitaries removed from mature tilapia in FW (4 males and 4 females, weighing 80-150 g). The pituitaries were sonicated as described above and kept
frozen at -20°C. Data from both sexes were combined, since their responses were not statistically significantly different from each other.

**Radioimmunoassays**

Tilapia PRL$_{188}$ and GH in the culture medium and pituitary tissues and in plasma samples were measured by homologous radioimmunoassays (Ayson *et al.*, 1993; Yada *et al.*, 1994). The iodination of PRL$_{177}$ was carried out by the Iodogen method (Salacinski *et al.*, 1981; Ayson *et al.*, 1993; Swanson, 1994). Micro-centrifuge tubes (1.5 ml) were coated with 100 μl Iodogen (Pierce Chemical Co., Rockford, IL, 40 μg/ml CH$_2$Cl$_2$). The solution was then evaporated with N$_2$. The iodogen-coated tubes were stored at -80°C. For the iodination, 10μl of elution buffer (0.1 M Tris-HCl, 0.1% Triton X-100, pH 7.2) were added to an iodogen-coated tube, followed by 5 μl of Na$^{125}$I (Amersham Pharmacia Biotech, Piscataway, NJ) and 5 μg of PRL$_{177}$ dissolved in 25 μl elution buffer. The reaction mixture was tapped gently and allowed to rest for 15 min, after which 100 μl of elution buffer was added. The mixture was then applied to a Sephadex G-75 column (0.7 x 30 cm, Pharmacia, Uppsala, Sweden), previously saturated with 0.1 M Tris-HCl containing 1% Triton X-100, and further washed with elution buffer. Fractions were collected after 10 drops in tubes containing 50 μl of RIA buffer. The fractions containing labeled PRL$_{177}$ were stored at -20°C. The radioimmunoassay procedure was the same as described by Ayson *et al.* (1993).

For the measurement of ACTH, a heterologous radioimmunoassay specific for human ACTH (1-39) was employed using antiserum 19 (anti-rabbit), as described by Lancha *et al.* (1994). Synthetic human ACTH (1-39) was iodinated by chloramine-T.
Assay procedures were essentially the same as those employed for tilapia PRLs and GH, using 10 mM phosphate buffer (pH 7.3) containing 0.1% triton X-100 and 1% BSA as assay buffer. Lyophilized $^{125}\text{I}\text{-ACTH}$, ACTH standard and ACTH antiserum were re-suspended in distilled water. ACTH standard was further diluted to 10 μg/ml with 5% acetic acid containing 1 mg/ml BSA. Antiserum was diluted with 50 mM EDTA in 10 mM phosphate buffer. Each assay tube contained 50 μl of standard or sample, 50 μl of $^{125}\text{I}\text{-ACTH}$ (10,000 cpm) in assay buffer, and 50 μl of anti-ACTH antibody (1: 15,000) in EDTA-phosphate buffer containing 1% normal rabbit serum. After incubation for 18 hr at 4°C, 100 μl of goat anti-rabbit IgG (Sigma, St. Louis, MO) in EDTA-phosphate buffer was added followed by 100 μl assay buffer. Tubes were centrifuged and counted in a gamma counter (Cobra II, Packard, Meriden, CT).

As shown in Fig. 1, serial dilutions of pituitary homogenate and culture medium of tilapia pituitary showed inhibition curves parallel to the ACTH standard. No cross-reaction was seen with proopiomelanocortin peptides of chum salmon (chum salmon α-MSH, desacetyl-α-MSH, β-MSH, β-endorphin I, β-endorphin II and N-terminal peptide, kindly provided by Prof. H. Kawauchi, Kitasato University); a slight cross-reaction with acetyl-α-MSH is in agreement with previous observation (Lancha et al., 1994). This antiserum was not used for the measurement of ACTH levels in tilapia plasma, since plasma from hypophysectomized fish cross-reacted equally with plasma from the intact fish. Treatment of the plasma with 5 N acetic acid did not remove the cross-reaction in the hypophysectomized fish plasma (unpublished observation).
Fig. 1. Competitive binding curves for ACTH standard, and serial dilution of pituitary homogenate and culture medium of tilapia pituitary. The proopiomelanocortin peptides of chum salmon (α-MSH, desacetyl-α-MSH, β-MSH, β-endorphin I, β-endorphin II and N-terminal peptide) did not show cross-reaction with ACTH standard, and are plotted in the upper right corner.
Desacetyl-α-MSH

-MSH

Endorphin

-Endorphin

NPP

α-MSH

culture medium

pituitary extract

0.063 0.25 1.00 μl

0.01 0.1 1 10 100 1000 ng/ml

B/B₀ (%)
Statistical analysis

Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test or Mann-Whitney U-test. Significance level was set at 95% (p<0.05). Calculations were performed using Minitab Statistical Software Package (State College, PA) and STATISTICA (StatSoft, Tulsa, OK). Data are expressed as means ± S.E.M.

RESULTS

Transfer experiment

As shown in Fig. 2A, plasma osmolality of the tilapia acclimated to SW (349 mOsmolal) was significantly (P < 0.001) higher than that of the fish kept in FW (312 mOsmolal). Plasma osmolality increased significantly 6 h after transfer from FW to 80% SW (443 mOsmolal, P < 0.001). Plasma osmolality decreased to the level of FW-acclimated fish (305 mOsmolal) 6 h following transfer from SW to FW.

The plasma of fish acclimated to FW had significantly higher levels of PRL_{177} and PRL_{188} (P < 0.05 and P < 0.001 respectively) than the plasma of fish acclimated to SW (Figs. 2B and 2C). When they were transferred from FW to 80% SW, a significant reduction in plasma PRL_{177} and PRL_{188} (P < 0.05 and P < 0.001 respectively) levels was observed after 6 h compared with FW-acclimated fish. Transfer from SW to FW
Fig. 2. Effects of transfer of tilapia from fresh water (FW) to 80% seawater (SW) and from SW to FW on plasma osmolality (A), PRL<sub>177</sub> (B), PRL<sub>188</sub> (C) and GH (D). Plasma samples were collected 6 h after transfer. Vertical bars indicate mean ± S.E.M. (n=10-12).

***, ††† Significantly different from FW or SW, respectively, at $P < 0.001$.

* Significantly different from FW, $P < 0.05$. 
elicited a marked increase in PRL$^{177}$ and PRL$^{188}$ levels ($P < 0.001$) after 6 h compared with levels in SW-acclimated fish. 

On the other hand, significant increases ($P < 0.001$) in plasma GH were observed only at 6 h after transfer from FW to 80% SW. However, there was no difference in plasma GH levels between fish acclimated to FW and those to SW (Fig. 2D). I could not determine changes in plasma levels of ACTH, since the antibody used cross-reacted with the plasma from hypophysectomized tilapia.

Effects of repeated blood withdrawal

In this experiment, FW-acclimated tilapia, weighing 300-500 g, were used. Blood samples were taken at 0, 0.5, 1, 4, 8, 12 h and 1, 2, 3, 5 and 7 days. Each sample consisted of 2-3% of total blood volume (blood volume = 7% of body weight, as estimated by Okimoto et al. (1994). As shown in Fig. 3A, a significant ($P < 0.01$) reduction in plasma osmolality was observed after 1 h. The osmolality kept decreasing during the next 24 hr, and returned to initial levels after 3 days. In association with the reduction in plasma osmolality, significant increases in plasma PRL$^{177}$ (Fig. 3B) and PRL$^{188}$ (Fig. 3C) were observed during the first 24 h. The maximum circulating levels, 65 ng/ml for PRL$^{177}$ and 175 ng/ml for PRL$^{188}$, were obtained after 12 h. On the other hand, there was no significant change in plasma GH levels throughout the course of the experiment (Fig. 3D).
Fig. 3. Effects of repeated blood withdrawal from male tilapia in fresh water on plasma osmolality (A), PRL\textsubscript{177} (B), PRL\textsubscript{188} (C) and GH (D). Plasma samples, consisting of 2-3% of total blood volume, were taken at 0, 0.5, 1, 4, 8 and 12 h, and 1, 2, 3, 5 and 7 days. Vertical bars indicate mean ± S.E.M. (n= 4-6).

*, **, *** Significantly different from first sampling (0 h) at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
Effects of medium osmolality on PRL, GH and ACTH release during long-term incubation

Entire pituitaries from FW tilapia were incubated either in hyposmotic (290 mOsmol) or hyperosmotic (365 mOsmol) medium for 7 days. The rates of release of both PRL$_{177}$ and PRL$_{188}$ were significantly ($P < 0.01$) greater in the hyposmotic medium (300-400 and 600-800 ng/100 g/h, respectively) than in the hyperosmotic medium (200 and 350 ng/100 g/h, respectively) for the first 12 hours (Figs. 4A and B). The rate of release gradually declined during the incubation, reaching approximately 100 ng/100 g/h for both PRLs after 7 days.

Reflecting the relatively high release rates during the first 12 h, the cumulative release of both PRLs was greater under hyposmotic conditions than under hyperosmotic conditions for the first 24 h (Figs. 5A and B). However, there was no significant difference in release under hyper- and hyposmotic conditions after 2 days and thereafter, with total PRL$_{177}$ and PRL$_{188}$ released reaching 20-25 and 40-50 μg/100 g, respectively after 7 days of incubation. On the other hand, amounts of PRL$_{177}$ and PRL$_{188}$ remaining in the pituitary after 7 days were significantly ($P < 0.01$) lower under hyposmotic conditions, whereas % release of PRLs during 7 days of incubation was significantly ($P < 0.01$) greater under the same conditions (Table 1).

The release of GH from the same pituitaries was relatively low (10-40 ng/100 g/h) compared with PRL secretion. The rate of release under hyposmotic conditions was significantly ($P < 0.01$) greater at 6 h of incubation than the rate under hyperosmotic conditions (Fig. 4C). After 24 h however, the rate of release became significantly ($P <$
Fig. 4. Effects of long-term incubations of whole pituitaries under hyperosmotic and hyposmotic conditions on the hourly release rate of PRL$_{177}$, PRL$_{188}$, GH and ACTH. Pituitaries were incubated for 7 days, and medium samples were taken at 6 and 12 h, and 1, 2, 3, 4, 5, 6 and 7 days. Vertical bars indicate mean ± S.E.M. (n= 6-8). Release rates of the hormones was normalized to fish body weight.

*, **, *** Significantly different from first sampling (0 h) at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
Fig. 5. Effects of long-term incubations of whole pituitaries under hyperosmotic and hyposmotic conditions on cumulative release of PRL177, PRL188, GH and ACTH. Pituitaries were incubated for 7 days, and medium samples were taken at 6 and 12 h, and 1, 2, 3, 4, 5, 6 and 7 days. Vertical bars indicate mean ± S.E.M. (n= 6-8). Cumulative release of the hormones was normalized to fish body weight.

*, ** Significantly different from first sampling (0 h) at \( P < 0.05 \) and \( P < 0.01 \), respectively.
0.01) lower than in hyperosmotic conditions. No effect of medium osmolality was seen thereafter, although the rate of release tended to be greater in hyperosmotic medium up to 3 days. On the other hand, cumulative release of GH was significantly \( (P < 0.05) \) greater during the first 12 h in hyposmotic medium, but more GH was released in hyperosmotic medium after 2 days and throughout the remainder of the incubation (Fig. 5C). After 7 days, cumulative release of GH was 7 \( \mu g/100 \text{ g} \) in hyperosmotic medium and 3.3 \( \mu g/100 \text{ g} \) in hyposmotic medium. However, no significant difference was observed in pituitary GH content after 7 days or in % release during 7 days of incubation between hyperosmotic and hyposmotic incubations (Table 1).

The release of ACTH was low (2-4 ng/100 g/h) compared with PRL, and there was no consistent effect of medium osmolality. After 12 h, release was significantly \( (P < 0.001) \) greater under hyposmotic conditions, but after 6 days, it was significantly lower \( (P < 0.01) \) (Fig. 4D). There was no difference in cumulative release, total hormone secreted in 7 days or final pituitary content (Fig. 5D, Table 1).

Pituitaries were also removed from FW-adapted fish to quantify hormone content in vivo. The total pituitary content of PRL\(_{177}\) was 14.4 ± 0.96 \( \mu g \)/100 g; PRL\(_{188}\) and GH were 17.1 ± 3.83 \( \mu g/100 \text{ g} \), and 6.43 ± 0.35 \( \mu g/100 \text{ g} \), respectively (n=8).
Table I: Effects of medium osmolality on pituitary content after 7 days, absolute hormone release, and on % hormone release during incubation for 7 days.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Content (µg/100 g)</th>
<th>365 mOsmolal</th>
<th>290 mOsmolal</th>
<th></th>
<th>Hormone released (µg/100 g)</th>
<th>365 mOsmolal</th>
<th>290 mOsmolal</th>
<th>% Release</th>
<th>365 mOsmolal</th>
<th>290 mOsmolal</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRL188</td>
<td>5.94 ± 0.60</td>
<td>2.81 ± 0.63**</td>
<td>44.7 ± 3.7</td>
<td>51.2 ± 4.8</td>
<td>87.9 ± 1.5</td>
<td>94.7 ± 1.2**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRL177</td>
<td>7.9 ± 1.0</td>
<td>2.86 ± 0.36**</td>
<td>21.4 ± 1.3</td>
<td>22.4 ± 2.4</td>
<td>73.5 ± 2.2</td>
<td>87.8 ± 2.1**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>3.87 ± 0.51</td>
<td>2.58 ± 0.44</td>
<td>7.02 ± 1.36</td>
<td>3.34 ± 0.28</td>
<td>63.6 ± 4.0</td>
<td>57.1 ± 4.6</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>0.49 ± 0.13</td>
<td>0.31 ± 0.07</td>
<td>0.48 ± 0.3</td>
<td>0.39 ± 0.07</td>
<td>63.4 ± 4.3</td>
<td>47.9 ± 7.4</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n = 6-8). Hormone content and released hormone were normalized to 100 g body weight of the fish.

