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The regulation of prolactin release from the pituitary of the tilapia, *Oreochromis mossambicus*, by cortisol and environmental salinity

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University of Hawai‘i, 1992
THE REGULATION OF PROLACTIN RELEASE FROM THE PITUITARY OF 
THE TILAPIA, OREOCROMIS MOSSAMBICUS, BY CORTISOL AND 
ENVIRONMENTAL SALINITY.

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE 
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE 
REQUIREMENTS FOR THE DEGREE OF 
DOCTOR OF PHILOSOPHY 
IN ZOOLOGY 
DECEMBER 1992

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I dedicate this dissertation to my mother and father, Kathleen and Herman Borski, for their constant support in all my endeavors – particularly this one, fish hormones.
ACKNOWLEDGEMENTS

I thank everyone of the Fish Endocrinology Laboratory 1986-1992 at the Hawaii Institute of Marine Biology, especially Greg Weber, Joanne Yoshikawa, Luis Santana, and Mette Hansen for all their contributions and assistance throughout the production of this thesis.

I thank Professor Howard A. Bern for his continued counsel throughout my undergraduate and graduate studies and for spawning my initial and continued interest in research.

I would like to thank Drs. N. Hal Richman, Richard S. Nishioka, Masatoshi Mita, and Lisa Helms and Profs. Chris Brown, Graham Young, Phillip Helfrich, Yoshitaka Nagahama, and Tetsuya Hirano for their valuable advice and discussions which led to the maturation of this dissertation topic and the continued development of my science career.

Finally, special gratitude goes out to my advisor, Professor E. Gordon Grau for teaching me science, for sharing the successes, and for helping me through the hurdles of my thesis research. Most of all, I thank Gordon for the opportunity to have worked and learned under his guidance.
ABSTRACT

Prolactin is an essential hormone in the freshwater osmoregulation of the euryhaline teleost fish, tilapia, Oreochromis mossambicus. Cortisol, on the other hand, is important in seawater adaptation in the tilapia. The present studies address how cortisol and environmental salinity regulate prolactin cell function in the tilapia.

During in vitro incubations, prolactin release is inhibited in a dose-related manner by cortisol. This action is mimicked by the synthetic glucocorticoid agonist dexamethasone but not by other classes of steroids tested. Perifusion studies indicate that physiological concentrations of cortisol inhibit prolactin release within 20 min. Cortisol reduces cAMP and Ca$^{2+}$ accumulation in the tilapia pituitary within 15 min, a time-course similar to the one over which cortisol inhibits prolactin release. These studies suggest that the rapid inhibition of prolactin release by cortisol is a specific glucocorticoid action that is mediated, in part, by the cAMP and Ca$^{2+}$ second-messenger systems.

Previous studies have shown that reductions in medium osmotic pressure, which reflect the blood osmotic pressure
of a tilapia adapting to fresh water, rapidly stimulate prolactin release, while elevations in medium osmotic pressure inhibit prolactin release. The present studies clearly indicate that exposure to reduced osmotic pressure increases intracellular free Ca\(^{2+}\) in single, tilapia prolactin cells. This hyposmolar-induced elevation in intracellular free Ca\(^{2+}\) occurs within 30 seconds, is sustained as long as the cells are exposed to hyposmotic medium, and can be reduced to prestimulated levels by exposure to hyperosmotic medium. Taken together, these data suggest a mediatory role for Ca\(^{2+}\) in the induction of prolactin secretion by osmotic pressure.

Consistent with prolactin's role in freshwater osmoregulation, the quantity of the two tilapia prolactins (tPRL\(_{188}\) and tPRL\(_{177}\)) and their release are greater from the pituitaries of freshwater tilapia compared with that from seawater fish. Nonetheless, the relative content of the two tilapia prolactins (tPRL\(_{188}/tPRL_{177}\)) in the pituitaries of freshwater tilapia was significantly higher (1.5:1) than that seen in seawater fish (0.75:1). These studies indicate that the processing of the tPRL\(_{188}\) and tPRL\(_{177}\) may be differentially sensitive to environmental salinity and that they may be undergoing functional divergence.
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LIST OF ABBREVIATIONS

cAMP .................. 3',5'-cyclic adenosine monophosphate
dbcAMP ................. N6,O2'-Dibutyryladenosine 3',5'-cyclic monophosphate
DMSO .................... Dimethyl sulfoxide
EGTA ..................... Ethylene glycol Bis-(B-aminoethyl ether) N,N,N',N'-Tetracetic acid
F ....................... Cortisol
FW ....................... Fresh water
IBMX ..................... 3-Isobutyl-1-methyl-xanthine
MEM ..................... Minimum essential medium
mOsmolal ............... Milliosmolal
mOsm .................... Milliosmolal
ODU ..................... Optical density unit
PAGE ..................... Polyacrylamide gel electrophoresis
PBS ....................... Phosphate buffer saline
PRL ....................... Prolactin
RIA ....................... Radioimmunoassay
RPD ....................... Rostral pars distalis
SDS ....................... Sodium dodecyl sulfate
SRIF .................. Somatostatin
SW .................... Seawater
TEP .................... Transepithelial potential
VIP ..................... Vasoactive intestinal peptide
CHAPTER I

INTRODUCTION

Prolactin (PRL) has been called "the most versatile of the various pituitary hormones in both number and diversity of the physiological processes it regulates" (Nicoll, 1982). The spectrum of PRL's actions throughout the vertebrates include effects on reproduction, parental behavior, growth and development, metabolism, and osmoregulation (Clarke and Bern, 1980; Loretz and Bern, 1982). It is PRL's regulation in regards to its osmoregulatory function which is the subject of this thesis.

Roles of Prolactin and Cortisol in Osmoregulation

The tilapia, Oreochromis mossambicus, is a euryhaline teleost fish that occupies habitats ranging from fresh water (FW) to salinities far in excess of full-strength seawater (SW). Prolactin and cortisol act in the regulation of the ability of this fish to adapt to changing salinities.

Prolactin plays a central role in the FW adaptation of the tilapia as well as other euryhaline teleost fishes.
Removal of the pituitary gland (hypophysectomy) and transfer to FW results in declining electrolyte concentrations and blood osmolality in the tilapia and other teleost fishes (Pickford and Phillips, 1959; cf. Clarke and Bern, 1980). Prolactin restores blood osmotic pressure by acting on virtually all osmoregulatory tissues including the gills, integument, urinary bladder, intestine, and kidney to reduce water permeability and increase sodium retention (Clarke and Bern, 1980; Hirano, 1986). Cortisol, on the other hand, has been shown to act in SW osmoregulation in many teleosts, including the tilapia, by reducing blood osmotic pressure. It does so by stimulating secretion of electrolytes and increasing ion and water permeability in osmoregulatory epithelia (Hirano and Mayer-Gostan, 1978; Foskett et al., 1983).

Regulation of PRL Cell Activity

Prolactin cell activity is enhanced when tilapia are maintained in FW and is reduced in SW fish (Dharmamba and Nishioka, 1968; Clarke et al., 1973; Nagahama et al., 1975; cf. Clarke and Bern, 1980). The alteration of PRL cell activity under these conditions appears to result, in part, from the direct actions of osmotic pressure. Following in vitro incubation, details of fine structure
suggest that prolactin cell activity is augmented when the osmotic pressure of the medium is reduced (Nagahama et al., 1975; Nishioka et al., 1988). Similarly, recent studies have shown that small reductions in medium osmotic pressure, well within the physiological range of the tilapia, increase PRL release within 10-20 min (Grau et al., 1986; Grau et al., 1987).

Prolactin cell function in the tilapia also appears to be sensitive to modification by cortisol. An in vitro study showed that a single, high dose of cortisol suppressed prolactin release during overnight incubations (Wigham et al., 1977).

The Tilapia PRL Cell as a Model for Studying PRL Cell Function

A principal impediment to the study of PRL cell function has been the difficulty in isolating the PRL cells, which in most vertebrates are interdispersed among other cell types of the pituitary. Currently, most investigators utilize mammalian clonal cell lines derived from PRL-secreting tumors (i.e. GH₃ cells) to study PRL cell regulation and stimulus-secretion coupling (Tashjian et al., 1970; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990). However, interpretation of these tumor
cell line studies is limited since one is never sure whether they are investigating normal PRL cell function or tumor cell function. The PRL cell of the tilapia has two advantages for studying PRL cell regulation. In most teleosts, PRL cells are segregated into a nearly homogeneous mass in the anterior-most portion of the adenohypophysis, the rostral pars distalis (RPD). For the tilapia, a conservative estimate is that this segregation is 95-99% complete allowing the PRL-secreting tissue or RPD to be separated easily for in vitro incubation in defined media for up to two weeks. A second important advantage of the tilapia PRL cell is that its release of hormone is directly responsive to small changes in medium osmotic pressure. Low osmotic pressure stimulates and high osmotic pressure inhibits PRL release. This osmosensitivity of the tilapia PRL cell makes it possible to control experimental baseline release, which greatly facilitates the study of potentially important stimulators and inhibitors of PRL secretion (Grau et al., 1982). Moreover, the tilapia PRL cell provides a useful model for studying the nature of the "osmoreceptor" and the intracellular regulation of an osmosensitive tissue with a known osmoregulatory output (e.g., PRL).
Role of Second Messengers in Stimulus-secretion Coupling

Stimulus-secretion coupling describes the processes by which an external cue (i.e., hormone, osmotic pressure) is transduced into an intracellular second message which, in turn, alters the rate of hormone or transmitter release. An increase in the concentration of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is a central event in stimulus-secretion coupling (Rubin, 1982; Rasmussen & Barrett, 1984; Rasmussen et al., 1984). Evidence suggests that an appropriate stimulus evokes a rise in [Ca\(^{2+}\)]\(_i\) in endocrine cells which results from both the influx of extracellular Ca\(^{2+}\) and from the release of Ca\(^{2+}\) from intracellular stores. While the release of Ca\(^{2+}\) from intracellular stores is believed to initiate the events leading to hormone secretion, the influx of Ca\(^{2+}\) through Ca\(^{2+}\) channels in the plasma membrane is thought to extend a biological response (e.g., PRL release). The influx of extracellular Ca\(^{2+}\) depends upon the opening of Ca\(^{2+}\) channels (e.g., stretch-, voltage-, ligand-gated) in the membrane. It is generally believed that as long as a stimulatory hormone (or nonhormonal signal) remains bound to its receptor, Ca\(^{2+}\) channels are more likely to open and...
thereby maintain hormone release. Evidence also suggests that inhibitors of hormone release reduce the probability that Ca^{2+} channels will open (cf. Rubin, 1982; Grau and Helms, 1989).

The cyclic nucleotides are a second important class of second messengers in endocrine cells. The most studied of these, cyclic adenosine-3',5'-monophosphate (cAMP), activates key enzymes which initiate a cascade of events leading to increased hormone release (Berridge, 1985; Levitzki, 1986). When a receptor is activated, such as when a hormone binds to its receptor, the production of cAMP from ATP is stimulated by an increase in adenylate cyclase activity. The increase in cAMP production, in turn, activates specific protein kinases, which are ultimately responsible for producing the appropriate biological response (e.g., PRL release). Resting conditions are restored by phosphodiesterase which catalyzes the conversion of cAMP to 5'AMP. The tilapia PRL cell appears to respond to osmotic pressure through the second-messenger systems.

*The Role of Ca^{2+} in Tilapia Prolactin Release*

As in mammals, several lines of evidence support the conclusion that osmotically-induced release of PRL from
the tilapia pituitary is dependent on Ca$^{2+}$ (Grau et al., 1981; Grau et al., 1982; Grau et al., 1986). Prolactin release is blocked when Ca$^{2+}$ is deleted from incubation media. Furthermore, the stimulatory effect of reduced osmotic pressure on PRL release is suppressed in the presence of Co$^{2+}$, a competitive inhibitor of Ca$^{2+}$-mediated processes, both chronically in static culture and acutely during perifusion incubation (Grau et al., 1986). The Ca$^{2+}$ ionophore, A23187, which raises [Ca$^{2+}$]$_i$, rapidly leads to a large increase in PRL release from the tilapia RPD (Grau et al., 1982, Helms, 1988). Finally, Richman and coworkers (1990) showed that reduced osmotic pressure stimulates the accumulation of extracellular $^{45}$Ca$^{2+}$ into the RPD of tilapia. Taken together, these studies suggest, only indirectly, that increases in PRL release invoked by reduced osmotic pressure might be mediated by rises in [Ca$^{2+}$]$_i$.

The Role of cAMP in Tilapia Prolactin Release

As with Ca$^{2+}$, there is now good evidence for an important role for cAMP in mediating PRL secretion, including the response to changes in osmotic pressure, in the tilapia. Exposure to dibutyryl-cAMP and/or the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine
(IBMX), increases PRL release during static incubation (Grau et al., 1982; Helms, 1988; Grau and Helms, 1989; Helms et al., 1991). In addition, forskolin, a potent stimulator of adenylate cyclase, produces increases in both cAMP levels in the tilapia RPD and PRL release. Thus, increases in intracellular cAMP are sufficient to stimulate PRL release. The accumulation of cAMP in the presence of IBMX is inversely related to medium osmotic pressure as is PRL release (Helms, 1988; Helms et al., 1991). This suggests that PRL release during exposure to reduced osmotic pressure may involve an increase in adenylate cyclase activity. Since cAMP levels do not change, except in the presence of IBMX, it would appear that this enhanced adenylate cyclase activity is balanced by a comparable increase in cAMP catabolism by phosphodiesterase (cf. Grau and Helms, 1989). Our laboratory has shown that somatostatin (SRIF) rapidly (≤ 10 min) inhibits PRL release (Grau et al., 1987). More recently, our laboratory showed that SRIF substantially reduced cAMP accumulation during 10 min incubations in the presence of IBMX, suggesting that its actions on PRL release are mediated, in part, by reductions in cAMP Helms et al. (1991). Also, it would appear that SRIF may act to reduce cAMP accumulation by reducing adenylate cyclase activity.
Two Tilapia Prolactins

The tilapia RPD synthesizes and releases two distinct PRL molecules which are encoded by different genes and are not derived through the differential processing of the same translation product (Specker et al., 1985a). Yamaguchi et al. (1988) have shown that the larger tilapia PRL has a molecular weight of 20.8 kDa and contains 188 amino acid residues (PRL\textsubscript{188}), whereas the smaller PRL molecule is 19.6 kDa and contains 177 amino acid residues (PRL\textsubscript{177}). The existence of two PRLs in the tilapia suggests that the two molecules may have arisen serendipitously through gene duplication, an event that appears to have lead to the divergence of the growth hormone/PRL proteins and the family of pituitary glycoprotein hormones (i.e., lutenizing, follicle-stimulating, and thyrotropin-stimulating hormones). Since, it appears that the two tilapia PRLs are translated from separate gene transcripts, yet are presumed to be derived from a common ancestral gene, one would speculate that these two hormones possess overlapping as well as distinct actions. However, besides the possible growth-promoting action of the larger PRL, the functional distinction between the two PRLs within the tilapia has not been elucidated. In fact, the two molecules showed
similar effects in the tilapia sodium-retaining assay, a well characterized bioassay that measures PRL activity (Specker et al., 1985a).

Research Objectives

A distinction between the roles of the two PRLs within the tilapia has not been discerned. The high sensitivity of the tilapia PRL cell to environmental salinity suggests that the two tilapia PRLs might be disparately regulated by environmental salinity. The first objective of my thesis research, therefore was to determine whether the processing of the two tilapia PRLs in the pituitary is differentially sensitive to environmental salinity (chapter 2).

Wigham et al. (1977) showed that a single, high dose of cortisol reduced PRL release from the tilapia RPD during 18-20 hr static incubations. It remains to be determined in the tilapia whether cortisol's effect is dose-dependent, and over what time-course it inhibits PRL release. One might suspect cortisol to have a rapid action in inhibiting PRL release since adjustments to acute changes in osmotic pressure are rapid. These are the questions that I have addressed in chapter 3. In addition, I wanted to determine whether cortisol's actions
might be mediated through the cAMP and/or Ca\(^{2+}\) second messengers.

Pharmacological and radiotracer studies suggest that hyposmolar-induced PRL release is mediated by rises in \([\text{Ca}^{2+}]_i\). A goal of my research was to determine, using the Ca\(^{2+}\)-sensitive probe fura-2, whether osmotic pressure alters \([\text{Ca}^{2+}]_i\) in optically-isolated single PRL cells (chapter 4).

The results from chapters 2 and 3 are published in the Journal of Experimental Zoology (vol. 264, pp. 46-54) and the Proceedings of the National Academy of Sciences, USA (vol. 88, pp. 2758-2762), respectively. Chapter 4 is in preparation for publication.
CHAPTER II

DIFFERENTIAL PROCESSING OF THE TWO PROLACTINS OF THE TILAPIA, OREOCHROMIS MOSSAMBICUS, IN RELATION TO ENVIRONMENTAL SALINITY

INTRODUCTION

Prolactin plays a central role in FW osmoregulation of the tilapia, Oreochromis mossambicus, and many other euryhaline teleost fishes. Prolactin acts by reducing both water and ion fluxes, thereby conserving salt and water (Pickford and Phillips, 1959; Clarke and Bern, 1980; Hirano, 1986). Prolactin cell function in fishes is thought to be controlled by several elements (cf. Nishioka et al., 1988). In the tilapia, these include the hypothalamic factors, SRIF, thyrotropin-releasing hormone, and vasoactive-intestinal peptide (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1982, 1986, 1987; Rivas et al., 1986; Barry and Grau, 1986; Kelley et al., 1988; Grau and Helms, 1989), as well as other hormones including urotensin II, cortisol and estradiol-17β, and testosterone (Wigham et al., 1977; Grau et al., 1982; Barry and Grau, 1986; Rivas et al., 1986; Borski et al., 1991, see Chapter 3).
Consistent with its central role in FW osmoregulation, histological studies show that PRL cell activity in the tilapia and other teleosts is enhanced by FW and is reduced by SW (Dharmamba and Nishioka, 1968; Abraham, 1971; Nagahama et al., 1973, 1975; Olivereau et al., 1981). In vitro studies suggest that in some teleosts, PRL cell activity may be altered directly by changes in blood osmotic pressure (cf. Clarke and Bern, 1980; Nishioka et al., 1988). In the tilapia, small physiological reductions in medium osmotic pressure, similar to those which occur as a fish adapts to FW, rapidly stimulate PRL release while conversely, elevated medium osmotic pressure inhibits PRL release (Grau et al., 1986, 1987).

Specker and colleagues (1985a) have shown that the anterior most portion of the tilapia pituitary, the RPD (95-99% PRL cells), synthesizes and releases two distinct PRL molecules. These hormones (tPRL\textsubscript{188} and tPRL\textsubscript{177}) are encoded by different genes and are not derived from the differential processing of the same translational product (Specker et al., 1985a, b; Yamaguchi et al., 1988). The degree of similarity between the amino acid sequences of the two tilapia PRLs is only 69%, which is no higher than that between the tilapia PRLs and carp or salmon PRLs (cf. Yamaguchi et al., 1988). This suggests that the two tila-
tilapia PRL molecules might exert separate actions and/or be independently regulated.