** Significantly different from 365 mOsmolal at P < 0.01.
DISCUSSION

The tilapia pituitary produces two distinct PRL molecules, PRL$_{177}$ and PRL$_{188}$, that are encoded by separate genes (Specker et al., 1985; Yamaguchi et al., 1988; Rentier-Delrue et al., 1989). It has been well documented that circulating levels of the two PRLs increase when euryhaline tilapia (O. mossambicus) are transferred from SW to FW, whereas there is a reduction when transferred from FW to SW or brackish water (Ayson et al., 1993; Yada et al., 1994). In the less euryhaline species, O. niloticus, Auperin et al. (1994) also reported a marked decrease in plasma PRL levels within 6 h after transfer to brackish water. Recently, Shepherd et al. (1999) have clearly shown that changes in plasma osmolality in vivo exert a direct regulatory action on PRL release and gene expression in the tilapia pituitary. In the present study, I have also observed a rapid reduction in plasma PRL$_{177}$ and PRL$_{188}$ after transfer to 80% SW within 6 h as well as a rapid increase after transfer from SW to FW. The changes in plasma PRL levels were inversely correlated with the changes in plasma osmolality, in agreement with previous reports (Ayson et al., 1993; Auperin et al., 1994; Yada et al., 1994).

While conducting experiments on plasma clearance of recombinant bovine GH in tilapia, it was noticed that repeated blood withdrawal leads to a marked increase in plasma PRL levels (more than 400 ng/ml). Thus, I hypothesized that the increase in plasma PRL after blood withdrawal is due to the reduction in plasma osmolality as a result of hemodilution. As shown in Fig. 3, repeated withdrawal of blood caused a dramatic increase in plasma PRL$_{177}$ and PRL$_{188}$, up to about 175 ng/ml and 65 ng/ml,
respectively, after the 6th sampling of 2-3% of total blood volume. The increase in plasma PRLs was again well correlated with the reduction in plasma osmolality. The effect seems to be specific to PRLs, as there was no significant change in plasma GH levels. Since increased stress and/or change in blood volume may also be involved in the pathway(s) responsible for the rise in PRL following repeated blood withdrawal, further studies are being conducted to examine if the reduction in plasma osmolality is due to hemodilution in order to maintain blood volume, and if such effect could be seen in SW acclimated fish as well.

The rostral pars distalis (RPD) of the tilapia pituitary is almost entirely composed of PRL cells, and provides an excellent model to study regulatory mechanisms of PRL secretion not only in fish but also in vertebrates in general (Nishioka et al., 1988; Grau and Helms, 1989; Grau and Helms, 1990; Grau et al., 1994). In many hormone-secreting cells, non-specific hormone release triggered by osmotic cell swelling has been observed (Lang et al., 1998; Strbak and Greer, 2000). In most of the studies, the spike in hormonal release following reduction in osmolality is short-lived (approx. 10 min), and medium osmolalities are often reduced to levels lower than those normally experienced by the animals. In contrast, the range of osmolality to which the tilapia PRL cell responds in vitro is well within the range of blood osmolalities observed in vivo. Previous studies have described the effects of reduced extracellular osmolality on PRL release, and it has been shown that shifts in osmolality as small as 15 mOsmolal, less than 5% of normal serum osmolality, are sufficient to markedly alter the activity of the tilapia PRL cell (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981; Grau et al., 1987; Nishioka et al., 1988; Helms et al., 1991; Borski et al., 1994; Grau et al., 1994). In the
present study, both PRL177 and PRL188 were stimulated in hyposmotic medium during the first day of culture, confirming previous reports.

In teleosts, GH is involved in the regulation of several physiological processes such as growth, osmoregulation, metabolism, reproduction and development (Donaldson et al., 1979; Bern and Madsen, 1992; McLean and Donaldson, 1993; Shepherd et al., 1997). GH and its mediator, insulin-like growth factor I (IGF-I), have also been shown to facilitate SW adaptation in several salmonids (McCormick et al., 1991; Sakamoto et al., 1991) as well as in the euryhaline tilapia, *O. mossambicus* (Sakamoto et al., 1997; Fruchtman et al., 2000) and the killifish, *Fundulus heteroclitus* (Mancera and McCormick, 1998). The SW-adapting effect of GH seems to be limited to the euryhaline species, as Auperin et al. (1995) failed to show hyposmoregulatory activity of GH in the less euryhaline tilapia, *O. niloticus*. In *O. mossambicus*, pituitary GH content and GH cell activity were found to be higher in SW tilapia when compared to FW tilapia (Borski et al., 1994). In this study, I observed a significant increase in plasma GH levels 6 h after transfer of fish from FW to 80% SW, whereas there was no change when they were transferred from 100% SW to FW. Furthermore, there was no significant difference between the fish acclimated to FW and those to SW, suggesting that GH is involved in maintaining hydromineral balance specifically during the course of SW adaptation. Alternatively, there could be an increase in the GH clearance rate in SW-acclimated fish that could account for the lack of difference in GH levels between FW and SW-acclimated fish (Sakamoto et al., 1990). These results are in good agreement with previous observations in *O. mossambicus* (Ayson et al., 1993; Borski et al., 1994; Yada et al., 1994).
In contrast to the well-established effects of cortisol in fish osmoregulation, particularly during the course of SW adaptation (see reviews by (Bern and Madsen, 1992; McCormick, 1995; McCormick, 2002), little is known about the effects of environmental salinity on ACTH secretion in teleosts. An acute stress, produced by crowding and confinement, increased plasma ACTH and cortisol in coho salmon (Sumpter and Donaldson, 1986) and brown trout (Norris et al., 1999). In the tilapia (O. mossambicus), Balm et al. (1994) reported that a confinement for 3 h resulted in high plasma cortisol and ACTH levels. Since stress for less than 12 min did not induce any change in plasma ACTH in spite of a marked increase in plasma cortisol, they concluded that both neuronal mechanisms and cortisol feedback may have regulated the pituitary-interrenal axis at the level of the interrenal. In this study, I was unable to measure plasma levels of ACTH, since the antibody used cross-reacted with hypophysectomized fish (Fig. 1). According to Balm et al. (1994), the rate of secretion of ACTH from the tilapia pituitary was 6-9 pg/min/pituitary. Since they used fish weighing about 18 g, the rate was 2-3 ng/100 g/h, which is comparable to the rate observed in this study (2-4 ng/100 g/h). In any case, the rate of ACTH secretion was low compared with the secretion rates of GH (20-40 ng/100 g/h) and of PRL (100-1000 ng/100 g/h). The difference in secretion rate among the three hormones may reflect the difference in the cell population producing each hormone (Nishioka et al., 1988). In humans, there was no difference in circulating ACTH levels from patients infused with 0.9% saline and those infused with 5% saline (Elias et al., 1997). In the present study, there was no difference in ACTH release between the pituitaries incubated in hyposmotic medium and those in hyperosmotic medium for 7 days, clearly indicating that tilapia ACTH cells do not respond to changes in extracellular
Therefore, any increases in cortisol that accompany SW adaptation will not be a reflection of hyperosmotic-induced direct activation of ACTH release at the level of the corticotroph.

During long-term culture (7 days), the release of PRL\textsubscript{177} and PRL\textsubscript{188} was greater in hyposmotic conditions only during the first 12 h, after which rates dropped to the same levels observed in the hyperosmotic incubations. Reflecting the high levels of release during the first 12 h, the cumulative release of both PRLs was greater in hyposmotic medium than in hyperosmotic medium for the first 24 h. On the other hand, GH release from the same pituitaries was relatively low compared with PRL release. No consistent effect of medium osmolality was seen during the first 24 h. There was an elevation of GH in hyposmotic medium during the first 6 h, which may be a result of a nonspecific effect of cell swelling. Unlike PRL release, however, the hyposmotic effect on GH release was not sustained, and there was no significant difference in cumulative release of GH during the first 24 h between hyper and hyposmotic medium. This result is consistent with a previous \textit{in vitro} study in this species (Helms \textit{et al.}, 1987). In the present study, however, release tended to be greater under hyperosmotic medium after 2 and 3 days, and reflecting the tendency, cumulative GH release was greater in hyperosmotic medium after 2 days and thereafter. In agreement with this observation, Borski \textit{et al.} (1994) reported greater GH cell activity in the tilapia in SW compared with those in FW. In salmonids, an opposite pattern has been shown, where rates of GH release were about 20-fold higher than PRL release up to 12 h and increased to over 200-fold thereafter (Suzuki \textit{et al.}, 1987; Yada and Hirano, 1992).
In the present study, the total pituitary content of PRL\textsubscript{177} and PRL\textsubscript{188} in FW fish was about 14 and 17 μg/100 g body weight, respectively, whereas about 20 and 50 μg/100 g of PRL\textsubscript{177} and PRL\textsubscript{188}, respectively, was released during the 7-day culture. This observation indicates that, at least for PRL\textsubscript{188}, most of the hormone released during the long-term incubation was synthesized during the incubation itself. Furthermore, 30% of the total PRL\textsubscript{177} and PRL\textsubscript{188} was released during the first day of the experiment under hyposmotic conditions, and only 17% and 20% of PRL\textsubscript{177} and PRL\textsubscript{188}, respectively, were released under hyperosmotic conditions during the same period. It has been shown that exposure to reduced osmotic pressure augments \textsuperscript{3}H-leucine incorporation into each of the two PRLs after 4-6 h, indicating that sustained exposure to reduced osmolality enhances the synthesis of tilapia PRLs (Nagahama \textit{et al.}, 1975; Yoshikawa-Ebesu \textit{et al.}, 1995). Reduced medium osmolality also increased mRNA levels of both PRLs compared to levels in tissues exposed to hyperosmotic medium (Yoshikawa, 1992). Furthermore, the mRNA levels of both PRLs were higher in pituitaries of FW-adapted tilapia than in SW fish, and decreased with increasing salinity, in both sham-operated and RPD-autotransplanted groups (Shepherd \textit{et al.}, 1999). Thus, the current results are in agreement with the effects of osmotic pressure on PRL gene expression.

In conclusion, the present results indicate that PRL release from the tilapia pituitary is stimulated both \textit{in vivo} and \textit{in vitro} as extracellular osmolality is reduced, but for no longer than 2 days. Of the hormones tested, PRL has shown the strongest relationship with alterations in extracellular osmolality. The release of GH, on the other hand, tends to increase temporarily with increasing osmolality, and ACTH seems to be relatively insensitive to the changes in extracellular osmolality.
CHAPTER III

Disparate release of prolactin and growth hormone from the tilapia pituitary in response to osmotic stimulation

INTRODUCTION

Osmoregulation in teleost fish is dependent on the interplay of various pituitary hormones, such as prolactin (PRL) and growth hormone (GH) (Manzon, 2002; McCormick, 2002). In euryhaline species such as the tilapia, Oreochromis mossambicus, PRL is known to promote freshwater adaptation, whereas GH is considered a seawater-adapting hormone (Hirano, 1986; McCormick, 2002). In vitro release of PRL from the tilapia pituitary is increased when extracellular osmolality is reduced (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981; Seale et al., 2002b, Chapter II), and circulating PRL levels are increased after transfer from seawater to freshwater (Yada et al., 1994; Seale et al., 2002b, Chapter II). In vitro release of GH, on the other hand, has been reported to increase when medium osmolality is increased (Helms et al., 1987), and circulating levels of GH were increased after transfer from fresh water to 80% seawater (Yada et al., 1994; Seale et al., 2002b, Chapter II). Because of their direct osmosensitivity and their ability to maintain hydromineral balance in fresh water, PRL cells are considered osmoreceptors. In the tilapia, the mediation between osmosensitivity and increased PRL release seems to occur through stretch-gated Ca\(^{2+}\)-permeant channels that respond to cell swelling (see Chapters IV and V). The effects of extracellular osmolality on GH release, however, have been studied only in long-term static cultures...
and it is still unclear to what extent GH cells are osmosensitive (Helms et al., 1987; Seale et al., 2002b, Chapter II).

The objectives of this study were to examine PRL and GH release from the tilapia pituitary in response to gradual changes in medium osmolality. The results indicate differential regulation of PRL and GH release in response to alterations in medium osmolality.

MATERIALS AND METHODS

Fish

Mature Mozambique 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 5000-liter tanks in fresh water, and fed twice daily with Purina Trout Chow (approximately 2% of body weight 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.

Static cultures

Pituitaries were removed after decapitation and placed individually in hyperosmotic (355 mOsmolal) 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. (1977). Whole pituitaries were cultured in 24-well culture plates kept at
26-28 °C on a gyratory platform (80 rpm) under a humidified atmosphere composed of 95% O₂ and 5% CO₂. Pituitaries were pre-incubated for 18-20 h in 300 µl of hyperosmotic medium (355 mOsmolal), and then rinsed once with hyperosmotic medium before initiating the experiment. Pituitaries were exposed to either 300, 355 or 400 mOsmolal medium, and medium samples (300 µl) were taken hourly for 4 hours. Medium samples were then diluted 1:10 with RIA buffer (0.01 M phosphate buffer, pH 7.3, containing 0.14 M NaCl, 1% BSA, 0.01% NaN₃, and 0.1% Triton X-100) and kept frozen until quantification by radioimmunoassays.

Cell dispersions and perifusion

Pituitaries were removed after decapitation and placed individually in hyperosmotic (355 mOsmolal) medium. The rostral pars distalis (RPD), comprised of nearly 100% PRL cells, was dissected and placed in groups of 3 in a 24-well culture plate with 500 µl of the medium (355 mOsmolal). From the same pituitaries, the proximal pars distalis (PPD), which contains GH-releasing cells, was separated from the pars intermedia (PI) by dissection, and treated in the same way as RPDs (Nishioka et al., 1985; Helms et al., 1987). Medium osmolality was adjusted by varying the concentration of NaCl. Tissues were pre-incubated overnight (18-20 h) at 26-28 °C on a gyratory platform (80 rpm) under a humidified atmosphere composed of 95% O₂ and 5% CO₂. After pre-incubation, PRL cells were dispersed with 0.125% trypsin (Sigma, St Louis, MO), dissolved in phosphate buffered saline (PBS, 355 mOsmolal), washed twice with PBS to remove trypsin, and plated on a poly-L-lysine (0.1mg/ml; Sigma) coated chamber that allows for the measurements of cell size and collection of perifusate for
quantification of PRL release (Chapter IV). Cells from the PPD were treated in a similar way, and the perifusate was specifically assayed for tilapia GH. For the experiment comparing PRL and GH responses to 300 mOsmolal medium, RPDs and PPDs were dispersed and cells were incubated in the same chamber. Media of different osmolalities were gravity-fed through the perifusion system, and changed through a manifold valve set upstream from the chamber. Samples of the perifusate were collected every 5 min throughout the experiment.