Aside from a possible growth-promoting effect of tPRL$_{188}$, no clear difference in the actions, regulation, or metabolism of tPRL$_{188}$ and tPRL$_{177}$ have been demonstrated in tilapia (Specker et al., 1985a, b). In fact, both tPRL$_{188}$ and tPRL$_{177}$ possess similar activity in the standard bioassay for tilapia PRL (Specker et al., 1985a, b). To my knowledge, no work has demonstrated that the two tilapia PRLs respond independently to changes in salinity. In the present studies, I investigated whether the long-term rearing or short-term acclimation in FW and SW could differentially alter the RPD content and subsequent in vitro release of the two PRLs in the tilapia.

MATERIALS AND METHODS

Rearing and Long-term Acclimation Study

Tilapia were reared from the stage of yolk-sac absorption for 7 months in 700 liter oval fiber glass tanks in FW or SW (25 ± 2°C) under a natural photoperiod. Fish were fed Purina trout chow twice daily (total ration = 4% of body weight). At the end of this rearing period,
pituitaries from FW and SW male tilapia were removed and RPD were dissected for determinations of the content of both PRLs in vivo and in vitro, and the subsequent response to altered osmotic pressure in vitro.

For the long-term acclimation study, male tilapia reared in FW for 7 months from the stage of yolk-sac absorption were transferred to SW for 49 days or transferred to FW for an additional 49 days. Conversely, fish reared in SW for 7 months were transferred to FW for 49 days or transferred to SW for an additional 49 days. At the end of this 49 day period the in vivo RPD content of tPRL\textsubscript{188} and tPRL\textsubscript{177} was determined in fish from all four groups.

These experiments were repeated over a two-year period with consistent results. However, only data from the second year are presented because all phases of the study were not performed in the first year.

Short-term Acclimation Study

Male tilapia, 15-20 cm long, were obtained from brackish water ditches on the North Shore of the island of Oahu, Hawaii, and were maintained for at least three weeks in FW prior to the start of the acclimation period. Fish were transferred to four tanks, containing FW (25 ± 2°C)
for 7 days. At the start of the acclimation (day 0), RPD were dissected from pituitaries of fish for in vivo PRL content and for in vitro PRL release determinations. Following this initial sampling, the salinity of two tanks was increased to 25% for 5 days, followed by 50% (5 days), 75% (4 days) and finally to 100% SW over a 14 day period. The fish were maintained in full-strength SW, and samples were taken 21 and 35 days after the start of the acclimation. Fish were sampled from the FW tanks during the same time periods.

In Vitro Studies

A modified Krebs bicarbonate-Ringer solution containing glucose (500 mg/liter), L-glutamine (290 mg/liter) and Eagle's minimum essential medium (50X MEM, 20 ml/liter: GIBCO; Grand Island, NY) was used for pituitary incubations (Wigham et al., 1977). The medium was gassed for 10 min with 95% O₂/5% CO₂ (pH = 7.25). Osmotic pressure, measured with a Wescor vapor osmometer (Wescor; Logan, UT), was adjusted to 300 and 355 mOsmolal (mOsm) using NaCl. Tilapia were decapitated, their pituitaries were removed and collected. The RPD were dissected from the pituitaries and each RPD was placed into a well of a Falcon 96-well culture plate containing
100 μl of hyperosmotic or hyposmotic medium. Tissues were placed in a chamber under a humidified atmosphere of 95% O₂/5% CO₂ (28 ± 1°C) and incubated on a gyratory platform (80 rpm) for 18-20 hr.

At the termination of the 18-20 hr incubations, media and tissue were placed in sodium dodecyl sulfate (SDS)-2-mercaptoethanol buffer, ultrasonically disrupted (Heat Systems W-385 sonicator, Heat Systems-Ultrasonics, Inc.; Farmingdale, NY), boiled in a water bath for 3 min, and frozen at -80°C (Laemmli, 1970; Specker et al., 1985a; Kelly et al., 1988).

The in vivo RPO content of the PRLs was determined from freshly dissected RPD transferred directly to the SDS-buffer. As above, the tissues were sonicated, boiled and frozen prior to hormone separation by gel electrophoresis.

**SDS Polyacrylamide Gel Electrophoresis**

The tilapia PRLs were separated in tissue and medium samples by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970) and Specker et al., (1985a). A vertical slab electrophoresis apparatus (Bio-Rad; Richmond, CA) was used. The samples containing the two tilapia PRLs were stacked in a 4% 37.5:1
acrylamide:bis-acrylamide gel and separated in a 15% 37.5:1 acrylamide:bis-acrylamide gel (12 cm long, 0.15 cm thick). All samples were electrophoresed at 30 mA constant current/gel for 4-5 hr using a voltage- and current-regulated power supply (ISCO; Lincoln, Nebraska). The gels were stained in 1 liter of Coomassie blue R-250 (10% methanol, 5% acetic acid) on a gyratory platform at room temperature for 15-18 hr. The gels were destained (10% methanol, 7% acetic acid, 83% dH₂O solution) until clearly discernable bands were observed and then placed in a storage solution of 7% acetic acid. Bands of both PRLs were quantified by using a densitometer and proprietary software (Hoefer Scientific, San Francisco, CA). All values are expressed in optical density units (ODU) measured by the densitometer. The optical densities of the stained PRL bands were linearly related to the amounts of the PRLs loaded onto the gel over a range extending from 0.5 to 3 times the amount of PRL we typically load. Prolactin release is expressed as a percentage of PRL released into the incubation medium divided by the total amount of the PRL in the incubation medium plus tissue.
Statistical Analysis

Differences among means were evaluated using analysis of variance (Crunch Software; San Francisco, CA) followed by the least-significant difference test for predetermined comparisons (Steele and Torrie, 1980). Experiments employing only two groups were analyzed using the Student's t-test. Statistical differences between the two tilapia PRLs within a treatment were analyzed using the paired Student's t-test.

RESULTS

In Vivo Content and In Vitro Release of tPRL\textsubscript{188} and tPRL\textsubscript{177} from the RPD of FW and SW Raised Tilapia

The RPD content of tPRL\textsubscript{188} was almost 5X higher in FW-reared tilapia than in SW-reared fish (Fig. 1; \(p < 0.001\)). In a similar way, the RPD content of tPRL\textsubscript{177} was greater in FW-reared fish compared to SW-reared fish (Fig. 1; \(p < 0.001\)). In FW, the RPD contained significantly more tPRL\textsubscript{188} than tPRL\textsubscript{177}, whereas in SW the reverse was true (Fig. 1; \(p < 0.01, p < 0.05\)).

In order to better illustrate the relative content of the two tilapia PRLs from FW- and SW-reared fish, we ex-
Figure 1. The content of tPRL$_{188}$ and tPRL$_{177}$ from the RPD of tilapia reared for 7 months in fresh water and seawater, measured in optical densitometric units (O.D.U.) (mean ± SEM; n = 5-6 RPD). ***, p < 0.001, **, p < 0.01, *, p < 0.05.
Figure 2. The relative content of the larger and smaller PRL expressed as a ratio (tPRL$_{188}$/tPRL$_{177}$) from the RPD of FW- and SW-reared tilapia (mean ± SEM; n = 5-6 RPD). ***, p < 0.001.
pressed the data as a ratio (tPRL\textsubscript{188}/tPRL\textsubscript{177}). This ratio was found to be strongly dependent on rearing salinity (Fig 2). The tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio was significantly higher (~1.5:1) in the RPD of FW-reared tilapia than that seen in the RPD of SW fish (~0.75:1; p < 0.001).

The tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio shifts in the tilapia RPD with environmental salinity. Previous studies have shown that a small reduction in medium osmotic pressure in vitro stimulates the release of PRL (Grau et al., 1982). This suggested that the shift in the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio with environmental salinity in vivo might result from the differential effects of osmotic pressure on the production, release, and/or metabolism of the two PRLs. I, therefore, undertook in vitro studies directed at determining whether exposure to physiological changes of medium osmotic pressure might differentially alter the pituitary content and/or release of the two tilapia PRLs from the RPD of FW- and SW-reared tilapia.

As in previous studies, hyposmotic medium stimulated the release of tPRL\textsubscript{177} from RPD of FW fish over levels observed in hyperosmotic medium during 18-20 hr incubations (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1982). Here I report that reduced osmotic pressure also increases the release of tPRL\textsubscript{188} in vitro. Reduced osmotic pressure also increases the release of
Figure 3. The effect of osmotic pressure (355 and 300 mosm) during 18-20 hr static incubation on the release of tPRL$_{188}$ and tPRL$_{177}$ (mean ± SEM) from the RPD ($n = 5-6$ RPD) of tilapia reared in fresh water and seawater for 7 months. ***, $p < 0.001$ and *, $p < 0.05$. 
Figure 4. The effects of osmotic pressure (355 and 300 mosm) on the relative quantity of tissue (A) and total (medium + tissue; B) tPRL_{188} and tPRL_{177} expressed as a ratio (tPRL_{188}/tPRL_{177}) during 18-20 hr static incubation. RPD from tilapia reared for 7 months in FW and SW were utilized for the incubations (mean ± SEM; n = 5-6 RPD). ***, p < 0.001.
both PRLs from RPD of SW fish (Fig. 3). Nevertheless, during exposure to either hyposmotic or hyperosmotic medium, the RPD of FW-reared tilapia released significantly more of both PRLs than did the RPD of SW-reared fish (Fig. 3; \( p < 0.001, p < 0.05 \)).

We also found that while variations in medium osmotic pressure did alter PRL release, the \( \text{tPRL}_{188}/\text{tPRL}_{177} \) ratio of the incubations remained unaltered from values established \textit{in vivo} in the rearing salinity. This was true whether considering the tissue content of the two PRLs alone (Fig. 4A; \( p < 0.001 \)), their medium content alone (not shown), or both in combination (Fig 4B; \( p < 0.001 \)).

\textit{Long-term Acclimation Study}

After it was determined that the \( \text{tPRL}_{188}/\text{tPRL}_{177} \) ratio shifts in the RPD of FW- compared to SW-reared tilapia (Fig. 2), my next objective was to determine whether the \( \text{tPRL}_{188}/\text{tPRL}_{177} \) ratio reverses when the rearing salinity is reversed. When tilapia were reared for 7 months in FW and transferred to SW and held for 49 days, the \( \text{tPRL}_{188}/\text{tPRL}_{177} \) ratio was reduced (\( \approx 0.4:1 \)) from that seen in tilapia that were retained in FW (\( \approx 1.5:1; \) Fig. 5; \( p < 0.05 \)). The \( \text{tPRL}_{188}/\text{tPRL}_{177} \) ratio in fish
Figure 5. The relative quantity (tPRL$_{188}$/tPRL$_{177}$) of tPRL$_{188}$ and tPRL$_{177}$ from RPD of tilapia reared in FW for 7 months and transferred to FW for an additional 49 days (FW-FW) or transferred to SW for 49 days (FW-SW). RPD of tilapia reared in SW for 7 months and transferred to SW for an additional 49 days (SW-SW) or transferred to FW for 49 days (SW-FW) were also analyzed for relative quantities of the two PRLs. (mean ± SEM; n = 5-6 RPD). **, p < 0.01.
Figure 6. The relative quantity (tPRL_{188}/tPRL_{177}) of tPRL_{188} and tPRL_{177} from RPD of tilapia acclimated to fresh water (solid line) or seawater (dashed line) for 21 days and 35 days (mean ± SEM; n = 6 RPD). ***, p < 0.001, **, p < 0.01.
transferred to SW (≈0.4:1) for 49 days is similar to that seen in SW-reared fish (≈0.5:1). By contrast, the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio of fish transferred from SW to FW for 7 weeks was increased (≈1.3:1) compared to that seen in fish retained in SW (≈0.5:1; p < 0.01) and was very close to the ratio seen in fish that were retained in FW.

**Short-term Acclimation Study**

The foregoing evidence shows that the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio in the tilapia RPD depends on the rearing salinity. This pattern is retained even after 18-20 hr incubations. In addition, the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio can be reversed, at least within 49 days, by reversing the rearing salinity. This observation led me to question whether the ratio of the two PRLs is altered during short-term acclimation (< 49 days) of fish to different salinities (i.e., FW and SW).

Figure 6 shows the in vivo tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio in the RPD from fish that were transferred from FW to SW for either 21 or 35 days. The tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio in the RPD of fish transferred from FW to SW (≈0.75:1) for 21 days was significantly reduced compared to the ratio observed in RPD of control fish that were transferred from FW to FW (≈1.2:1; p < 0.01). This pattern was similar to
that in fish transferred from FW to SW and held for 35 days (Fig. 6; p < 0.001).

The RPD of tilapia acclimated to FW for either 21 or 35 days released significantly more of both forms of PRL during exposure to either hyperosmotic or hyposmotic medium for 18-20 hr than did the RPD from fish acclimated to SW (Fig. 7B,C; p < 0.01, p < 0.001). Similar to the pattern seen in incubations of RPD from fish raised for 7 months in FW and SW (Fig. 3), RPD from fish acclimated to FW and SW for 21 and 35 days released more tPRL\textsubscript{188} and tPRL\textsubscript{177} when incubated in hyposmotic medium than when held in hyperosmotic medium (Fig. 7A-C). There were no differences between tPRL\textsubscript{188} and tPRL\textsubscript{177} release from the RPD under our incubation conditions, regardless of the acclimation salinity of the fish. As previously observed with rearing salinity, we also found that while variations in medium osmotic pressure did alter PRL release, the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio of the incubations remained unaltered from values established from RPD of FW- and SW-acclimated tilapia in vivo. This was true whether considering the tissue content of the two PRLs alone, their medium content alone, or both in combination (data not shown).
Figure 7. The effect of osmotic pressure (355 and 300 mosm) on the release of tPRL_{188} and tPRL_{177} during 18-20 hr static incubation of RPD from tilapia acclimated to fresh water (Day 0, A) and seawater for 21 (B) and 35 days (C; mean ± SEM; n = 5-6 RPD). ***, p < 0.001, **, p < 0.01.
A Day 0

![Graph showing % PRL Release vs. Fresh Water concentration for Day 0 with two bars for each concentration level, IPRL-188 and IPRL-177.]

B Day 21

![Graph showing % PRL Release vs. Fresh Water and Seawater concentrations for Day 21 with three bars for each concentration level, IPRL-188, IPRL-177, and an asterisk indicating statistical significance.]

C Day 35

![Graph showing % PRL Release vs. Fresh Water and Seawater concentrations for Day 35 with three bars for each concentration level, IPRL-188, IPRL-177, and asterisks indicating statistical significance.]

DISCUSSION

Clarifying the functional significance of tilapia having two PRLs has attracted considerable interest since they were first described by Specker et al., (1984, 1985a, b). Other than a preliminary observation that tPRL_{188} alone may promote an increase in the weight and length of juvenile FW tilapia, no physiological distinction between the two PRL molecules has yet been reported (cf. Specker et al., 1985a, b). My findings suggest, however, that the processing of the two tilapia PRLs may be differentially sensitive to environmental salinity. The relative RPD content or ratio (tPRL_{188}/tPRL_{177}) of the larger to smaller PRL molecule shifts from a higher (> 1) value in FW fish to a lower (< 1) value in SW tilapia when fish are reared from the stage of yolk-sac absorption for 7 months (Figs. 1, 2). The tPRL_{188}/tPRL_{177} ratio can be reversed within 49 days when tilapia reared in FW and SW are later acclimated to SW and FW, respectively (Fig. 5). Moreover, this alteration of the ratio occurs not only in tilapia reared for 7 months in FW and SW, but also appears in tilapia reared in brackish water and acclimated for 21 days in FW and SW (Fig. 6).

Prolactin cell function has been shown repeatedly to be augmented in FW tilapia compared with SW tilapia.
Dharmamba and Nishioka (1968) showed that both the area of the RPD and the size of its individual PRL cells, are greater in the pituitaries of FW-acclimated tilapia compared with SW-acclimated tilapia. Clarke (1973) also showed that the RPD of FW tilapia contain more tPRL_{177} than the RPD of SW tilapia. In addition, details of fine structure and changes in the rate of $^{3}$H-leucine incorporation suggest that PRL cell activity is enhanced in FW tilapia compared with SW tilapia (Nagahama et al., 1975). I report here, for the first time, that the content of tPRL_{188}, like that of tPRL_{177}, is higher in the RPD of FW tilapia compared with the RPD of SW tilapia. My data are in agreement with the idea that both PRLs play essential roles in FW adaptation in tilapia and a variety of other teleost fishes (cf. Clarke and Bern, 1980; Specker, 1985a).

Both PRLs are present in greater quantities in RPD from FW tilapia than from those of SW fish. However, when comparing FW tilapia (reared and acclimated) with SW tilapia, I found that the change in RPD content of tPRL_{188} ($\approx 78\%$) always exceeded that of tPRL_{177} ($\approx 53\%$; data not shown; Chi square test, $p < 0.05$). This suggests that there is a significantly more pronounced shift in the quantity of tPRL_{188} than tPRL_{177} in the RPD of the tilapia exposed to alterations in environmental salinities.
Whether this shift in the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio reflects modifications in the production, secretion or possibly the degradation of both or one of the PRLs has been determined recently. Subsequent to my investigations, studies show that more tPRL\textsubscript{188} than tPRL\textsubscript{177} is synthesized in RPD of FW-acclimated tilapia compared with RPD of SW-acclimated fish, whereas the reverse is true for SW fish (personal communication, Yoshikawa and Grau).

Not only was the content of the two PRLs in the RPD of FW tilapia higher than that observed in SW fish, but so too was the ability of FW RPD to release the two hormones during exposure to either hyperosmotic or hyposmotic medium in vitro. Nevertheless, exposure to reduced osmotic pressure augmented the release of both PRLs from RPD of FW and SW fish over levels observed during exposure to hyperosmotic pressure. Overall, release of both PRLs in vitro appeared to be equally sensitive to medium osmotic pressure (Fig. 3).

The tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio established in vivo in FW and in SW, respectively, was not altered by changes in medium osmotic pressure after the RPD were incubated for 18-20 hr in vitro. I found this to be true whether examining RPD content of the two PRLs alone, their medium content alone, or both in combination. It would appear then that the shift in the tPRL\textsubscript{188}/tPRL\textsubscript{177} ratio seen with
a change in environmental salinity does not result from the differential release of the two tilapia PRLs elicited directly by physiological changes in osmotic pressure, at least under conditions described herein.