**Gradual alterations in medium osmolality**

Dispersed PRL and PPD cells were prepared as described above. Medium osmolality was reduced by gradually replacing hyperosmotic medium (355 mOsmolal) with dilute medium (133 mOsmolal). The volume in the syringe (10 ml) was kept constant by adding medium at a rate of 100 µl/min, approximately the same rate as perifusate was collected. This was done to ensure a constant flow rate. After 30 min in hyperosmotic medium, dilute medium (133 mOsmolal) was added for 80 min. Reduction in perifusate osmolality occurred at a rate of about 2 mOsmolal/min. Gradual increases in medium osmolality were attained by replacing hyposmotic medium (300 mOsmolal) with concentrated medium (522 mOsmolal). After 30 min of preincubation in hyperosmotic medium, followed by 30 min in hyposmotic medium (300 mOsmolal), concentrated medium was added at a rate of 100 µL/min, inducing a gradual rise in medium osmolality at a rate of about 2 mOsmolal/min. Samples were collected every 5 min and kept at -20°C. Osmolality of the perifusate was measured by vapor pressure osmometer (Wescor, Logan, UT) and used to correlate with PRL and GH release.
measured by radioimmunoassays. The data from the experiment in which medium osmolality was gradually decreased were combined with the data from the experiment in which medium osmolality was gradually increased. Cell volume measurements were taken every 5 min by recording the cells with a video camera mounted on the microscope and connected to a computer, as described elsewhere (Chapter IV).

**Radioimmunoassays**

The tilapia pituitary secretes two distinct PRL molecules that are encoded by separate genes (Specker et al., 1985; Yamaguchi et al., 1988). The release of the two PRLs, designated PRL177 and PRL188, was measured by homologous radioimmunoassays (Ayson et al., 1993; Yada et al., 1994; Seale et al., 2002b, Chapter II). Since PRL177 and PRL188 release showed identical patterns in response to changes in medium osmolality, only the PRL188 response is presented for clarity. For dispersed cell cultures, values obtained as ng/ml were converted to a percent change from the baseline set at 100%, which was determined from the average of the first 5 time points.

**Statistical analysis**

A comparison between PRL and GH release peak responses of dispersed cells to hyposmotic medium was performed using a paired t-test. Values for PRL and GH release were obtained in ng/ml and converted to a percent change from baseline. Comparisons among treatments from whole pituitary cultures were performed using a 1-way ANOVA at each time point. Hormone release was log transformed for statistical analysis and is expressed in µg/h ± S.E.M. In order to assess the effects of gradual changes in
osmolality, values for PRL and GH release were obtained in ng/ml and converted to a percent change from baseline. Cell volumes were also converted to percent change from baseline. For PRL cell or PPD cultures, the mean hormone release, cell volume and osmolality were graphed together. Linear regression and non-linear regression analyses was used to analyze the relationship between osmolality vs. hormone release. Significance level was set at 95% (P < 0.05). Calculations were performed using Minitab Statistical Software Package (State College, PA) and Sigma Plot (Chicago, IL).

RESULTS

*Effects of extracellular osmolality on PRL and GH release from dispersed cells*

To examine the effects of reduced extracellular osmolality on PRL release, dispersed PRL cells were perfused first with hyperosmotic medium (355 mOsmolal) and then exposed to hyposmotic medium (300 mOsmolal) for 20 min. Prolactin and GH release were measured from dispersed PPDs cultured together with PRL cells. After hyposmotic stimulation, PRL release was quickly elevated to 13-fold above the baseline, after 5 min, and then declined to initial levels after 15–20 min. Growth hormone, on the other hand rose 3-fold above baseline after 5 min, and then declined to initial levels after 15-20 min (Fig. 6). The peak release of PRL release after hyposmotic stimulation was significantly higher than that of GH (P < 0.01).
Fig. 6. Effects of 15% reduction in medium osmolality (from 355 to 300 mOsmolal) on PRL and GH release measured from dispersed pituitary cells. Prolactin and GH release were measured from dispersed PRL cells incubated together with dispersed PPD cells. Hormone release was expressed as percent change from the baseline. Horizontal bars represent the time in which the solution reached the cells. After exposure to 300 mOsmolal medium, PRL release (filled circles) increases 13-fold above baseline whereas GH release (open circles) increases 3-3.5-fold above baseline. Stars indicate significance of the peak PRL release when compared to peak GH release. Each point represents the mean change in PRL or GH release (n=6; **P < 0.01)
Fig. 7. Effects of 15% reduction or 15% increase in medium osmolality (from 355 to 300 or 400 mOsmolal) on PRL and GH release measured from whole pituitary glands. Hormone release was measured hourly for 4 hours and was expressed in ng/h. Arrows indicate when shifts in osmolality took place. Prolactin release increased in response to hyposmotic medium only, whereas GH release increased in response to both hyposmotic and hyperosmotic medium. Points represent mean ± S.E.M (n=10; *P < 0.05, **P < 0.01 and ***P < 0.001).
A PRL

B GH

Time (h)
Effects of extracellular osmolality on PRL and GH release from whole pituitaries

In the next experiment, effects of changes in extracellular osmolality on PRL and GH release were examined in the whole pituitary (Fig. 7). Pituitaries were incubated in 300, 355 and 400 mOsmolal media. After overnight pre-incubation in control (355 mOsmolal) medium, pituitaries were rinsed and incubated for 1 h under the same condition (355 mOsmolal). Then one group was retained in control medium while the other groups were exposed to either 300 or 400 mOsmolal media. Exposure to 300 mOsmolal significantly increased PRL release during the following 3 h (Fig. 7A). No change in PRL release was seen when pituitaries were exposed to 400 mOsmolal medium. Growth hormone release, on the other hand, significantly increased after exposure to 400 mOsmolal medium from the first hour of exposure (Fig. 7B). Exposure to hyposmotic medium significantly increased GH release from the second hour of exposure, but to a lower degree.

Effects of a gradual reduction or increase in osmolality on cell volume, PRL and GH release

This experiment was designed to test the response of PRL cells and PPD cells to gradual changes in osmolality. In the first part, osmolality of the pre-incubation medium (355 mOsmolal) was gradually reduced by adding dilute medium (133 mOsmolal) at a rate of about 2 mOsmolal/min. In the second part, cells were pre-incubated in hyperosmotic medium for 30 min followed by hyposmotic medium (300 mOsmolal) for 30 min. Medium osmolality was then gradually increased by adding concentrated medium (522 mOsmolal) at a rate of about 2 mOsmolal/min. The data for both
Fig. 8. Effects of a gradual decrease or increase in medium osmolality on cell volume and PRL or GH release from dispersed cells. Prolactin and PRL cell volume was measured from dispersed PRL cells, whereas GH and PPD cell volume was measured from dispersed PPD cells (which include GH cells). Medium osmolalities, PRL release and cell volumes were measured every 5 min, for 80 min, from the same group of dispersed cells and were plotted 3-dimensionally. Prolactin release increased in direct proportion to cell volume and in inverse proportion to medium osmolality (A). Growth hormone increased after both gradual increases and decreases in osmolality (B). Each point represents the mean osmolality, cell volume (n = 15-20) and hormone release of 4 replicate runs from 2 combined experiments.
increasing and decreasing medium osmolalities were combined for both PRL cells and PPD cells. As osmolality decreased gradually, cell volume and PRL release increased, at a similar rate showing a linear 3-dimentional relationship (Fig. 8A). A significant linear regression ($r^2=0.82$, $P < 0.001$) was obtained between the entire range of medium osmolalities and PRL release. The relationship between medium osmolality, PPD cell volume and GH release was not linear (Fig. 8B). As with PRL cells volume, PPD cell volume gradually increased as osmolality decreased. However, GH release increased as osmolality was either reduced or increased from 355 mOsmolal.

**DISCUSSION**

In the present study, the effects of osmolality on *in vitro* release of PRL and GH were compared using whole pituitary and dispersed pituitary cells of the tilapia, *Oreochromis mossambicus*. The release of both PRL and GH increased in response to hyposmotic medium, although the rise in PRL was more pronounced. Unlike PRL release, GH release was also increased in response to increases in extracellular osmolality. It is well established that PRL release increases after a decrease in extracellular osmolality in the tilapia (Nagahama *et al.*, 1975; Wigham *et al.*, 1977; Grau *et al.*, 1981; Grau *et al.*, 1994; Seale *et al.*, 2002b, Chapter II). This response has been shown to be closely tied to cell swelling (Chapter IV), and is consistent with the role of PRL in promoting freshwater adaptation (for review see Manzon, 2002). In the present experiments, this response was confirmed in both dispersed cells and in intact pituitaries.
Prolactin cells are easily isolated as they constitute nearly 100% of the rostral pars distalis (RPD) (Nishioka et al., 1988; Nishioka et al., 1993). Growth hormone cells, on the other hand, are interspersed among thyrotropin and gonadotropin cells in the proximal pars distalis (PPD) of the pituitary (Ueda et al., 1985; Huang and Specker, 1994; Norris, 1997; Villaplana et al., 2000; Pandolfi et al., 2001). In this study, PPD cells were dispersed and incubated with PRL cells to examine the effects of extracellular osmolality on PRL and GH release under identical conditions. The release of PRL and GH in response to extracellular osmolality was also examined in whole pituitaries, which may approximate in vivo conditions to a greater extent when compared with dispersed cells. Responses of PRL and GH release to changes in medium osmolality were identical between dissociated cells and whole pituitaries, although these responses occurred over a longer period in the latter case. The present observation provides direct evidence that the sensitivity to extracellular osmolality is a property of individual cells, rather than being dependent on the whole tissue.

The actions of PRL on hydromineral balance are mediated through the osmoregulatory organs such as the gills, kidney and intestine, mainly by decreasing water permeability and increasing the uptake of ions (Manzon, 2002; McCormick, 2002). Prolactin cells are remarkably sensitive to physiologically relevant reductions in extracellular osmolality, a response that is consistent with its physiological actions. Growth hormone has also been shown to be involved in fish osmoregulation, and has been implicated in seawater adaptation in tilapia (Sakamoto et al., 1997; McCormick, 2002). Consistent with this notion, the pituitary GH content, the volume of the GH-immunostained region and the activity of GH cells were found to be higher in seawater
tilapia than in freshwater fish (Borski et al., 1994). Helms et al (1987) reported that GH release from tilapia PPDs incubated for 18-20 h in 355 mOsmolal medium was significantly higher than from those incubated in 320 or 285 mOsmolal media. A reduction in osmolality did not affect GH release over this time course. This pattern was observed in fish weighing 60 g, but no difference in GH released was observed using PPD from larger fish (120 g). In the present study, large fish (200-400 g) were used, and GH responded to both reductions and elevations in medium osmolality within minutes in dispersed cells, and after 1 h in whole pituitaries. This discrepancy in time-course could be a result of the longer time necessary for the medium to fully equilibrate with all cells of whole pituitaries under static culture conditions compared with dispersed cells which were perfused. When intact RPDs were perfused under the same conditions as dispersed PRL cells, it took 30-40 min for them to respond to hyposmotic medium, whereas dispersed PRL cells responded within 5 minutes (Seale, unpublished observations). In a previous study (Seale et al., 2002b, Chapter II), GH release was observed in whole pituitaries cultured under hyperosmotic (365 mOsmolal) and hyposmotic media (290 mOsmolal) for 7 days. After the first determination (6 h), the rate of GH release was significantly higher in hyposmotic medium compared to hyperosmotic medium, but over time, the rate of GH release increased in the pituitaries incubated under hyperosmotic medium. From 2 days of incubation, cumulative GH release was higher in pituitaries exposed to hyperosmotic conditions when compared to those in hyposmotic medium. The present results, therefore, confirm the short-term (hours) effect of hyposmotic medium on GH release, and for the first time, describes the short-term stimulation of GH in response to an elevation in medium osmolality.
The rapid increase in the release of PRL after exposure to reduced osmolality seems to occur as a consequence of the cell swelling-induced rise in intracellular Ca\(^{2+}\) that occurs within minutes of hyposmotic stimulation (Chapter IV). In the present experiment, PRL release from dispersed PRL cells increased up to 13-fold above baseline within 5 min after hyposmotic stimulation. This rapid and marked response is consistent with the osmosensitive and osmoreceptive properties previously described for tilapia PRL cells (Grau and Helms, 1989; Weber et al., 2002), Chapters IV and V). However to a lesser extent, GH cells also responded to a physiologically relevant decrease in extracellular osmolality. This response is unlikely to have physiological significance, since there was no change in circulating GH levels after fish were transferred from seawater to freshwater within 6 h, when a significant increase in PRL levels was observed (Seale et al., 2002b, Chapter II). On the other hand, exposure to a 15% increase in osmolality (400 mOsmolal) elicited greater GH release from whole pituitaries than the 15% reduction in osmolality. This response is consistent with GH’s role in seawater adaptation and is in agreement with previous findings relative to the responses of GH to elevations in medium osmolality (Helms et al., 1987; Seale et al., 2002b, Chapter II). This GH response pattern to both hyperosmotic and hyposmotic medium, is also observed when medium osmolality is gradually increased or gradually reduced. Prolactin release, on the other hand, is inversely proportional to extracellular osmolality and cell volume over the entire range of osmolalities tested (250-450 mOsmolal).

Recent evidence suggests that the mediation of cell-swelling-induced PRL release in tilapia occurs via stretch-activated Ca\(^{2+}\)-permeable channels (Chapter V). Stretch-gated channels that are sensitive to small alterations in extracellular osmolality have been
previously described in several cell types (Morris, 1998). Osmoreception in rat vasopressin cell, for example, is believed to be mediated via stretch-inactivated ion channels that increase the firing rate of magnocellular neurons in response to increases in medium osmolality (Bourque, 1998). It is possible that the response of GH cells to hyperosmotic medium is mediated by stretch-inactivated ion channels. The small but significant response of GH to hyposmotic conditions, observed in vitro, may be a consequence of a similar transduction pathway in response to reductions in osmolality as observed with PRL release. Thus, in addition to stretch-inactivated ion channels, the stimulation of GH release by hyposmotic medium may indicate the occurrence of stretch-activated channels, possibly in lower numbers than in PRL cells. The concurrence of stretch activated and stretch-inactivated ion channels has been previously described in molluscan neurons (Morris and Sigurdson, 1989).