Studies in our laboratory have shown that SW-reared tilapia grew almost 2X faster than FW-reared tilapia (Kuwaye et al., 1991, in press). I considered the possibility that factors associated with increased growth rate or fish size might account for the shift in tPRL_{188}/tPRL_{177} ratio. However, I found no correlation between the size of individual fish and the tPRL_{188}/tPRL_{177} ratio, at least when comparing individuals from a particular salinity (i.e., FW or SW; data not shown). Furthermore, the tPRL_{188}/tPRL_{177} ratio in the RPD of fish acclimated to SW for 21 days was 0.75:1, much lower than its FW controls (ratio = 1.2:1), even though the average weight of fish in the two groups was similar (223 and 220 g, respectively). Therefore, I conclude that the shift in the tPRL_{188}/tPRL_{177} ratios in FW and SW fish is not likely to be related to differences in fish size or growth rate. Differences in age also do not appear to be a critical factor, since all FW- and SW-reared tilapia were of the same age. Clearly then, it appears that the alterations in the relative content of the two PRLs in vitro and in vivo are a result of variations in
environmental salinity and are not due to differences in the age or growth rate of the tilapia being compared.

I was unable to detect any differences between the release of the two tilapia PRLs under my culture conditions. This finding is in agreement with that of Specker and colleagues (1985a), who showed no variations in the release or synthesis of the two PRLs during exposure to reduced osmotic pressure. In vitro studies have shown that the release of both tilapia PRLs responded similarly to cortisol, estradiol-17β, urotensin II, vasoactive intestinal peptide, testosterone, SRIF, aldosterone, 11-deoxycorticosterone, 17α,20β-dihydroxyprogesterone, and progesterone (Rivas et al., 1986; Kelly et al., 1988; Borski et al., 1991, see chapter 3). Nevertheless, all studies to date have been restricted to the investigation of PRL cell regulation by hypothalamic and/or other endocrine factors in FW fish only. Our data showing alterations in the relative quantity of the two PRLs in FW and SW tilapia invites further investigation into potentially important regulators of PRL secretion and synthesis in SW tilapia.

The demonstration of a shift in the RPD content of the two PRLs in FW and SW tilapia leads one to speculate among several possibilities that these molecules might have distinct actions in the osmoregulation of the
tilapia. This does not appear to be the case, however. To our knowledge, the only example of the two tilapia PRLs having differential roles in osmoregulatory physiology is that they possess different abilities to alter whole-animal transepithelial potential (TEP) of the red-spotted newt. This study indicates that receptors in the newt can discriminate between two heterologous PRLs (Specker et al., 1989). Although the tilapia PRLs might differentially alter certain electrophysiological characteristics of the newt skin, both PRLs were equally active in the tilapia Na⁺ retaining bioassay (Specker et al., 1989). Also, the two PRLs were indistinguishable with regard to their effects on several aspects of tilapia osmoregulation, including the restoration of whole-animal TEP, plasma osmolality, [Na⁺] and [Cl⁻] in hypophysectomized FW fish, as well as Na⁺, K⁺-ATPase activity (Young et al., 1988). In addition, heterologous receptor studies have shown that both PRLs were equipotent in displacing ovine PRL in kidney and gill membranes of the FW tilapia (Dauder et al., 1990). In light of these findings, it appears that the two tilapia PRLs similarly regulate the osmoregulatory processes of the tilapia, although not all aspects of PRL's actions in osmoregulation have been investigated.
It is possible that the existence of two PRL molecules might be advantageous for regulating other physiological processes in the tilapia. The alteration in the proportion of the two tilapia PRLs in response to environmental salinity may reflect a stage of readiness for the preferential modulation of release of one of the PRLs by factors which regulate processes other than osmoregulation, such as reproduction or metabolism.

Two studies have been aimed at addressing whether the two tPRLs might differentially act on reproduction. Thus far the issue remains unclear. Rubin and Specker (1988) found that both molecules exert similar actions, either alone or in combination, on the production of testosterone by the testis of FW tilapia in vitro. Unfortunately their effects were not examined in SW tilapia.

Of considerable interest is the finding by Tan et al. (1988) that only the larger tilapia PRL molecule was effective in stimulating estradiol-17β secretion from oocytes of the guppy, Poecilia reticulata. This suggests that the two PRLs may not be equipotent in this aspect of reproduction. Nevertheless, the critical test of this notion must be undertaken in the tilapia, Oreochromis mossambicus, a species for which we have no evidence at present.
The existence of two PRLs in the tilapia leads one to postulate the evolution of separate and distinct actions. Until now, however, no clear differences in the regulation of secretion and/or metabolism of the two PRLs in the tilapia have been demonstrated. I now have evidence, however, that some component in the processing of the two tilapia PRLs (in their synthesis, secretion, and/or intracellular degradation) is differentially correlated to environmental salinity. This suggests that the pituitary content of the two PRLs of the tilapia is individually regulated. This disparate control at the level of the pituitary argues that the two PRLs of the tilapia may indeed possess distinct but possibly overlapping actions.
CHAPTER III

CORTISOL RAPIDLY REDUCES PROLACTIN RELEASE AND cAMP AND $^{45}\text{Ca}^{2+}$ ACCUMULATION IN THE TILAPIA PITUITARY IN VITRO

INTRODUCTION

The tilapia, *Oreochromis mossambicus*, is a euryhaline cichlid fish that occupies habitats ranging from fresh water to salinities far in excess of full-strength SW. Prolactin and cortisol act in the regulation of the remarkable ability of this fish to adapt to extreme and sometimes rapidly changing salinities.

Prolactin plays a central role in the FW osmoregulation of the tilapia and many euryhaline teleost fishes, acting to reduce both water and ion fluxes (Pickford and Phillips, 1959; Clarke and Bern, 1980; Hirano, 1986). Cortisol, a major corticosteroid in teleosts, including the tilapia, acts in SW osmoregulation by reducing blood osmotic pressure (Foskett et al., 1983).

In the tilapia, PRL-producing cells are segregated into a nearly homogeneous mass, the RPD of the pituitary. The tilapia RPD synthesizes and releases two distinct prolactin molecules (~19.6 kDa and 20.8 kDa; Clarke, 1973;
Specker et al., 1985; Yamaguchi et al., 1988). Both hormones display similar biological activities, though the mechanisms which regulate the smaller PRL have been more thoroughly studied (cf. Specker et al., 1985a).

Prolactin cell activity is enhanced when tilapia are maintained in FW and is reduced in SW (Nagahama et al., 1975). The alteration of PRL cell activity under these conditions appears to result, in part, from the direct actions of osmotic pressure. During in vitro incubation, details of fine structure suggest that PRL cell activity is augmented when medium osmotic pressure is reduced (Nishioka et al., 1988). Similarly, recent studies in our laboratory have shown that small reductions in medium osmotic pressure, well within the physiological range of the tilapia, increase prolactin release within 10-20 min (Grau et al., 1986). The response of the tilapia PRL cell to small changes in osmotic pressure makes the tilapia RPD an especially valuable model for studying the intracellular regulation of less accessible and more complexly structured osmosensitive tissues (Leng et al., 1982).

The requirements of FW and SW osmoregulation clearly differ and are in many ways opposite. The relative importance of PRL in FW osmoregulation and cortisol in SW osmoregulation suggests that PRL cell function might be
altered by cortisol. Indeed, a preliminary in vitro study showed that a single, possibly pharmacological dose of cortisol suppressed PRL release during 18-20 hr static incubations (Wigham et al., 1977). Moreover, the ability of the tilapia PRL cell to react quickly to osmotic stimulation led me to question whether it might also respond rapidly to cortisol.

Here, I present evidence that cortisol exerts a rapid, dose-related inhibition of the release of the smaller of the two tilapia prolactins. This action is accompanied by similarly rapid reductions in the accumulation of cAMP and extracellular $^{45}\text{Ca}^{++}$ and is specific to cortisol and its synthetic agonist dexamethasone.

MATERIALS AND METHODS

Static Incubations

Male tilapia, 15-20 cm long, held for at least 3 weeks in fresh water (25 ± 2°C) were decapitated. Each RPD was dissected and placed in Krebs bicarbonate-Ringer (355 mOsm) containing glucose, glutamine, and Eagle's MEM (Wigham et al., 1977). The osmotic pressure of the incubation medium was adjusted to 300 mOsm (hyposmotic) or
355 mOsm (hyperosmotic) with NaCl. Tissues were incubated on a gyrotory platform (80 rpm) for 18-20 hr at 28 ± 1°C under a humidified atmosphere of 95% O₂/5% CO₂.

At termination, tissues were ultrasonically disrupted and both tissues and incubation media were subjected separately to SDS/PAGE (Kelley et al., 1988; Laemmli, 1970) or polyacrylamide disc gel electrophoresis (Nagahama et al., 1975; Wigham et al., 1977). Coomassie brilliant blue (R-250)-stained PRL bands were quantified densitometrically. Prolactin release was normalized as a percentage of the total PRL measured in the RPD and medium.

All steroids (Sigma) were dissolved in absolute ethanol to 1 mM and diluted in hyposmotic medium to concentrations described in the text. The Ca²⁺ ionophore A23187 (CalBiochem) was solubilized in dimethyl sulfoxide to 5 mM and further diluted to 1 μM in hyposmotic medium containing 50 nM cortisol. N⁶,O²'-Dibutyryl cAMP (dbcAMP; Sigma) and 3-Isobutyl-1-Methyl-Xanthine (IBMX; Sigma) were added directly to the incubation media at 1 mM and 0.1 mM, respectively. Media in control experiments received equal volumes of ethanol or dimethyl sulfoxide.
**Perifusion Incubation**

For perifusion, RPD were preincubated individually under conditions described above for 48 hours in hyperosmotic medium containing \(^3\)H leucine (New England Nuclear) at 6 \(\mu\)Ci/ml (1 \(\mu\)Ci = kBq). The perifusion apparatus has been described previously (Grau et al., 1986). Eighteen RPD containing \(^3\)H-leucine-labeled PRL were transferred to each incubation chamber. Perifusion medium (28 ± 1°C) was identical to the preincubation medium, but without \(^3\)H-leucine. Before each experiment, RPD were perifused for 2-3 hr in hyperosmotic medium until the spontaneous release of \(^3\)H-PRL was stable. Samples of perifusate were collected at 10 min intervals. Prolactin release was quantified by a direct counting method previously described and validated (Grau et al., 1987). We have found that the magnitude of the \(^3\)H PRL response to experimental manipulation during perifusion incubations is proportional to the level of \(^3\)H PRL release that is established in hyperosmotic medium during the period prior to stimulation. For this reason, the average activity of \(^3\)H PRL in the last 3 fractions collected from each perifusion chamber immediately before each experiment was used to normalize \(^3\)H PRL release. This resulted in a considerable reduction in the
variability of the responses observed among replicate perifusion incubations.

$^{45}$Ca$^{2+}$ Accumulation into the La$^{3+}$-Resistant Pool of the RPD

For studies of Ca$^{2+}$ accumulation, six RPD were loaded into each perifusion chamber in hyperosmotic medium for 2 hr. The rate of Ca$^{2+}$ accumulation was characterized using a method previously described and validated (Richman et al., 1990). In brief, RPD were exposed to $^{45}$Ca$^{2+}$ (12 μCi/ml) for 15 min, then rinsed with 2 ml of ice-cold saline (355 mOsm) for 30 sec, and finally, perifused with ice-cold 4.2 mM LaCl$_3$ for 7 min to displace extracellular $^{45}$Ca$^{3+}$. Each RPD was then placed in 250 μl of 1 M NaOH, sonicated, and neutralized with 250 μl of 1 N HCl. $^{45}$Ca$^{2+}$ activity was normalized to tissue protein (bicinchoninic acid protein assay, Pierce Chemical Co.).

cAMP Accumulation in the RPD

Individual tilapia RPD were incubated for 2 hr in 500 μl of hyperosmotic medium. Experimental media (500 μl) were then introduced to RPD for 15 min along with IBMX, which was added to suppress the breakdown of cAMP.
Following the experiment, tissues were fixed with 250 μl of ice-cold 6% trichloroacetic acid in distilled water. Tissues were prepared for radioimmunoassay (RIA) by sonicating in trichloroacetic acid, centrifuging for 3 min (Beckman microcentrifuge B), and extracting the supernatant 4 times with 1 ml of water-saturated ethyl ether. Samples were dried in a 70-80°C water bath under an atmosphere of 99.9% N₂. All samples were acetylated and cAMP levels were determined according to the protocol for the New England Nuclear cAMP RIA kit, previously validated for use with tilapia RPD (Helms et al., 1991). Direct measurements of cAMP were closely correlated with levels normalized to tissue protein (p < 0.001); hence only direct measurements have been reported (Helms et al., 1991).

Plasma Cortisol Determinations

Male tilapia were raised from the period of yolk-sac absorption for 4 months in 700 l oval tanks containing FW or SW. In order to minimize stress-induced elevations in plasma cortisol levels I used the blood sampling method of Young (1986). Tilapia were not approached for at least 12 hours prior to blood sampling. Fish were netted in one sweep and exposed to a 300 mg/liter solution of tricaine
methanesulfonate (Sigma) buffered with sodium bicarbonate. Blood samples from the severed caudal vein were collected in heparinized microcapillary tubes. Plasma was isolated by centrifugation and stored at -80°C. Plasma cortisol concentrations were determined by radioimmunassay according to the method of Young (1986), modified for tilapia.

**Statistical Analysis**

Differences among means were determined using analysis of variance (Crunch Software, San Francisco) followed by the least significant difference test for predetermined comparisons (Steele and Torrie, 1980). Experiments with only two groups were analyzed using the unpaired Student's t test.

**RESULTS**

*Effect of Cortisol and Dexamethasone on PRL Release from the RPD During Static Incubation*

Cortisol produced a dose-related inhibition of PRL release from the tilapia RPD during 18-20 hr of in vitro incubation in hyposmotic medium (Fig. 8A). This effect
was significant for doses ranging from 10 nM (p < 0.05) to 1 μM (p < 0.001), with maximum inhibition occurring at 50 nM (p < 0.001). In an otherwise identical experiment, we wanted to determine whether cortisol's actions are shared by its synthetic agonist, dexamethasone. Dexamethasone was more effective than cortisol, inhibiting PRL release in a dose-related manner starting at 1 nM (p < 0.001; Fig. 8B).

**Time-Course of the Inhibition of PRL Release by Cortisol**

The time required for cortisol to reduce PRL release was investigated. As in previous studies, [3H]PRL release increased within 10-20 min after introduction of hyposmotic medium. This response was rapidly suppressed by 200 nM cortisol (p < 0.001). Release diverged from control levels immediately and was significantly reduced within 20 min (p < 0.05; Fig. 9A). Cortisol was also effective at 50 nM, although to a lesser degree and after a longer delay (50 min; p < 0.05; Fig. 9B).
Figure 8. Effect of cortisol (A) and dexamethasone (B) on PRL release from the tilapia RPD during 18-20 hr of static incubation in hyposmotic medium. Values are expressed as mean ± SEM (for A, n = 12-18 RPDs, except 20 nM dose, n = 6; for B, n = 8 RPDs). Asterisks denote values significantly different from control (C) values (*, p < 0.05; ***, p < 0.001).
Glucocorticoid, nM

% PRL Release

A

B

0 10 20 50 100 1000

C 0.1 1
Figure 9. Effect of cortisol on the release of $[^3\text{H}]$PRL from RPD during perifusion incubation. The last 30 min of the initial 2- to 3-hr perifusion in hyperosmotic medium are shown between −30 and 0 min. Hyposmotic medium was introduced to all chambers immediately following time 0 maintained until the end of the experiment. Cortisol was introduced (arrow) into half of the perifusion chambers either immediately after the 30-min fraction (A) or 50 min fraction (B). The increase in $[^3\text{H}]$PRL release in response to hyposmotic medium was reduced within 20 min by 200 nM cortisol (A) and within 50 min by 50 nM cortisol (B). Closed squares, hyposmotic medium; closed circles, cortisol. Asterisks denote significant differences (*, p < 0.05; **, p < 0.01). Each point represents the mean ± SEM of 5 perifusion chambers with 18 RPDs per chamber.
Ca²⁺ Ionophore A23187 Blocks the Inhibition of PRL Release by Cortisol

During 18-20 hr of static incubation, 50 nM cortisol reduced PRL release in both hyposmotic (p < 0.001) and hyperosmotic (p < 0.05) media (Fig. 10). This inhibition of PRL release was blocked by the Ca²⁺ ionophore A23187 (p < 0.001).

Effect of dbcAMP and IBMX on the Inhibition of PRL Release by Cortisol

Previous studies (Grau et al., 1982) have shown that the addition of the membrane-permeant derivative of cAMP (dbcAMP) and the phosphodiesterase inhibitor IBMX, substances which stimulate the cAMP messenger system, can also increase PRL release from the tilapia RPD. During 18-20 hr of static incubation in hyposmotic medium, 50 nM cortisol significantly reduced PRL release (p < 0.01; Fig. 11). Dibutyryl cAMP and IBMX completely blocked this inhibition (p < 0.01).
Figure 10. Effect of Ca$^{++}$ ionophore A23187 (1 μM) on the inhibition of PRL release by 50 nM cortisol (F) in hyposmotic (300 mosm) and hyperosmotic (355 mosm) media during 18-20 hr of static incubation (mean ± SEM; n = 11-12 RPDs). C, control. Asterisks denote significant differences (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
Figure 11. Effect of dbcAMP (1 mM) and IBMX (0.1 mM) on the inhibition of PRL release by 50 nM cortisol (F) in hyposmotic medium during 18-20 hr of static incubation (mean ± SEM; n = 7 RPDs). Asterisks denote significant differences (**, p < 0.01).
Cortisol Decreases the Accumulation of $^{45}\text{Ca}^{2+}$ into the La$^{3+}$-Resistant Ca$^{++}$ Pool and Reduces the Accumulation of cAMP in the RPD

The following experiments were directed toward determining whether cortisol might alter cellular Ca$^{2+}$ metabolism. To this end, we investigated the possible effects of cortisol on the accumulation of extracellular $^{45}\text{Ca}^{2+}$ into the La$^{3+}$-resistant Ca$^{++}$ pool of the RPD. The RPD tissues were perifused in hyperosmotic medium alone or with 50 nM cortisol. After 15 min, the osmotic pressure of all media were reduced and $^{45}\text{Ca}^{2+}$ radiotracer was added. Cortisol significantly reduced the accumulation of $^{45}\text{Ca}^{2+}$ into the RPD in 15 min ($p < 0.01$; Table 1). The rapid actions of cortisol on PRL release and on $^{45}\text{Ca}^{2+}$ accumulation suggested that it might also act on cAMP metabolism. With IBMX added to suppress phosphodiesterase activity, exposure of RPD to reduced osmotic pressure for 15 min significantly increased cAMP accumulation in the RPD over levels in hyperosmotic medium ($p < 0.001$, data not shown). This increase was substantially reduced by 50 nM cortisol ($p < 0.01$; Table 1).
Table 1

Effects of cortisol on cAMP and $^{45}$Ca$^{2+}$ accumulation in RPDs incubated for 15 min

<table>
<thead>
<tr>
<th></th>
<th>cAMP, pmol/RPD</th>
<th>$^{45}$Ca$^{2+}$, dpm/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$0.17 \pm 0.01$ (5)</td>
<td>$20.36 \pm 1.30$ (6)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>$0.12 \pm 0.01***$ (5)</td>
<td>$14.05 \pm 1.42**$ (6)</td>
</tr>
</tbody>
</table>

RPDs were incubated in hyposmotic (300 mosm) media in the absence or presence of cortisol (50 nM). Values represent mean ± SEM with the number of RPDs in parentheses. Cyclic AMP and $^{45}$Ca$^{2+}$ accumulation data were obtained from separate experiments. For cAMP, incubation mixtures contained 0.1 mM IBMX. $^{45}$Ca$^{2+}$ data represent accumulation into the La$^{3+}$-resistant Ca$^{2+}$ pool. Double asterisks denote $p < 0.01$ for differences from control.
Specificity

It was of interest to determine whether the inhibition of PRL release by cortisol was specific to this SW-adapting hormone or whether the inhibitory response might be a general steroid hormone effect. Cortisol (50 nM) significantly reduced PRL release from the RPD during 18-20 hr of static incubation in hyposmotic medium (p < 0.001; p < 0.05; Table 2). In contrast, estradiol-17β and testosterone stimulated PRL release, while cholesterol, 17α,20β-dihydroxy-4-pregnen-3-one, progesterone, aldosterone, and 11-deoxycorticosterone were without effect (50 nM; p < 0.05; Table 2).