The present findings indicate that both PRL and GH cells are osmosensitive. While PRL release increased in response to decreases in osmolality in close association with cell swelling, GH release was stimulated in response to both decreases and increases in osmolality, and its release may be regulated by both cell swelling and shrinkage. Therefore, while the hyperosmotically-induced GH release is consistent with the role GH plays in seawater adaptation, it is unlikely that the short-term GH stimulation in response to hyposmotic media is physiologically relevant. On the other hand, PRL release in response to hyposmotic stimulation is consistent with its role in freshwater adaptation. The mechanisms mediating the osmosensitive properties of GH cells remain to be investigated.
CHAPTER IV

Cell volume increase and extracellular calcium are needed for increased prolactin release from the tilapia pituitary in response to hyposmotic medium

INTRODUCTION

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 (Hirano, 1986; Brown and Brown, 1987; Bern and Madsen, 1992; McCormick, 2002). Consistent with its osmoregulatory activity, PRL release from the tilapia pituitary increases as extracellular osmolality is decreased, both *in vivo* and *in vitro* (Nagahama et al., 1975; Grau et al., 1981; Yada et al., 1994; Shepherd et al., 1999; Seale et al., 2002b, Chapter II). The release of PRL increases in direct relation to the reduction in osmotic pressure (Grau et al., 1981; Grau et al., 1987; Nishioka et al., 1988). Thus, PRL release is governed by extracellular osmolality, the factor that PRL regulates at the organismic level.

These osmoreceptive properties of the tilapia PRL cells facilitate the investigation of the mechanisms by which osmotic signals are transduced into osmoregulatory responses. Furthermore, of particular advantage, PRL cells are arranged into a nearly homogeneous tissue, comprising nearly 100% of the rostral pars distalis (RPD), allowing
PRL cells to be easily isolated for *in vitro* studies (Nishioka *et al.*, 1993; Grau *et al.*, 1994).

Under hyposmotic conditions, most cells swell and then undergo what has come to be called a regulatory volume decrease (RVD) (Lang *et al.*, 1998). In perifusion 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 (Grau *et al.*, 1987). Furthermore, this elevation in PRL release from whole pituitaries incubated in hyposmotic medium is maintained for up to 12 h when compared with those maintained in hyperosmotic medium (Seale *et al.*, 2002b, Chapter II). 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 (Borski, 1992). 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.

It has been suggested in several studies using mammalian renal cells and cell lines, 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\(^{2+}\) (Wong and Chase, 1986; McCarty and O'Neil, 1991; Urbach *et al.*, 1999). Increases in PRL release are dependent on extracellular [Ca\(^{2+}\)] and can be induced experimentally by increasing [Ca\(^{2+}\)] using the Ca\(^{2+}\) ionophore, A23187 (Grau *et al.*, 1981; Grau *et al.*, 1982; Grau *et al.*, 1986). Since hyposmotic medium alone is sufficient stimulus for a rise in [Ca\(^{2+}\)]\(_i\) (Grau *et al.*, 1994), 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 perifusion system utilizing dispersed PRL cells permitted switches in media of different osmotic concentrations and ionic compositions with minimal mixing. By video imaging the cells during perifusion, the time-course of hyposmotically-induced increase in cell volume could be observed together with the increase in PRL release into the perifusate. A similar chamber, containing PRL cells exposed to the same conditions, allowed the determination of the changes in [Ca$^{2+}$]$_i$ from fura-2-AM loaded cells. Thus, I was able to show for the first time that a cell volume increase, occurring rapidly in response to hyposmotic media, 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$^{2+}$]$_i$, cell volume and PRL release after hyposmotic stimulation, was 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 5000-liter tanks in fresh water, and fed twice daily with Purina Trout Chow (approximately 2% of body weight, 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 perifusion

Pituitaries were removed after decapitation. The rostral pars distalis (RPD), comprised 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 mOsmolal) 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. (1977). Medium osmolality was adjusted by varying the concentration of NaCl. Tissues were pre-incubated overnight (18-20 h) at 26-28 °C on a gyratory platform (80 rpm) under a humidified atmosphere composed of 95% O₂ and 5% CO₂. After pre-incubation, PRL cells were dispersed in 0.125% trypsin (Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS, 355 mOsmolal), washed twice with PBS to remove trypsin from the cells (Borski, 1992), and plated on a poly-L-lysine (Sigma; 0.1 mg/ml) coated chamber. The chamber consisted of two rectangular cover slips (22 x 40 mm) held together with 100% silicone at the extremities, with cut hypodermic needles (23G) forming the inlet and outlet. The chamber volume ranged from 250 to 300 μl, and accommodated an average of 537,000 ± 53,000 PRL cells (n=12). The inlet of the chamber was connected to syringes (60 ml) containing media. The chamber was mounted on the stage of a microscope equipped with a video camera. Media of various compositions were gravity-fed through the syringes, and switched through a manifold-valve set upstream of the chamber (Hyde et al., 2002). The flow rate ranged from 60 to 80 μl/min. The time lapse
between reaching the chamber and completely replacing the previous chamber solution ranged between 1.5 and 2.5 min. The dead time between changing the valve and the outflow of perifusate was 4-5 min. Thus the time difference between cell volume and PRL release measurements was defined by the temporal difference between the average time between reaching and clearing the chamber and the time taken to reach the point where the perifusate was collected. The mean time difference was 2 min and 9 ± 6 sec (n=4), and was incorporated in the figures to correctly express the time in which PRL cell volume and PRL release responded to a change in medium. The perifusate was collected manually every 5 min in 0.5 ml centrifuge tubes that had been previously weighed. Tubes containing perifusate were then re-weighed and the volume in each sample determined. These volumes were used to determine the flow rate and to calculate the amount of PRL in each sample after radioimmunoassay. Perfusion experiments for cell volume and PRL release determinations were replicated at least 4 times.

Cell size

Cell images were captured every 5 min with a video camera and stored in a computer (Macintosh IIcx). The microscope was equipped with a 100 x oil-immersion objective lens (Nikon, Japan) and the total magnification of the image, as seen on the screen, was 1200 x. The cross-sectional areas of cells were estimated by tracing each cell from digitally captured images. Images were processed with the NSF Scion Image software. Areas (A) were obtained in pixels and then transformed into μm² as determined by viewing a stage micrometer. Cell volume (V) was estimated from the area by: \( r = \sqrt{A/\pi} \), and \( V = \frac{4}{3} \pi (r^3) \)
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 5 time points in pretreatment medium.

**Intracellular Ca^{2+} concentration**

The procedure for fura-2 loading in dispersed PRL cells has been described previously (Hyde et al., 2002). Briefly, PRL cells are dispersed as described above, but placed on round coverslips (22 mm diameter) previously coated with poly-L-lysine (0.1 mg/ml) and were pre-incubated overnight in 355 mOsmolal 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 cover slips with cells were rinsed and placed in baseline medium (355 mOsmolal) for 30 min prior to data recording. Individual cover slips were mounted in a metal chamber that allows the cells to be perifused with media of different compositions (Borski, 1992). The chamber was mounted on the stage of an inverted microscope (Nikon).

Measurements of the fura-2 ratio were made in single cells from images captured digitally using an intensified CCD camera using a 40x or 100x oil-immersion objective lens mounted on the inverted microscope. Calcium signals, with an emission fluorescence of 500 nm, were analyzed using the proprietary software, Image-1/FL (Universal Imaging Corporation, West Chester, PA). Images were acquired at 340 and 380 nm excitation wavelengths and then background-subtracted from a field devoid of cells. Measurements were taken every 15 sec for up to 2 h. All data were expressed as the relative intensity of 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) (Gryniewicz et al., 1985). Data expressed as a ratio (340/380) resulted directly from changes in intracellular Ca\(^{2+}\) concentration. An approximate estimate of [Ca\(^{2+}\)\(_i\)] was obtained according to the formula provided by Gryniewicz et al. (1985) and the Kd value of 465.9, which has been used for goldfish gonadotropes and somatotropes incubated at 28 °C (Johnson et al., 1999).

The calibration procedure described by Kao (1994) 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 \(\mu\)M digitonin, respectively, to fura-2 AM-loaded PRL cells. The calibration equation was then plotted with the Image-1/FL software, and [Ca\(^{2+}\)\(_i\)] estimates 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 \(3-10 \times 10^{-8}\)M and when depolarized with high K\(^+\) show a ratio of 2-3, corresponding to a [Ca\(^{2+}\)\(_i\)] of \(1-2.5 \times 10^{-6}\)M. Perfusion experiments for [Ca\(^{2+}\)\(_i\)] determinations were replicated at least 3 times.

**Radioimmunoassays**

The tilapia pituitary secretes two distinct PRL molecules, PRL\(_{177}\) and PRL\(_{188}\), that are encoded by separate genes. The quantity of both PRLs in the perifusate was measured by homologous radioimmunoassays (Ayson et al., 1993; Yada et al., 1994; Seale et al., 2002b, Chapter II). In the present experiment PRL\(_{177}\) and PRL\(_{188}\) release showed highly similar patterns in response to a reduction in osmolality (Fig. 9). Thus, for clarity, only the PRL\(_{188}\) response is presented. Values obtained as ng/ml were converted
Fig. 9. Effects of sequential 15% reductions in medium osmolality (from 355 to 300 mOsmolal) on the release of PRL_{177} and PRL_{188}. Prolactin release is expressed as percent change from the baseline. Release of both PRLs from the same preparations of dispersed PRL cells increased in a similar way after medium osmolality was reduced. Both PRLs also responded identically to all other treatments in this study, and for clarity only PRL_{188} is shown in subsequent figures. Open and closed circles represent mean percent change for PRL_{177} and PRL_{188}, respectively (n= 4) ± S.E.M.
to a percent change from the baseline set at 100%, which was determined from the average of the first 5 time points under pretreatment conditions (355 mOsmolal).

**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, 5 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 hyposmotic 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 reduction in medium osmolality on cell volume and PRL release*

Effects of hyposmotic medium on PRL release and cell volume were examined over the time-course of 80 min (Fig. 10A). The increase in cell volume was osmometric
with respect to the reduction in medium osmolality. That is, as the osmolality was reduced by 15%, the peak volume increase amounted to approximately 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 further below). The second PRL determination (but not the first) following change to hyposmotic medium consistently showed a several-fold increase in PRL, on average 700% for a 15% decrease in medium osmolality. A peak in PRL release occurred at the 3rd determination (15 min) reaching as much as 11-fold above baseline release. The rate of PRL release then declined to reach a plateau of 2-3-fold 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 returning to hyperosmotic (355 mOsmolal) medium, cell volume declined to original size within a single measurement, while PRL release declined but more slowly.

While a 15% reduction in osmolality falls well within the physiological range tolerated by this species, a 30% reduction is extreme (Fig. 10B). Cell volume increased rapidly after medium osmolality was reduced from 355 to 248 mOsmolal, increasing to 30% above the baseline at the 1st 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 mOsmolal medium reached an average of 600% at the 2nd determination (10 min) and a peak of 10-fold at the 3rd determination (15 min). The time-course of the peak response and the magnitude of PRL
Fig. 10. Effects of 15% (A) and 30% (B) reduction in medium osmolality (from 355 to 300 mOsmolal and from 355 to 248 mOsmolal, respectively) on cell volume change and PRL release measured from the same preparation of dispersed PRL cells. Prolactin release and cell volume were expressed as percent change from the baseline. Horizontal bars represent the 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 mOsmolal medium, cell volume (open squares) increased 15% above baseline, closely followed by a 10-11-fold increase in PRL release (filled circles). B) Exposure to 248 mOsmolal medium increases cell volume (open diamonds) 30% above baseline, also followed by a 10-fold increase in PRL release (closed triangles). Each point represents the mean change in PRL release or cell volume ± S.E.M. (4 replicate runs for PRL release and 16 cells for volume measurements). C) After the peak induced by a 30% reduction in osmolality (248 mOsmolal) the slope of cell volume decline is significantly (P < 0.001) steeper than that of a 15% reduction (300 mOsmolal). The slopes were compared by multiple regression analysis and are shown as solid traces.
release are similar to that observed after a 15% decrease in medium osmolality. As with the response to a 15% reduction in medium osmolality, the PRL release declined to an elevated plateau of approximately 2-fold over baseline, that was sustained throughout the remaining exposure to 248 mOsmolal medium. Both cell volume and PRL release returned to baseline levels after medium osmolality was returned to 355 mOsmolal.

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. 10C). 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 negative slope ($P < 0.01$) for cell volume after a 30% drop in osmolality, but not after a 15% drop, is seen as early as 35 min after exposure to hyposmotic medium.

Intracellular $Ca^{2+}$ oscillations

The baseline $[Ca^{2+}]_i$ of PRL cells perifused with 355 mOsmolal medium was expressed as 340/380 ratios, and classified into 3 main patterns (Fig. 11). Quiescent or silent cells, exhibiting low spontaneous variation from baseline (Fig. 11A), accounted for
Fig. 11. Patterns of basal $[Ca^{2+}]_i$ in tilapia PRL cells. Traces represent the ratio 340/380 of single cells under control (355 mOsmolal) medium and directly reflect $[Ca^{2+}]_i$. Measurements under control medium were taken every 15 sec 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 patterns of $[Ca^{2+}]_i$ in a spontaneously active high-frequency oscillator (observed in 60 of 186 cells) and (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.
60% of the cells studied (111 of 186 cells). The remainder showed spontaneous activity that could be further classified into either of two patterns: spontaneous high-frequency oscillators (B) and low frequency-oscillators (C). The cells exhibiting the last two patterns accounted for 32 and 8% of the total cells studied, respectively. 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 mOsmolal, $[\text{Ca}^{2+}]_i$ quickly increased (within 1-2 min) as indicted by the increase in 340/380 ratios (Fig. 12). Both oscillating and silent cells responded to the hyposmotic stimulus, and the traces presented in Figs. 12A 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 (Figs. 12C 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 5-10 min and subsided either to an elevated plateau (45% of cells) or baseline levels (39% of cells). The remaining 16% of cells did not show an increase in 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 (Richman *et al*., 1990; Hyde *et al*., 2002), and this
Fig. 12. Effects of hyposmotic medium (300 mOsmolal) on \([\text{Ca}^{2+}]_i\) in single PRL cells. Arrows indicate the time 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) The mean ratio of cells from a single representative experiment, in which medium osmolality was changed from 355 mOsmolal to 300 mOsmolal, switched back to 355 mOsmolal and re-exposed to 300 mOsmolal, followed by high K⁺ hyperosmotic medium (355 mOsmolal). Each point represents mean ± S.E.M. (n=10) 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.
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. Depolarization of the membrane was induced by exposing the cells to medium containing high \([\text{K}^+]\) (56 mM KCl).

The mean of ratios from each observed time point was taken from cells (\(n=10\)) recorded during the same experimental run (Fig. 12 E). 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 switching 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 baseline, high \(\text{K}^+\) medium (355 mOsmolal) was introduced and elicited a sharp and strong increase in the mean ratio.

Effects of \(\text{Ca}^{2+}\) deletion on PRL release and cell volume

To examine the involvement of extracellular \(\text{Ca}^{2+}\) in hyposmotically-induced PRL release, PRL cells were exposed to hyposmotic medium devoid of \(\text{CaCl}_2\) ("\(\text{Ca}^{2+}\)-deleted"). Cells were initially perifused with normal hyperosmotic medium (355 mOsmolal, with 2 mM \(\text{CaCl}_2\)) and after switching to normal hyposmotic medium, PRL release increased over 3-fold above the baseline. A significant \((P < 0.05)\) decrease in net PRL release over a period of 25 min was observed in hyposmotic \(\text{Ca}^{2+}\)-deleted medium when compared with the normal hyposmotic control (Fig. 13A). Results similar to the ones described above were observed to \(\text{Ca}^{2+}\)-depleted hyperosmotic medium containing 2 mM EGTA. For clarity, only the responses to \(\text{Ca}^{2+}\)-deleted media are shown.
Fig. 13. Effects of Ca$^{2+}$-deleted hyposmotic medium (300 mOsmolal) on PRL release and cell volume measured from the same preparation of dispersed PRL cells. Horizontal bars indicate the time in which the medium reached the cells. Ca$^{2+}$-deleted hyposmotic medium was prepared by omitting 2 mM CaCl$_2$ present in normal hyposmotic medium.

A) The release of PRL increased in response to hyposmotic medium (300 mOsmolal, filled circles), but was significantly reduced in Ca$^{2+}$-deleted hyposmotic medium (open circles). Vertical bars indicate mean net PRL release change over 25 min ± S.E.M (n= 5 and 7 for control and Ca$^{2+}$-deleted, respectively).

*Significantly different from control (300 mOsmolal) at $P < 0.05$.

B) The change in PRL cell volume in response to hyposmotic medium (closed triangles) was not significantly different from that of Ca$^{2+}$-deleted hyposmotic medium (open triangles). Vertical bars indicate mean net cell volume change over 30 min ± S.E.M (n= 10 and 15 for control and Ca$^{2+}$-deleted hyposmotic medium, respectively.)
Prolactin cell volumes increased osmometrically in response to hyposmotic medium regardless of the presence or absence of Ca\(^{2+}\) (Fig. 13B). In both normal hyposmotic or Ca\(^{2+}\)-deleted hyposmotic media, RVD was not observed during 30 min following a 15% drop in osmolality, based on simple regression analysis. As mentioned previously, RVD becomes measurable 35 min after medium osmolality is reduced by 15%. In all treatments, cell volume increased approximately 10-15% above the baseline established in hyperosmotic medium, thus clearly indicating that deletion of Ca\(^{2+}\) from the incubation medium reduced hyposmotically-induced PRL release but not cell swelling.

**Effects of Ca\(^{2+}\) deletion on [Ca\(^{2+}\)]\(_i\)**

The changes in [Ca\(^{2+}\)]\(_i\) in response to Ca\(^{2+}\)-deleted hyposmotic medium clearly indicate a role for extracellular Ca\(^{2+}\) in triggering the hyposmotically-induced PRL release. Exposure to Ca\(^{2+}\)-deleted hyposmotic medium (300 mOsmolal) immediately after normal hyperosmotic medium (355 mOsmolal, with 2 mM CaCl\(_2\)) produced no change in 340/380 ratio in 9 out of 11 cells. This pattern, followed by an increase in [Ca\(^{2+}\)]\(_i\) in normal (i.e. Ca\(^{2+}\)-containing) hyposmotic medium, is exemplified by the single cell recording in Fig. 14A. Two of 11 cells responded to Ca\(^{2+}\)-deleted hyposmotic medium with a transient increase in [Ca\(^{2+}\)]\(_i\). A switch from normal hyperosmotic medium to Ca\(^{2+}\)-deleted hyperosmotic medium also produced either a transient increase (23% of cells) or no change in [Ca\(^{2+}\)]\(_i\) (77% of 52 cells). The subsequent exposure of these same cells to Ca\(^{2+}\)-deleted hyposmotic medium did not produce a transient increase in [Ca\(^{2+}\)]\(_i\) in 98%
Fig. 14. Effects of Ca$^{2+}$-deleted hyperosmotic and hyposmotic medium (355 and 300 mOsmolal respectively) on [Ca$^{2+}$]$_i$ in single PRL cells. Arrows indicate the time in which the medium reached the cells and the treatment being perifused. A) [Ca$^{2+}$]$_i$ trace of a single PRL cell that did not respond to Ca$^{2+}$-deleted hyposmotic medium and recovered by increasing the 340/380 ratio after exposure to normal hyposmotic medium. This pattern is representative of 9 out of 11 cells. B) [Ca$^{2+}$]$_i$ trace of a single PRL cell that transiently and strongly increased [Ca$^{2+}$]$_i$ after switching from normal hyperosmotic medium to Ca$^{2+}$-deleted hyperosmotic medium, but did not change [Ca$^{2+}$]$_i$ following exposure to Ca$^{2+}$-deleted hyposmotic medium. This response was seen in 12 out of 52 cells. After recovery, responsiveness to normal hyposmotic medium or high K$^+$ was observed in 75% and 87% of cells, respectively, although to different magnitudes.
of the cases. An example of a cell responding to Ca\(^{2+}\)-deleted hyperosmotic medium, followed by no response to Ca\(^{2+}\)-deleted hyposmotic medium is shown in Fig. 14B. This cell also responded strongly to high K\(^+\) in normal hyperosmotic media, at the end of the experiment. Of all cells subjected to Ca\(^{2+}\)-deleted medium, 75% exhibited a recovery in their ability to respond to normal hyposmotic medium by increasing [Ca\(^{2+}\)]\(_i\) and 87% responded to high K\(^+\) in normal hyperosmotic media at the end of the experiments by increasing [Ca\(^{2+}\)]\(_i\).

Effects of Ca\(^{2+}\) depletion on [Ca\(^{2+}\)]\(_i\)

Of the 39 cells exposed to Ca\(^{2+}\)-depleted hyperosmotic medium (devoid of CaCl\(_2\) and containing 2 mM EGTA) after pretreatment in normal hyperosmotic medium, 48% responded with a transient increase in [Ca\(^{2+}\)]\(_i\), but none of them showed an increase in [Ca\(^{2+}\)]\(_i\) after subsequent exposure to Ca\(^{2+}\)-depleted hyposmotic medium. Only 53% of these cells recovered by responding to normal hyposmotic medium after removal of EGTA, but 18 out of 19 cells that were exposed to high K\(^+\), under normal hyperosmotic medium, increased [Ca\(^{2+}\)]\(_i\).

DISCUSSION

The present findings clearly indicate the importance of extracellular Ca\(^{2+}\) in hyposmotically-induced PRL release. When Ca\(^{2+}\) is deleted from the incubation medium, a reduction in osmolality and the subsequent increase in cell volume do not lead to a significant increase in PRL release. In the presence of extracellular Ca\(^{2+}\), however, an
increase in cell volume appears 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 by which an increase in cell volume triggers a rise in $[\text{Ca}^{2+}]_i$ and subsequent PRL release, relies on the availability of extracellular $\text{Ca}^{2+}$. Therefore, extracellular $\text{Ca}^{2+}$ entry would appear to be essential for the full response of PRL secretion in hyposmotic environment, although the participation of intracellular $\text{Ca}^{2+}$ stores in this process remains to be examined.

This is the first report to correlate changes in cell size and PRL release in an osmoreceptive model system, in 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 involved with vasopressin and oxytocin release in mammals (Bourque, 1998; Weber et al., 2002). 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, which are involved in the osmoreceptive process.

In the present perifusion incubations, dispersed PRL cells responded within minutes to decreases in medium osmolality. Prolactin cells were pre-incubated in hyperosmotic medium (355 mOsmolal) in order to observe the effects of a physiologically relevant reduction in medium osmolality. In tilapia, blood osmolality ranges between 320 and 340 mOsmolal for both freshwater- and seawater-acclimated fish (Yada et al., 1994; Seale et al., 2002b, Chapter II). 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 fresh water 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 mOsmolal in the blood osmolality are commonly observed in tilapia that transfer successfully between freshwater and seawater (Grau et al., 1994; Seale et al., 2002b, Chapter II). Thus, the changes of hyperosmotic medium (355 mOsmolal) to hyposmotic medium (300) 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%. Cell volume did not decrease over the first 35 min of hyposmotic stimulation. Thereafter, cell volume returned gradually toward baseline over the next 80 min. 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 what is called a regulatory volume decrease (RVD) (Macknight et al., 1994; Lang et al., 1998).

In many cell types, RVD is observed within 20 min of hyposmotic stimulation (Sato et al., 1990; McCarty and O'Neil, 1991; Wang et al., 1991; Banderali and Roy, 1992; Macknight et al., 1994; Urbach et al., 1999). 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 to those typically used by others, i.e., 25-50% (Sato et al., 1990; McCarty and O'Neil, 1991; Sato et al., 1991b; Wang et al., 1991; Banderali and Roy, 1992; Macknight et al., 1994). Therefore, in addition to testing a physiologically relevant 15% change, the cells were subjected to a 30% decrease in osmolality to examine PRL cell volume changes and PRL release. This produced a 30% increase in cell volume,
which in turn led to a regulatory volume decrease that was not 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 (Lang et al., 1998). Many of these studies, however, are based on in vitro experiments which often extrapolate the conditions observed in vivo. In normal and tumor-derived GH4C1 and MMQ rat pituitary cells, for example, regulatory volume decrease was observed within 10 min after exposure to 27% hyposmotic medium (Strbak and Greer, 2000). On the other hand, pancreatic β-cells may exhibit RVD after a reduction in osmolality as low as 10% (Miley et al., 1997). In the latter case, however, the physiological significance of RVD is thought to be 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 volume decrease in tilapia PRL cells exposed to small, physiological changes (15%) in extracellular osmolality is likely to 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.
(Seale et al., 2002b, Chapter II). 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, I 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.

The effect of reduced osmolality on PRL release in the tilapia is currently believed to be mediated by a signal transduction system linked to changes in $[\text{Ca}^{2+}]_i$. Measuring the uptake and loss of $^{45}\text{Ca}^{2+}$ in the RPD, Richman et al. (1990; 1991) have shown that intracellular $\text{Ca}^{2+}$ metabolism is modified in response to extracellular osmolality and is directly linked to the stimulation of PRL release. Through the use of the $\text{Ca}^{2+}$-sensitive fluorescent dye, fura-2, it has been observed that $[\text{Ca}^{2+}]_i$ rises rapidly after a reduction in the osmolality (300 mOsmolal) of the perifusion medium. It has been previously reported that 25% of the tilapia PRL cells show spontaneous oscillatory activity, whereas the remainder are silent (Grau et al., 1994). The baseline of the oscillatory cells was elevated, while amplitude of oscillations was decreased, at the onset of hyposmotic stimulation. This is consistent with my current findings, in which the majority of cells in hyperosmotic medium were silent (60%). Regardless of the oscillation pattern, PRL cells responded to hyposmotic medium by elevating $[\text{Ca}^{2+}]_i$. This rise in $[\text{Ca}^{2+}]_i$ varied in magnitude among experiments. Although this may represent natural fluctuations 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 to induce cell depolarization and over 90%
of the cells studied responded with a sharp increase in $[\text{Ca}^{2+}]_i$. In addition to silent and spontaneously active cells, low frequency oscillation patterns are described for the first time in the tilapia PRL cell. Intracellular $\text{Ca}^{2+}$ oscillations have been described in many cell types, including mammalian PRL cells (Stojilkovic and Catt, 1992; Villalobos et al., 1998) and goldfish GH cells (Yunker and Chang, 2001). Oscillations have been implicated in modulating specific signal transduction pathways (Stojilkovic and Catt, 1992; Putney Jr. and Bird, 1993; Johnson and Chang, 2000b), although there is little evidence as to how this modulation occurs. In goldfish somatotropes, 88% of the cells were quiescent, and low-frequency oscillations were not reported (Yunker and Chang, 2001). In mammalian PRL cells, four patterns were described, including low frequency oscillations (Villalobos et al., 1998).

This study also examined whether the short-term rise in $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]$. Indeed, the osmotically-induced release of hormones from rat anterior pituitary cells seems to occur independently of extracellular $\text{Ca}^{2+}$ availability (Strbak and Greer, 2000). 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 (Strbak and Greer, 2000). The entry of extracellular $\text{Ca}^{2+}$ following cell swelling has been described in several cases (Wong and Chase, 1986; McCarty and O'Neil, 1991; Urbach et al., 1999). Increases in $[\text{Ca}^{2+}]_i$ are often attributed to activation of regulatory volume decrease. This does not appear to be the case in the tilapia PRL cell. For example, in *Xenopus* renal A6 cell lines, removal of
extracellular Ca\(^{2+}\) from the medium prevented a full regulatory volume decrease (Urbach et al., 1999). In the present study, cell volume remained elevated during hyposmotic stimulation in both Ca\(^{2+}\)-deleted as well as normal medium, suggesting that extracellular Ca\(^{2+}\) does not affect cell volume.