Plasma Cortisol Concentrations in FW and SW Tilapia

To be sure that the concentrations of cortisol used in my in vitro studies are physiological I measured circulating cortisol levels in FW and SW tilapia. The concentration of cortisol in the plasma of SW tilapia (154 ± 23.7 nM) was significantly higher than levels measured in the plasma of FW tilapia (63 ± 9.9 nM; p < 0.01; Fig. 12).
Table 2

Effects of various steroids on PRL release during 18-20 hr incubations in hyposmotic media (300 mOsmolal)

<table>
<thead>
<tr>
<th>% PRL Release</th>
</tr>
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<tbody>
<tr>
<td>Experiment 1</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Cortisol</td>
</tr>
<tr>
<td>Progesterone</td>
</tr>
<tr>
<td>17α,20β-Dihydroxy-4-pregnen-3-one</td>
</tr>
<tr>
<td>Aldosterone</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
</tr>
</tbody>
</table>

| Experiment 2  |
| Control       | 49.95 ± 1.85 (7) |
| Cortisol      | 26.26 ± 1.96*** (7) |
| Cholesterol   | 53.66 ± 3.84 (4) |
| Testosterone  | 64.69 ± 5.85* (7) |
| Estradiol-17β | 70.15 ± 4.47** (7) |

RPD were incubated in media in the absence or presence of steroids (50 nM). Values represent mean ± SEM with the number of RPDs in parentheses. Asterisks indicate significant differences from control (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
Figure 12. Cortisol concentrations from the plasma of tilapia reared in fresh water (FW) and seawater (SW) for 4 months (mean ± SEM; n = 4).
DISCUSSION

This work shows that physiological concentrations of cortisol rapidly inhibit the response of the tilapia PRL cell to reduced osmotic pressure. Prolactin release declined immediately from control levels after the introduction of cortisol, becoming significantly reduced within 20 min. This inhibition was accompanied by reductions in tissue cAMP levels and in \(^{45}\text{Ca}^{2+}\) accumulation, actions that are compatible with a mediating role for these two messenger systems. Our results demonstrate that a naturally occurring glucocorticoid, cortisol, rapidly suppress the activity of a previously stimulated endocrine tissue. They also show that a steroid hormone rapidly reduces the activity of an osmosensitive tissue, the tilapia RPD.

Comparably rapid effects of other steroid hormones have been reported (Dufy et al., 1979; Duval et al., 1983; Hua and Chen, 1989). Indeed, the stimulation of both PRL release and cAMP production by vasoactive intestinal peptide (VIP) from dissociated rat pituitary cells was rapidly inhibited by the synthetic glucocorticoid agonist, dexamethasone. This effect required exposure to dexamethasone prior to the introduction of VIP, but
pretreatment for as little as 15 min was effective (Rotsztejn et al., 1981). These findings are not directly comparable to those reported here because of the reversed order of stimulation and inhibition. Nevertheless, considered together, both investigations suggest that glucocorticoids may have a variety of rapid actions on the PRL cells of at least two vertebrate species.

Considerable effort has been directed toward the characterization of steroid hormone action. Glucocorticoid actions can be observed in many tissues only after lag periods ranging from several hours to days (Leung and Munck, 1975; Durant et al., 1986). This fits well with the notion that steroid hormones act primarily by altering gene expression (Gorski and Gannon, 1976; Katzenellenbogen, 1980). Nevertheless, steroid hormones can also have rapid genomic and membrane-associated (nongenomic) actions (Ringold et al., 1977; Dufy et al., 1979; Duval et al., 1983; Ucker and Yamamoto, 1984; Hua and Chen, 1989; Orchinik et al., 1991). For example, dexamethasone stimulates transcription of the mouse mammary tumor virus within 10 min (Ringold et al., 1977; Ucker and Yamamoto, 1984). While it appears that in most cases steroid hormones act by regulating gene expression, sometimes very rapidly, the rapid effects observed here (see also refs. Dufy et al., 1979; Duval et al., 1983; Hua
and Chen, 1989; Orchinik et al., 1991) are accompanied by changes in Ca\(^{2+}\) and cAMP metabolism that are unlikely to be mediated through transcription, a possibility, however, that is not precluded by the results of the present investigation.

To my knowledge, the modification of Ca\(^{2+}\) metabolism by cortisol, or any corticosteroid, has not been previously reported. This action and the inhibition of cAMP metabolism provide support to the notion that cortisol may act on the tilapia PRL cell, in part, through mechanisms that are similar to those characterized for peptide hormones.

An increase in intracellular free Ca\(^{2+}\) is thought to be a central event in the initiation of endocrine secretion. This rise in Ca\(^{2+}\) can come from both intracellular and extracellular sources. The idea that cortisol may act through effects on intracellular Ca\(^{2+}\) finds support in our observation that its suppression of PRL release is blocked by A23187, which increases intracellular Ca\(^{2+}\). The inward movement of extracellular Ca\(^{2+}\) is, in turn, thought to be dependent upon the opening of membrane channels operated either by membrane receptors or by changes in membrane potential. Inhibitors of hormone release may reduce the probability that Ca\(^{2+}\)
channels will open (Rubin, 1982; Rasmussen and Barret, 1984).

Richman et al. (1990) found that changes in the movement of $^{45}$Ca$^{2+}$ into the La$^{3+}$-resistant (presumed intracellular) pool of the tilapia RPD were closely correlated to changes in PRL release. Thus, exposure to hyposmotic medium or medium with depolarizing K$^+$ concentration increased the accumulation of $^{45}$Ca$^{2+}$ in the La$^{3+}$-resistant pool along with PRL release. In the present study, I found that $^{45}$Ca$^{2+}$ entry into the La$^{3+}$-resistant pool was significantly reduced by exposure to cortisol for 15 min. The suppression by cortisol of $^{45}$Ca$^{2+}$ accumulation into the tilapia RPD in 15 min is less than the time required for cortisol to reduce PRL release (within 20 min). This suggests that cortisol might reduce PRL release by reducing the influx of extracellular Ca$^{2+}$ through plasma membrane-associated Ca$^{2+}$ channels. This idea is supported by the observation that cortisol can rapidly hyperpolarize (within 2 min) guinea pig neurons (Hua and Chen, 1989). This response was abolished by the glucocorticoid receptor antagonist RU38486 and appeared to be mediated through binding sites on synaptic plasma membranes. Other steroid hormones, including estrogen and glucocorticoids, have also been shown to bind to receptor or receptor-like plasma membrane components of pituitaries.
and other types of tissues (Pietras and Szego, 1977; Koch et al., 1978; Towle and Sze, 1983; Orchinik et al., 1991).

The cAMP messenger system, like Ca²⁺, is widely implicated in the mediation of hormone action (Levitski, 1986). In our study, cAMP in the RPD was reduced by cortisol within 15 min, an interval that is comparable to that over which cortisol reduces PRL release. Since phosphodiesterase activity was suppressed by IBMX, it would appear that cortisol may reduce cAMP by reducing adenylate cyclase activity. Similar observations and conclusions have been obtained from studies on the induction by progesterone of meiotic division in Xenopus oocytes (Schorderet-Slatkine et al., 1978; Sadler and Maller, 1985).

Several lines of investigation strongly suggest that the rapid suppression by cortisol of PRL release from the tilapia RPD is glucocorticoid-specific. First, under my culture conditions, cortisol and dexamethasone were the only steroids tested that significantly reduced PRL release (Table 2). Even aldosterone, which possesses some glucocorticoid activity, lowers PRL release, but not significantly under control levels (Table 2). These data are in agreement with a previous study that showed no effect of aldosterone on VIP-induced PRL release in rats (Rotsztejn et al., 1981).
Cortisol appears to be an important extrahypothalamic regulator of PRL secretion in mammals and fishes. Cortisol suppresses PRL release from GH3 cells in vitro, an effect that was related to dose (Tashjian et al., 1977; Melmed, 1984; Pragar et al., 1988). Likewise, PRL release was inhibited in vitro by high doses of cortisol in two teleost fishes, including the tilapia and coho salmon (Wigham et al., 1977; Kelley et al., 1990).

I have shown that cortisol directly and rapidly alters PRL release from the osmosensitive tilapia RPD. The idea that cortisol, a SW-adapting hormone in the tilapia and other teleost fish, may be an important inhibitor of PRL release has special appeal since PRL has been shown repeatedly to play a central role in FW osmoregulation (Pickford and Phillips, 1959; Clarke and Bern, 1980; Hirano, 1986). In my studies, cortisol was effective in vitro in reducing PRL release at concentrations in the range we have measured in the plasma of FW tilapia (40-100 nM) and SW-reared tilapia (100-200 nM; Fig. 12).