The deletion of Ca\(^{2+}\) from hyposmotic medium significantly reduced the hyposmotically-induced PRL release, indicating that extracellular Ca\(^{2+}\) is a crucial component for the initiation of this signal transduction. This response to hyposmotic Ca\(^{2+}\)-deleted medium has been previously described from intact RPDs perifused under similar conditions (Richman et al., 1991). The small (less than 2-fold) response in PRL release observed in Ca\(^{2+}\)-deleted hyposmotic media might suggest the participation of other second messenger systems and intracellular Ca\(^{2+}\) stores in the transduction pathway. Alternatively, this transient response may be a reflection of compensatory release of Ca\(^{2+}\) from intracellular stores, since it occurred after depleting extracellular Ca\(^{2+}\) with either EGTA or with nominally free Ca\(^{2+}\) media. In some cells, a transient peak in [Ca\(^{2+}\)]\(_i\) was observed after exposure to hyperosmotic or hyposmotic Ca\(^{2+}\)-deleted and Ca\(^{2+}\)-depleted media, followed by a decline or return to baseline ratio. Since these transient responses occurred in both hyper and hyposmotic media deprived of Ca\(^{2+}\), they may represent compensatory Ca\(^{2+}\) release from intracellular stores. This compensatory release has also been suggested in goldfish somatotropes (Johnson and Chang, 2000a), but the mechanism underlying such response remains to be clarified.

To increase our knowledge on the actual role that intracellular Ca\(^{2+}\) stores may play during hyposmotically-induced PRL release, I carried out a series of preliminary short-term (1 hour) static cultures employing different pharmacological agents that target
intracellular Ca\textsuperscript{2+} stores (Fig. 15). TMB-8 is a widely employed inhibitor of Ca\textsuperscript{2+} release from intracellular stores, and has been described to inhibit the GnRH-induced GH and GTH release in the goldfish (Johnson and Chang, 2000a; Johnson \textit{et al.}, 2000). Xestospongin-C is a selective, membrane-permeant blocker of inositol 1,4,5- triphosphate (IP\textsubscript{3}) receptors (Gafni \textit{et al.}, 1997), and has been shown to inhibit sGnRH-induced GTH release in goldfish gonadotropes (Johnson \textit{et al.}, 2000). Another method of directly evaluating the role of intracellular stores is to prevent their Ca\textsuperscript{2+} loading with inhibitors of sarco-endoplasmic reticulum Ca\textsuperscript{2+}-activated ATPases (SERCA), such as cyclopiazonic acid (CPA) (Mason \textit{et al.}, 1991; Demaurex \textit{et al.}, 1992), which has been shown to inhibit GnRH-induced GTH release in goldfish gonadotropes (Johnson \textit{et al.}, 2000). In this experiment, exposure of dispersed PRL cells to TMB-8, xestospongin-C and CPA, at the same concentrations as those employed in goldfish pituitary cells, did not prevent the hyposmotically-induced increase in PRL release after 1 hour (Fig. 15). The naturally occurring intracellular signaling molecule, lysophosphatidic acid (LPA), is known for its ability to evoke an IP\textsubscript{3}-mediated increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Jalink \textit{et al.}, 1990; Pietruck \textit{et al.}, 1997). Extracellular LPA produces cellular responses by binding to a G protein-coupled receptor (Fukushima \textit{et al.}, 1998). In the present study, LPA stimulated PRL release in hyperosmotic medium within 1 hour (Fig. 15). Furthermore, this LPA-induced stimulation of PRL release was inhibited by pre-incubation with CPA (Seale, unpublished observation). A schematic diagram showing the actions of the pharmacological agents employed in this study is shown in Fig. 16. Taken together these results indicate that while PRL release can be induced by the activation of IP\textsubscript{3}-dependent pathways, intracellular Ca\textsuperscript{2+} stores do not seem to be essential in the short-term (within 1 h).
hyposmotically-induced PRL release. Further studies are required to further characterize the involvement of intracellular Ca\(^{2+}\) stores in hyposmotically-induced PRL release as well as PRL gene expression and synthesis.

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 mOsmolal decrease in medium osmotic pressure increases membrane stretch to a degree equivalent to 18 mm of Hg, well within the range that stretch-sensitive ion channels are responsive to (Morris and Sigurdson, 1989). 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\(^{2+}\) to this process has been identified, further study of the nature and control of this transduction pathway is necessary. 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\(^{2+}\) entry and a subsequent increase in PRL release is tested in the following chapter.
Fig. 15. Effects of pharmacological agents targeting intracellular Ca\textsuperscript{2+} stores on the release of PRL from dispersed PRL cells. Cells were dispersed with trypsin, rinsed with trypsin inhibitor and phosphate buffered saline (Chapter IV). After an overnight incubation in hyperosmotic medium (355 mOsmolal), cells were exposed to control conditions for 1 hour, then exposed to the treatments during the 2nd hour. Paired t-tests were used to compare the responses before and after exposure to each treatment. Prolactin release is expressed in ng/h and the x axis shows the different treatments and medium osmolalites associated with each group. Prolactin release in hyperosmotic medium did not change between 1 and 2 hours of incubation. Exposure to hyposmotic medium (300 mOsmolal) significantly increased PRL release. Exposures to TMB-8 (100 \( \mu \)M), xestospongin-C (1 \( \mu \)M), and CPA (10 \( \mu \)M) did not inhibit stimulation of PRL release by hyposmotic medium. LPA significantly increased PRL release in hyperosmotic medium, and CPA inhibited this response. Bars represent mean ± S.E.M. (n=20 and 30 for hyperosmotic and hyposmotic controls, respectively, and n=10 for other treatments; *P < 0.05, ns=not significant).
PRL (ng/h)

- 355
- 300
- TMB-8 (100µM)
- Xestospongin-C (1µM)
- CPA (10µM)
- LPA (1µM)
- LPA (1µM) + CPA (10µM)

1 h

2 h

* Significant difference
Fig. 16. Illustration of the reported effects of LPA, TMB-8, xestospongin-C and CPA on intracellular Ca\(^{2+}\) stores. Following hyposmotically-induced cell swelling, Ca\(^{2+}\) is hypothesized to enter the cell via stretch-activated ion channels. A rise in \([\text{Ca}^{2+}]_i\) can induce Ca\(^{2+}\) release from the endoplasmic reticulum (ER). A variety of pharmacological agents that act on ER Ca\(^{2+}\) stores were employed in this experiment to indicate if such stores are involved in short-term (1 h) hyposmotically-induced PRL release. TMB-8 inhibits Ca\(^{2+}\) release from the ER, CPA inhibits uptake of Ca\(^{2+}\) by the ER by inhibiting SERCA pumps and xestospongin-C is a specific blocker of IP\(_3\) receptors. LPA binds to G protein-coupled receptors in the membrane surface and activates the IP\(_3\) signalling pathway. + and − signs represent stimulatory or inhibitory effects respectively. LPA= lysophosphatidic acid, Rec= receptor, Gp= G protein, PIP\(_2\)= phosphatidyl inositol bisphosphate, DAG= diacylglycerol, IP\(_3\)= inositol 1,4,5- triphosphate, CPA= cyclopiazonic acid, SERCA= sarco-endoplasmic reticulum Ca\(^{2+}\) activated ATPase, IP\(_3\) rec= IP\(_3\) receptor.
CHAPTER V

Evidence that the signal transduction for osmoreception is mediated by stretch-activated ion channels in the euryhaline tilapia, *Oreochromis mossambicus*

INTRODUCTION

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 (Lang *et al.*, 1998). At an organismal 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. Tilapia PRL cells are an excellent example of the integration of the 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 (PRL release) can be measured simultaneously with other parameters involved in the osmoreceptive process, such as cell size, in a cell of known identity (Weber *et al.*, 2002). Prolactin cells are easily isolated and dissociated, and are remarkably sensitive to changes in extracellular osmolality whether as dispersed cells or kept as intact tissue (Nagahama *et al.*, 1975; Bern, 1980; Grau *et al.*, 1981; Nishioka *et al.*, 1988; Borski, 1992; Grau *et al.*, 1994; Weber *et al.*, 2002; Chapter IV). Thus, small
decreases in extracellular osmolality result in a robust elevation in PRL release (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1994). This direct sensitivity to changes in extracellular osmolality is observed both in vivo and in vitro (Yada et al., 1994; Shepherd et al., 1999; Seale et al., 2002b, Chapter II). This response is physiologically relevant, since in the tilapia and other teleost fish PRL is a key osmoregulatory hormone that promotes freshwater adaptation (Hirano, 1986; Manzon, 2002; McCormick, 2002). Thus, the sensitivity of the PRL cell to changes in medium osmolality coupled to its ability to maintain osmotic balance in the whole organism characterizes this cell type, by definition, as an osmoreceptor (Weber et al., 2002). In the previous Chapter I have shown the close relation between hyposmotically induced cell swelling, rise in intracellular Ca\textsuperscript{2+} and PRL release. Thus, it has been suggested that hyposmotically-induced cell swelling activates extracellular Ca\textsuperscript{2+} entry through stretch-activated ion channels (Grau and Helms, 1989; Richman et al., 1991; Grau et al., 1994, Chapter IV).

Stretch-activated Ca\textsuperscript{2+} and K\textsuperscript{+} channels have been identified in several cell types (Lang et al., 1998). 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 (Guharay and Sachs, 1984; Lansman et al., 1987; Sackin, 1987; Morris and Sigurdson, 1989; Morris, 1998). Although mechanosensitive channels have been described in a wide range of cell types (for review see Morris, 1998), only in vasopressinergic and oxytocinergic neurons in the rat hypothalamus have stretch-inactivated ion channels been described which respond directly to physiologically relevant increases in osmolality (Bourque and Oliet, 1997).
The objectives of the present study were to determine whether stretch-activated Ca\textsuperscript{2+}-permeant channels are involved in hyposmotically-induced PRL release from dispersed tilapia PRL cells. The lanthanide ion, gadolinium (Gd\textsuperscript{3+}), is widely utilized to block stretch-activated cation channels in general, including Ca\textsuperscript{2+} channels (Hamill and McBride, 1996), and its effects on hyposmotically-induced PRL cell swelling, rise in [Ca\textsuperscript{2+}], and PRL release were examined. Furthermore, hyposmotic stimulation during exposure to nifedipine and high [K\textsuperscript{+}] 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\textsuperscript{2+}], were determined from fura-2-AM loaded cells. This is the first study to indicate that stretch-activated ion channels play a crucial role in the signal transduction of a hyposmotic stimulus into a physiologically relevant osmoregulatory response.

MATERIALS AND METHODS

Fish

Tilapia (Oreochromis mossambicus) were obtained from a population maintained at the Hawaii Institute of Marine Biology. They were kept in 5000-liter tanks in fresh water under natural photoperiod. They were fed twice daily with Purina Trout Chow (approximately 2% of body weight per day). Water temperature was 22–26\degree C. All experiments were conducted in accordance with the principles and procedures approved...
Cell dispersions and perifusion

Pituitaries were removed from sexually mature tilapia (200-600 g) after decapitation. Each pituitary was placed individually in hyperosmotic (355 mOsmolal) 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. (1977). The rostral pars distalis (RPD), comprised of nearly 100% PRL cells, was dissected and placed in groups of 3 in a 24-well culture plate with 500 µl of hyperosmotic medium (355 mOsmolal). Medium osmolality was adjusted by varying the concentration of NaCl. Tissues were pre-incubated overnight (18-20 h) at 26-28 °C on a gyratory platform (80 rpm) under a humidified atmosphere composed of 95% O₂ and 5% CO₂. Cells were dispersed and plated as previously described and perifused in a chamber that allows for the measurement of cell size and collection of perifusate for quantification of PRL release (Chapter IV). Media of different compositions were gravity-fed through the chamber, and changed using a manifold valve set upstream from the chamber. Samples of the perifusate were collected every 5 min throughout the time course of the experiment.

There are technical considerations for using Gd³⁺ to identify stretch-activated channels. Gadolinium avidly binds to phosphate and bicarbonate, present in most incubation solutions, reducing the effective concentration of these ions and possibly leading to false negative conclusions (Caldwell et al., 1998). To employ Gd³⁺ in my studies effectively, I validated the utility of my incubation system for studying the
hyposmotically-induced PRL release culture system in bicarbonate- and phosphate-free medium, containing HEPES (25 mM) to buffer the medium.

Reagents

Stock solutions of nifedipine (Sigma) were dissolved in dimethyl sulfoxide (DMSO). An equivalent amount of DMSO (1:1000) was added to the control medium. Gadolinium chloride (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. This alteration in medium composition did not affect PRL cell volume.

Intracellular Ca²⁺ concentration and cell size

The methodology for measuring [Ca²⁺]ᵢ in tilapia PRL cells has been previously described (Chapter IV). Intracellular Ca²⁺ concentration was monitored by fluorescence imaging with the Ca²⁺-sensitive dye fura-2. Prolactin cells, plated on poly-L-lysine-coated circular cover slips, were loaded with the membrane-permeant acetoxyethyl ester derivative of fura-2, fura-2-AM. Individual cover slips were mounted in a chamber that allows the cells to be perifused 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 CCD camera interfaced with an inverted microscope (Nikon). Images were acquired at 340 nm and 380 nm and were averaged
and then background-subtracted by use of images at each wavelength from a field devoid of cells. All data were expressed as the relative intensity of 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). Shifts in this ratio (340/380) result directly from changes of \([\text{Ca}^{2+}]_i\) (Gryniewicz \textit{et al.}, 1985). These shifts are independent of dye concentration, cell thickness, and optical efficiency of the instrument.

The procedure for measuring cell size has been described previously (Chapter IV). Briefly, the cross-sectional area of each cell was determined by tracing cells individually. All cell volume data were expressed as a percent change from baseline. The baseline was set at 100% and consisted of the mean of the first 5 values obtained at the beginning of each run. Perifusion experiments for either cell volume and PRL release determinations or \([\text{Ca}^{2+}]_i\) determinations were replicated at least 3 times.

\textit{Radioimmunoassays}

The tilapia pituitary secretes two distinct PRL molecules that are encoded by separate genes (Specker \textit{et al.}, 1985; Yamaguchi \textit{et al.}, 1988). The release of the two PRLs, designated PRL\(_{177}\) and PRL\(_{188}\), was measured using homologous radioimmunoassays (Ayson \textit{et al.}, 1993; Yada \textit{et al.}, 1994; Seale \textit{et al.}, 2002b, Chapter II). Since the patterns of PRL\(_{188}\) and PRL\(_{177}\) were identical in response to hyposmotic medium and other treatments in all the experiments, only the release of PRL\(_{188}\) is presented in the present study (see Chapter IV). Values obtained as ng/ml were converted to a percent change from the baseline set at 100%, which was determined from the average of the first 5 time points.
Statistical analysis

Because the absolute levels of PRL release varied among incubations, values for PRL release were obtained in ng/ml and normalized as the 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 Minitab Statistical Software Package (State College, PA). Data are expressed as means ± S.E.M.

RESULTS

Effects of Gadolinium on PRL release, cell volume change and $[Ca^{2+}]_i$

Gadolinium (Gd$^{3+}$) is widely utilized to block stretch-activated ion channels including Ca$^{2+}$ channels (Hamill and McBride, 1996). In the following experiments the medium was buffered with HEPES instead of NaHCO$_3$ and KH$_2$PO$_4$, since these ions have strong affinity for Gd$^{3+}$ (Caldwell et al., 1998). The lack of NaHCO$_3$ and KH$_2$PO$_4$ in the medium did not affect either cell volume or PRL release (Fig. 17A). PRL release rose rapidly when medium osmolality was reduced to 300 from 355 mOsmol/l ($P < 0.01$ for the first exposure and $P < 0.05$ for the recovery). Gadolinium at 100 µM substantially reduced the response to hyposmotic medium; there was only a slight, albeit significant ($P < 0.05$) increase in PRL release (Fig. 17B). Cell volume increased about 15% after
exposure to both normal and Gd$^{3+}$-containing hyposmotic medium. Addition of 1 mM GdCl$_3$, however, completely blocked the hyposmotically-induced PRL release. Prolactin release increased during exposure to normal hyposmotic medium at the end of the experiment (Fig. 17C). The increase in cell volume at the introduction of hyposmotic medium was not altered by exposure to GdCl$_3$.

To determine whether suppression by Gd$^{3+}$ of the hyposmotically-induced increase in PRL release were attributable to a blockage of extracellular Ca$^{2+}$ entry, [Ca$^{2+}$]$_i$ was measured in PRL cells treated with Gd$^{3+}$. The absence of NaHCO$_3$ and Na$_2$PO$_4$ did not alter the hyposmotically-induced rise in [Ca$^{2+}$]$_i$ (Fig. 18A). Exposure to 1 mM GdCl$_3$ in hyposmotic medium blocked the rise in [Ca$^{2+}$]$_i$, but did not affect subsequent [Ca$^{2+}$]$_i$ responses to hyposmotic medium after its removal in 10 out of 12 cells (Fig. 18B). Thus, Gd$^{3+}$ uncoupled PRL release from changes in cell volume apparently by blocking the rise in [Ca$^{2+}$]$_i$. This finding supports the notion that extracellular Ca$^{2+}$ entry through stretch-activated channels is a critical step for hyposmotically-induced increases in PRL release.
Fig. 17. Effects of gadolinium (Gd\(^{3+}\)) on hyposmotically-induced stimulation of PRL release and cell volume change from dispersed PRL cells. A: Effects of hyposmotic (300 mOsmolal) medium on PRL release (filled circles) and cell volume (open squares). Repeated exposure to hyposmotic medium significantly \((P < 0.01\) and \(P < 0.05\) respectively, for first and second exposures) increased net PRL release. B and C: Effects of 0.1 and 1mM Gd\(^{3+}\) on hyposmotically-induced PRL release and cell volume change. Hyposmotically-induced PRL release was completely inhibited by 1mM Gd\(^{3+}\), but not by 0.1 mM Gd\(^{3+}\). Cell volume increased as medium osmolality was reduced and was not affected by Gd\(^{3+}\). Data are expressed as mean ± SEM (n= 4 replicate experiments for PRL release; n=16-20 for cell volume determinations).

*, **, *** Significantly different from baseline in 355 mOsmolal at \(P < 0.05\), \(P < 0.01\) and \(P < 0.001\), respectively.
Fig. 18. Effects of gadolinium on the hyposmotically-induced rise in $[\text{Ca}^{2+}]_i$ of single PRL cells. Arrows indicate the time that the medium reached the cells and the treatment being perfused. All recordings were taken from cells incubated in bicarbonate- and phosphate-free medium, containing HEPES (25 mM). A: $[\text{Ca}^{2+}]_i$ trace of a single PRL cell that increased the 340/380 ratio after switching from hyperosmotic medium (355 mOsmolal) to hyposmotic medium (300 mOsmolal), and maintained elevated $[\text{Ca}^{2+}]_i$ throughout the remainder of hyposmotic exposure. B: $[\text{Ca}^{2+}]_i$ trace of a single PRL cell that increased the 340/380 ratio after exposure to hyposmotic medium, and declined to baseline levels within 5 min. C: $[\text{Ca}^{2+}]_i$ trace of a single PRL cell exposed to hyperosmotic and hyposmotic medium containing 1mM Gd$^{3+}$ followed by recovery in normal hyperosmotic and hyposmotic medium. Gadolinium blocked the hyposmotically-induced rise in $[\text{Ca}^{2+}]_i$, but did not prevent the rise in $[\text{Ca}^{2+}]_i$ induced by normal hyposmotic medium after recovery. The trace shown is representative of 10 cells.
Effects of nifedipine, high K\(^+\) and hyposmotic medium on PRL release, cell volume and [Ca\(^{2+}\)]\(_i\).

The following experiments were designed to further investigate the nature of the ion channels that participate in hyposmotically-induced PRL release. Nifedipine, an L-type Ca\(^{2+}\) channel blocker, did not prevent an increase in PRL release in hyposmotic medium, suggesting that this type of voltage-gated Ca\(^{2+}\) channel is unlikely to be involved in hyposmotically-induced PRL release (Fig. 19).

Depolarizing concentrations of KCl produce a strong but brief stimulation of PRL release in the tilapia (Richman et al., 1990; Hyde et al., 2002). When 56 mM KCl was added to hyperosmotic medium (355 mOsmolal), PRL release increased within 5 min, and returned to baseline levels after 15 min (Fig. 20A). It has been found previously that PRL cells remain unresponsive to further stimulation by high [K\(^+\)] until they are allowed to repolarize in normal medium. Extended pre-exposure to high [K\(^+\)], however did not block the rise in PRL release produced by exposure to hyposmotic medium. When pre-exposure to high [K\(^+\)] was increased to 1 h before hyposmotic stimulation, the rise in PRL release was similar to that observed under non-depolarizing conditions (Fig. 20B). 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 media was perfused. These results suggest that channels, other than voltage-gated channels, may mediate the PRL cell response to reduced osmolality.
Fig. 19. Effects of nifedipine on hyposmotically-induced PRL release. Prolactin release is expressed as percent change from the baseline. The exposure of dispersed PRL cells to hyposmotic medium (300 mOsmolal) containing 10 μM nifedipine (open circles) did not prevent hyposmotically-induced PRL release. This response was identical to that of cells exposed to normal hyposmotic medium (solid circles). Each point represents the mean ± S.E.M (n= 4).
Fig. 20. Effects of depolarizing conditions on hyposmotically-induced PRL release and cell volume from dispersed PRL cell preparations. Bars indicate the time that media reached the cells. Prolactin release (filled circles) and cell volumes (open squares) are expressed as the percent change from baseline and the former is plotted in log scale for clarity. A: Dispersed PRL cells were exposed to hyperosmotic medium containing 56 mM KCl for 30 min before switching to hyposmotic medium in the same conditions. Prolactin release increased in response to high K⁺ and hyposmotic medium. 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 switching to hyposmotic medium in the same conditions. Prolactin release increased in response to high K⁺ and hyposmotic medium. Cell volumes increased only with reductions in medium osmolality. Each point represents the mean ± S.E.M (n= 4 for PRL release, and n= 17-19 for cell volumes).
Intracellular [Ca\textsuperscript{2+}] rose briefly after exposure to high [K\textsuperscript{+}] in a manner that was similar to the pattern observed with PRL release (Fig. 21). Under depolarizing conditions, the introduction of hyposmotic medium produced a second high and sustained rise in [Ca\textsuperscript{2+}]. Intracellular [Ca\textsuperscript{2+}] gradually declined to pretreatment levels after the reintroduction of normal hyperosmotic medium. A final exposure to hyposmotic medium with normal non-depolarizing [K\textsuperscript{+}] again produced a rise in [Ca\textsuperscript{2+}]. This pattern was observed in all of the 24 cells examined. These results suggest that the rise in [Ca\textsuperscript{2+}] after hyposmotic stimulation does not involve the entry of Ca\textsuperscript{2+} through voltage-gated channels.
Fig. 21. Effects of depolarizing conditions on hyposmotically-induced increase in [Ca^{2+}]. Arrows indicate the time that the medium reached the cells and the treatment being perifused. The trace represents [Ca^{2+}] from a single PRL cell. [Ca^{2+}] sharply increased with 56 mM KCl and after 2 min, gradually declined. [Ca^{2+}] increased again when the cells were exposed to hyposmotic (300 mOsmolal) high K⁺ medium. After recovery in normal hyperosmotic medium (355 mOsmolal), cells responded to hyposmotic medium by increasing [Ca^{2+}]. The trace shown is representative of 24 cells.
DISCUSSION

This study is the first report 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\(^{2+}\)]\(_i\) and the rise in PRL release, but did not prevent PRL cells from swelling. Furthermore, neither depolarizing concentrations of KCl, or the L-type voltage-gated Ca\(^{2+}\) channel blocker, nifedipine, occluded the hyposmotically-induced PRL release, suggesting that this type of channel is not involved in this transduction process.

In the present experiments, possible mechanisms linking an increase in cell volume to extracellular Ca\(^{2+}\) entry were investigated. Mechanosensitive channels, detected by single-cell recordings, have been described in many cell types, including those from bacteria, fungi, plants and animals (Morris, 1998). Surprisingly, however, most of these channels, with one exception thus far, have physiological roles or a functional significance ascribed to them. In rats, neurosecretory cells with true osmoreceptor properties utilize stretch-inactivated cation channels to transduce a hyperosmotic stimulus into an increase in magnocellular neuron firing rate (Bourque, 1998). The osmotic control of oxytocin and vasopressin likely represents an important osmoregulatory response in mammals, since vasopressin at least is involved in sodium excretion and water retention (Verbalis et al., 1986; Honda et al., 1990; Verbalis et al., 1991; Bourque, 1998). I have hypothesized that a similar mechanism operates in the tilapia PRL cell. Specifically, I propose that changes in cell volume would 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 probability of the opening of stretch-activated Ca\textsuperscript{2+}-permeant channels. In the present study I used osmotic, ionic and pharmacological manipulations to investigate this hypothesis while observing its effects on PRL release and [Ca\textsuperscript{2+}]\textsubscript{i}.

My finding that Gd\textsuperscript{3+} uncouples hyposmotically-driven cell swelling from the increase in PRL release is particularly interesting since removing extracellular Ca\textsuperscript{2+} from the medium also reduces PRL release, without preventing cell swelling (Chapter IV). Although Gd\textsuperscript{3+} has also been found to block other types of Ca\textsuperscript{2+} channels, it currently provides the best-available pharmacological tool 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 (Hamill and McBride, 1996). Gadolinium has also been used effectively to block stretch-inactivated channels that are believed to be involved in mammalian osmoreception (Oliet and Bourque, 1996). In the present study, Gd\textsuperscript{3+} blocked the hyposmotically-induced rise in [Ca\textsuperscript{2+}]\textsubscript{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 Ca\textsuperscript{2+} signal, thus suggesting that the entry of extracellular Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{i} in PRL cells (Hyde \textit{et al.}, 2002), as well as PRL release from the cultured RPD (Grau \textit{et al.}, 1986; Richman \textit{et al.}, 1990). The L-type Ca\textsuperscript{2+} channel, the
most widely determined type of voltage-gated Ca\(^{2+}\) channel in endocrine cells, can be activated by depolarization of the membrane (Stojilkovic and Catt, 1992). Nifedipine is a selective blocker of L-type voltage-gated Ca\(^{2+}\) channels, and has been shown to block the high K\(^+\) and BayK-induced PRL release in tilapia PRL cells after 4 h (Hyde et al., 2002) and significantly reduce the PRL response to high K\(^+\) after 10 min (Seale, unpublished observation). It was predicted that if nifedipine were able to block hyposmotically-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 hyposmotic medium, suggesting that L-type channels are not involved in hyposmotically-induced PRL release.

Many voltage-gated Ca\(^{2+}\) channels (VGCC), with the exception of the L-type, become inactivated after depolarization (Catterall, 1996), thus depolarizing the membrane with high K\(^+\) would not be expected to lead to any sustained rise of [Ca\(^{2+}\)]\(_i\) via these channels. If hyposmotically-induced PRL release is dependent on VGCCs, then depolarizing conditions should prevent subsequent responses to hyposmotic medium. In the present experiments, the stimulation of PRL release by hyposmotic medium was unaffected by previous and sustained exposure to depolarizing [K\(^+\)]. Thus, the observed hyposmotically-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 hyposmotically-induced rise in PRL release in either depolarizing or normal media. 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 (Johnson and Chang, 2000a). 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 VGCCs. In a similar manner, the current data provide evidence that the signal transduction for the hyposmotic signal in the tilapia PRL cell is mediated by stretch-activated Ca\(^{2+}\)-permeant channels.

This study provided several lines of evidence that support the involvement of stretch-activated Ca\(^{2+}\) channels in mediating the hyposmotically-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 (Bourque and Oliet, 1997; Bourque, 1998), this is the first study to provide evidence that an endocrine system that plays a key role in whole organism osmotic homeostasis in response to a hyposmotic stimulus is transduced by stretch-activated ion channels. While 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.
CHAPTER VI

CONCLUSIONS AND FINAL REMARKS

The maintenance of a stable osmotic environment within cells is essential for maintaining the structure of macromolecules, such as proteins, and hence is a fundamental aspect of homeostasis in all organisms. The euryhaline tilapia, Oreochromis mossambicus, with its ability to withstand a wide range of salinities, provides an excellent model to investigate the physiology underlying adaptation to different osmotic environments. The prolactin (PRL) cell of the pituitary is one of the main elements involved in this adaptive process, and it can be easily isolated for in vitro studies. Prolactin is among the most versatile hormones with respect to its array of actions, having over 300 distinct biological activities throughout vertebrates (Bole-Feyt et al., 1998). In tilapia, as well as other euryhaline teleost fishes, PRL plays a central role in promoting FW adaptation (Clarke and Bern, 1980; Hirano, 1986). The importance of this hormone for survival in FW is underlined by the original finding by Pickford and Phillips (1959) that PRL injection prevented death in FW fish after pituitary gland removal (hypophysectomy) in Fundulus heteroclitus, a euryhaline teleost. Since PRL is a central factor in the ability for restoring hydromineral balance in FW, it is not unexpected that PRL cells might be directly sensitive to alterations in extracellular osmolality. However, there are several factors known to stimulate PRL release, including the hypothalamic peptides: gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH) and PRL releasing peptide (PrRP) (Barry and Grau, 1986; Weber et al., 1997; Seale et al., 2002a). Together with these hypothalamic factors, in the normal course of
events PRL release is also modified by steroids from the interrenal and gonadal tissues, all which interact with one another and the osmotic regulation of PRL cell function. All of these modes of control are integrated in a fashion that allows the fish to meet diverse challenges and opportunities. It has been hypothesized that the simple osmotic control of PRL release may stem from the ability of certain endocrine cells to sense environmental stimuli directly as a consequence of their having been exposed to the environment during their early evolutionary history (Bern, 1980). Layered upon this direct environmental control of hormone release, more recently derived modes of control, such as hypothalamic control, would then have evolved to fine-tune the regulation of PRL release in accordance with the acquisition of additional biological activities by the hormone (Grau and Helms, 1990; Grau et al., 1994). Nevertheless, this primordial environmental control of PRL release has been shown to be fundamental to FW adaptation, as evidenced by the sustained osmoregulatory abilities of tilapia whose pituitaries were transplanted to an ectopic site (Shepherd et al., 1999). The absence of hypothalamic control did not prevent the elevation of PRL gene expression in transplanted pituitaries and the elevation in circulating PRL levels in the same fish after transfer to FW. In fact, PRL gene expression and circulating levels in tilapia with transplanted pituitaries were similar to those found in sham-operated fish. This intrinsic osmosensitivity and ability to transduce a reduction in extracellular osmolality into the release of a hormone capable of restoring hydromineral balance in FW characterizes the PRL cell as an osmoreceptor. The overall objectives of my dissertation research were to increase our current understanding about osmoreception by investigating the mechanism(s) by which an osmotic stimulus is transduced into PRL release in the tilapia.
The uniqueness of PRL cell osmosensitivity

One of the specific objectives of my dissertation research was to determine if other pituitary cell types, such as the GH cell, could respond to alterations in extracellular osmolality in the same way as PRL cells. Although to a different degree, both PRL and GH cells of the tilapia pituitary can directly respond to changes in extracellular solute concentration. While PRL exhibits a remarkable responsiveness to reductions in extracellular osmolality (cf. Bern, 1980; Nishioka et al., 1988; Grau et al., 1994), GH has been shown to increase in response to elevations in osmolality after 18 hours of incubation (Helms et al., 1987). For this reason, I decided to characterize the extent to which PRL cells are unique in responding to reductions in extracellular osmolality. I found that PRL release is not only more sensitive to a decrease in extracellular osmolality but also longer-lasting, when compared with the responses of other pituitary hormones such as GH and corticotropin (Chapter II). This unique response was also seen in vivo, as circulating PRL levels, but not GH, were increased greatly after a transfer from SW to FW. In Chapter III, I have shown that PRL release from dispersed cells increases up to 13-fold above baseline upon hyposmotic stimulation, whereas the release of GH was only increased by 3-fold. In the same study, I demonstrated that by gradually reducing medium osmolality (from 355 to 250 mOsmolal) cell size and PRL release gradually increased in close coordination. Conversely, when osmolality was gradually increased from 300 to 450 mOsmolal, cell size and PRL release gradually decreased. In the case of GH, the relationship between medium osmolality and cell volume was similar to that seen...
in PRL cells, but GH release increased gradually after reductions and after elevations in medium osmolality.

Although GH release is induced by both reductions and elevations in medium osmolality *in vitro*, we have a good understanding only of the physiological significance of the elevation of GH release in hyperosmotic medium, since an increase in circulating GH can be seen after transferring tilapia from FW to SW, but not from SW to FW, in accord with the fact that GH is a SW-adapting hormone. Thus, PRL cells, as exemplified by those of the tilapia, are unique in their sensitivity to reductions in extracellular osmolality as well as in their ability to control osmoregulatory activity. This property led me to examine in more detail how PRL cells transduce osmotic stimuli into hormone release.

*Role of extracellular Ca\(^2+\) and intracellular Ca\(^2+\) stores in PRL cell osmoreception*

In the tilapia, the PRL cell provides an excellent model with which to investigate osmoreception. Besides being highly osmosensitive, it represents the only model in which cell volume and hormone release could be measured from the same preparation of cells of known identity. Working with this model, I developed a technique for examining cell volume changes and PRL release from the same preparation of cells, and analysing the results together with measurements of \([\text{Ca}^{2+}]_{i}\). I have shown that the rapid increase in PRL release in response to reductions in medium osmolality is largely dependent on extracellular Ca\(^2+\) (Chapter IV). The question of whether a rise in Ca\(^2+\) from intracellular stores was also involved in hyposmotically-induced PRL release has been originally presented by Richman et al. (1991), reporting that depleting extracellular Ca\(^2+\)
with EGTA markedly diminished PRL release in response to hyposmotic medium. In the present study, this observation was confirmed, using both $\text{Ca}^{2+}$-deleted and $\text{Ca}^{2+}$-depleted medium (containing EGTA). In order to explain the small rise in PRL release after hyposmotic stimulation in $\text{Ca}^{2+}$-deleted medium, I suggested that this it could be related to a compensatory $\text{Ca}^{2+}$ release from intracellular stores, since $[\text{Ca}^{2+}]_i$ increased in response to hyperosmotic $\text{Ca}^{2+}$-deleted medium as well (Chapter IV). Furthermore, by employing several pharmacological reagents that target intracellular $\text{Ca}^{2+}$ channels, I provided some preliminary evidence that the short-term (within 1 h) hyposmotically-induced PRL release is not dependent on intracellular $\text{Ca}^{2+}$ stores.

*The osmotic signal transduction in the PRL cell is mediated by stretch-activated ion channels*

Findings from studies on a variety of cell types suggest that the response of the tilapia PRL cell to osmotic stimuli might be mediated through changes in the activity of stretch-gated ion channels (see Bourque, 1998). These channels, which are found in many and perhaps all cells, are regulated by tension on the cell membrane which varies as cell volume changes (Morris, 1998). Cell volume, in turn, is highly sensitive to extracellular osmotic concentration and volume changes evoked by osmotic stress appear to be corrected through the activation or inactivation of stretch-sensitive channels that allow specific osmolytes to re-equilibrate across the cell membrane (Lang et al., 1998). Evidence also suggests that the firing rate of magnocellular (putative vasopressin) neurons of rats is governed to an important degree by the activity of stretch-inactivated cation channels (Oliet and Bourque, 1993). Thus, it has been hypothesized that stretch-
activated ion channels played a central role in the transduction of osmotic stimuli in the tilapia PRL cell (Richman et al., 1990; Grau et al., 1994). To the degree that this were true, PRL release would be tied to cell size rather than to external osmolality per se.

The relationship between cell size and PRL release was determined by Weber et al. (2002) in a series of experiments that demonstrated the close association between cell volume increase and PRL release. When medium osmolality was reduced from 355 mOsmolal to 300 mOsmolal, PRL cell size increased rapidly, followed by an increase in PRL release. When medium osmolality was held at 355 mOsmolal, but with 55 mOsmolal of NaCl replaced by 55 mOsmolal of mannitol, neither cell size nor PRL release changed. This is because mannitol is membrane-impermeant, and osmotic balance is maintained across the cell membrane. By contrast, when 55 mOsmolal of NaCl was replaced by 55 mOsmolal of urea, cell size slowly increased, followed closely by an equally slow increase in PRL release. The rise in PRL release occurred despite the fact that medium solute concentration remained unchanged. The slower rate of increase in cell size after the partial substitution of NaCl with urea is explained by the fact that while cell membranes are permeable to urea, it penetrates more slowly than H₂O. The slower inward movement of urea also determines the rate at which osmotically-driven water enters the cell and in consequence the rate at which cell size increases. This close temporal correspondence between changes in cell size and PRL release suggests that the two are coupled. This is expected if PRL release changes with osmotic gradient and not osmolality. In the present dissertation I have shown the close correspondence between medium osmolality, cell volume and PRL release while gradually changing medium osmolality over a broad range (between 250 and 450 mOsmolal; Chapter III). The slow
change in osmolality was followed by a proportionally slow change in cell volume and PRL release, corroborating to the observations obtained during the urea substitution experiment.

I then investigated the mechanisms by which a hyposmotically-induced increase in cell volume could allow extracellular Ca\(^{2+}\) to enter the cell. The PRL cell is highly sensitive to depolarizing conditions, such as high [K\(^+\)], and responds by increasing [Ca\(^{2+}\)]; and PRL release (Grau et al., 1986; Richman et al., 1990; Hyde et al., 2002). These responses indicate that PRL cells are excitable and possess voltage-gated Ca\(^{2+}\) channels (VGCCs). In mammals, L-type and T-type VGCCs are believed to be the predominant types of VGCCs in the pituitary cells (Stojilkovic and Catt, 1992). Whether hyposmotically-induced rises in [Ca\(^{2+}\)], and PRL release rely on VGCC remains to be investigated. Thus, one of the indirect methods for addressing the hypothesis that hyposmotically-induced PRL release occurs by Ca\(^{2+}\) entering the cells through stretch-activated ion channels that open in response to cell swelling, is to provide evidence that by blocking or suppressing VGCCs, hyposmotically-induced PRL release would not be affected. I presented evidence that neither L-type VGCCs nor VGCCs that inactivate under depolarizing conditions (such as T-type), are involved in hyposmotically-induced PRL release. Furthermore, I presented direct evidence that stretch-activated ion channels are involved in the osmotic signal transduction by successfully blocking hyposmotically-induced Ca\(^{2+}\) entry and PRL release with Gd\(^{3+}\) (Chapter V). The current explanation of a mechanism for osmoreception in tilapia is illustrated in Fig. 22, and has been constructed based on the evidence presented in Chapters IV and V. According to this stimulus-secretion coupling model, the rapid (minutes) increase in PRL release is dependent on the
Fig. 22: This diagram illustrates my current hypothesis of the osmoreceptor, based on the data presented in this dissertation. Under hyperosmotic conditions, the probability that stretch-activated ion channels are open is low, and PRL release is maintained at a low level. Exposure to hyposmotic conditions leads to water entry and subsequent cell swelling, which in turn increases the probability that stretch-activated ion channels are open, allowing $\text{Ca}^{2+}$ to enter the cell. The increase in $\text{Ca}^{2+}$ then triggers an increase in PRL secretion.
Hyperosmotic

Hyposmotic

• = Ca$^{2+}$

Stretch-activated Ca$^{2+}$ channels

PRL cell

PRL

H$_2$O

PRL
entry of extracellular Ca\textsuperscript{2+} through stretch-activated ion channels following cell swelling. A variety of pharmacological agents have been employed, and despite the diversity of described actions for any single agent, the interpretation of the data derived from the use of several approaches strongly supports the mechanism presented above for short-term (minutes) hyposmotically-induced PRL release. The sustained (hours-days) hyposmotically-induced PRL release, on the other hand, appears to be better explained by the observed stimulation of PRL gene expression and PRL synthesis that follows a decrease in extracellular osmolality. The investigation of whether VGCCs and intracellular Ca\textsuperscript{2+} stores participate during sustained PRL release in response to hyposmotic stimulation was beyond the scope of this dissertation, but it is likely that a variety of mechanisms that control intracellular global or localized Ca\textsuperscript{2+} levels are involved in sustained PRL release. A change in frequency of Ca\textsuperscript{2+} oscillations, for example, could represent a signal for increasing PRL gene expression. Recent studies not only indicate that Ca\textsuperscript{2+} oscillation frequencies can be specifically decoded by Ca\textsuperscript{2+} signal-effector proteins such as calmodulin kinase II (Dolmetsch et al., 1998), but also demonstrate that specific frequencies can activate certain transcription factors (De Koninck and Schulman, 1998).

The presence and activity of mechanosensitive channels has been typically studied by patch-clamping cells. Indeed, the first direct demonstration of mechanosensitive channels arose from the observation that applying pressure to a patch induced a current (Guharay and Sachs, 1984). Other studies have employed electrophysiological approaches in combination with pharmacological treatments to identify mechanosensitive channels in a variety of cell types, but to date there has been
no direct evidence of the physiological importance of stretch-activated channels as a transducer for osmoreception. Furthermore, none has directly linked the activation of mechanosensitive ion channels to the release of an osmoregulatory hormone activated by physiologically relevant decreases in osmolality. In the rat hypothalamus, stretch-inactivated channels have been proposed to regulate the osmotic signal transduction for vasopressin and oxytocin release (Oliet and Bourque, 1993; Bourque, 1998). These hormones are involved in osmoregulation by increasing water uptake in the kidney, and are released in response to elevations in blood osmolality. Prolactin release in the tilapia, on the other hand, increases in response to decreases in extracellular osmolality, in accordance with its role in FW adaptation. Therefore, I believe that the tilapia PRL cell model may have broad applicability to the understanding of osmoreception. With the rat magnocellular neuron model it can provide useful tools to study how cells adapt to osmotic changes, and such understanding is an essential first step in the development of treatment for osmotic imbalances.

In summary, I have described in this dissertation the effects of extracellular osmolality on PRL cell volume, \([Ca^{2+}]_i\), and PRL release over a range of time courses, that allowed for the construction of a general model for osmoreception in tilapia. During this process I have reported 1) the uniqueness in the response of PRL cells to extracellular osmolality, 2) the dependency on extracellular Ca\(^{2+}\) of hyposmotically-induced PRL release, 3) the involvement of stretch-activated ion channels during osmoreception, and 4) evidence that intracellular Ca\(^{2+}\) stores are not directly involved in rapid hyposmotically-induced PRL release. These studies have provided a framework for
future research geared at understanding osmoreception and its implications in the tilapia and other euryhaline species.
REFERENCES