In nature, tilapia are often confronted with rapid and sometimes unpredictable changes in environmental salinity. If transient increases in cortisol occur in tilapia, as they do in the eel during SW adaptation (Hirano, 1980; Nishioka et al., 1985), it would not be
surprising that cortisol may serve the dual role of rapidly reducing the secretion of a FW osmoregulatory hormone, PRL (in concert with elevated osmotic pressure), while simultaneously modifying the activity of osmoregulatory tissues.

In conclusion, cortisol acts rapidly on the tilapia RPD in vitro to reduce PRL secretion, possibly through actions on the cAMP and/or Ca\(^{2+}\) second-messenger systems. The rapidity with which cortisol reduces PRL release invites further detailed investigations of the mechanisms mediating the actions of cortisol.
CHAPTER IV

EFFECT OF OSMOTIC PRESSURE ON CHANGES IN INTRACELLULAR FREE Ca\(^{2+}\) CONCENTRATION IN SINGLE DISPERSED PROLACTIN CELLS FROM THE TILAPIA, OREOCHROMIS MOSSAMBICUS.

INTRODUCTION

Control of PRL

Prolactin release in teleosts fishes and in mammals is thought to be regulated through the interplay of several factors (for review see Lambert et al., 1990; Nishioka et al., 1988). In the euryhaline teleost, tilapia, Oreochromis mossambicus, PRL appears to be chronically inhibited by several hypothalamic factors (Nishioka et al., 1988). One such neuropeptide, SRIF, rapidly reduces PRL release from the RPD during perifusion incubations (< 10 min) and does so in a dose-related manner at least during long-term static incubations (18-20 hr; Grau et al., 1982; Rivas et al., 1986).

In addition to hypothalamic control, evidence suggests that PRL cell function in the tilapia may also be directly regulated by extracellular osmotic pressure. Consistent with PRL's role in FW osmoregulation, small
decreases in osmotic pressure, well within the physiological range of the tilapia, stimulate PRL cell activity in vitro (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981). Likewise, perifusion incubations have shown that reductions in medium osmotic pressure rapidly augment PRL release from the tilapia RPD (Grau et al., 1987). Conversely, elevations in medium osmotic pressure, which reflect the blood osmotic pressure of fish adapting to SW, inhibit PRL release.

**PRL Cell Osmosensitivity**

The ability of tilapia PRL cells to respond directly to small physiological changes in osmotic pressure of the surrounding milieu provides an excellent model for studying the mechanisms mediating PRL cell function (Grau and Helms, 1989). The unique, nearly homogeneous arrangement of PRL cells (95-99% PRL cells) in the RPD of tilapia allows easy isolation and in vitro incubation in defined medium. Because of the osmosensitivity of the tilapia PRL cell, baseline PRL release can be set high or low in order to study potentially important regulators of PRL secretion in the tilapia. Moreover, the tilapia PRL cell has proved to be a useful model for understanding the
intracellular mechanisms which transduce extracellular signals into a secretory response.

Role of Ca$^{2+}$ in PRL Release

My interests have focused principally on the intracellular mechanisms involved in mediating PRL release in the tilapia. Calcium is a mediator of several cellular events including stimulus-secretion coupling in a variety of endocrine cell types (Alberts et al., 1983). Evidence suggests that an appropriate stimulus evokes an elevation in intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) which is thought to arise from both the release of Ca$^{2+}$ from intracellular sources and from the influx of extracellular Ca$^{2+}$. The influx of extracellular Ca$^{2+}$ occurs through channels that are stretch-activated or are responsive to receptor-ligand interactions or changes in transmembranal voltage. (cf. Rubin, 1982; Rasmussen and Barrett, 1984; Rasmussen et al., 1984; Berridge, 1985; Stoliojak et al, 1992). Prolactin secretion in teleost fishes appears to be transduced, in part, through the second messenger, Ca$^{2+}$ (Grau et al., 1982; Grau and Helms, 1989; Taraskevish and Douglas, 1978; MacDonald and KcKeown, 1985). In the tilapia, PRL release from the RPD, in vitro, is blocked when extracellular Ca$^{2+}$ is deleted.
from the incubation medium or when Co$^{2+}$, an antagonist of Ca$^{2+}$-mediated processes, is added to the incubation medium (Grau et al. 1981, 1982, 1986; Helms, 1988, Richman et al., 1991). Moreover, Richman et al. (1987) showed that reductions in medium osmotic pressure augment the accumulation of extracellular $^{45}$Ca$^{2+}$ into the tilapia RPD within 15 min, a period which correlates with the time over which reduced osmotic pressure elevates PRL release (Grau et al., 1986). These findings suggest indirectly that the stimulatory effects of reduced osmotic pressure on PRL release may be mediated, in part, through increases in the influx of extracellular Ca$^{2+}$. Prolactin release is elevated during exposure to the Ca$^{2+}$ ionophore, A23187, which effectively increases cytosolic Ca$^{2+}$ (Grau et al., 1982; Helms, 1988; Borski et al., 1991). Taken together, these isotopic and pharmacological studies suggest that PRL release from the tilapia RPD is associated with changes in $[\text{Ca}^{2+}]_i$ which may be derived from extracellular and/or intracellular Ca$^{2+}$ pools. To date, however, there is no direct evidence that the stimulatory effects of reduced osmotic pressure or other factors (e.g., thyrotropin-releasing hormone) on PRL release in any teleosts are mediated by increases in $[\text{Ca}^{2+}]_i$.

The present studies were undertaken to determine whether reductions in medium osmotic pressure alter
[Ca\(^{2+}\)]_i over a time-course which is consistent with that over which it stimulates PRL release in the tilapia (10-20 min). I describe methods for the viable incubation of dispersed PRL cells from the tilapia RPD and for measuring changes in [Ca\(^{2+}\)]_i in single, PRL cells employing microspectrofluorometric techniques in combination with the Ca\(^{2+}\)-sensitive probe, fura-2.

MATERIALS AND METHODS

Materials

Male tilapia, 10-20 cm long, were obtained locally from brackish water and maintained in FW tanks (25 ± 2°C) for at least 3 weeks prior to experiments at the Hawaii Institute of Marine Biology on Coconut Island, Oahu, Hawaii. Fish were fed twice daily with Purina trout chow. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Somatostatin was dissolved directly in incubation medium to a concentration of 300 nM.
**Cell Dispersion.**

Tilapia were decapitated, and their pituitaries were removed and collected. The RPD were dissected from the pituitaries, placed singly in 0.5 ml phosphate buffer saline (PBS; 0.5 M NaH$_2$PO$_4$, Na$_2$HPO$_4$; pH = 7.25; 355 mOsm) containing 0.25% porcine trypsin 1-300 (US Biochem., Cleveland, OH). After 45 min of exposure to trypsin at 28 ± 1°C, the PRL cells were dispersed by 5-10 gentle passages through a 1 ml pipette. The cells were centrifuged (250 X g, 5 min, 25 ± 1°C), the supernatant was decanted, and the cells were resuspended and triturated in 1 ml of PBS containing no trypsin. This rinse period was repeated 3 times and the cells were resuspended in a final solution of Kreb's bicarbonate buffer (see below; 355 mOsm). Following dispersion of RPD, PRL cell viability was assessed by the trypan blue (0.2%) exclusion test. The PRL cells were plated out onto a glass cover slip coated with 0.1 mg/ml of the attachment factor poly(L-lysine) (cf. Cooke et al., 1989; Letourneau, 1975). Cells were then incubated in modified Kreb's bicarbonate Ringer (355 mOsm; see below) for at least 12 hr prior to determinations of [Ca$^{2+}$]$_i$. 
Incubations

Dispersed PRL cells and RPD were incubated in a modified Kreb's bicarbonate ringer prepared according to Wigham et al. (1977) and consisting of (in meq/liter); CaCl₂, 4.2; MgSO₄, 2.8; KCl, 2.35; KH₂PO₄, 1.25; NaHCO₃, 25; glucose, 500 mg/liter; L-glutamine, 290 mg/liter, and Eagle's minimal essential medium without L-glutamine, 20 ml/liter 50X (GIBCO, Grand Island, NY). Medium was gassed with 95% O₂/5% CO₂ for 10 min (pH = 7.25). The osmotic pressure of the incubation medium was adjusted with a Wescor vapor osmometer (Wescor, Logan, UT) to 355 mOsm (hyperosmotic) or 300 mOsm (hyposmotic) using NaCl. Dispersed cells (equivalent of 1 RPD/well) and individual RPD were placed separately in Falcon 24-well plates containing 300 µl of hyperosmotic medium. The dishes containing RPD and dispersed PRL cells were incubated for 12 hr under a humidified atmosphere of 95% O₂/CO₂ on a gyratory platform (80 rpm; 28 ± 1°C). Following this preincubation period medium was replaced with either hyperosmotic or hyposmotic medium with or without somatostatin. All tissues and dispersed cells were subsequently incubated for 18 hr under a humidified atmosphere of 95% O₂/5% CO₂ on a gyratory platform (80 rpm; 28 ± 1°C). At termination, medium and tissue or cells were
ultrasonically disrupted (Heat Systems-Ultrasonics, Model W-370, Plainview, NY). Tissues or cells and incubation media were subjected separately to polyacrylamide disc gel electrophoresis as previously described (Wigham et al., 1977). According to methods described by Wigham et al. (1977) and validated by Nagahama et al. (1975) prolactin was quantified and validated by densitometric analysis using a Beckman DU-7 spectrophotometer (Beckman Instruments, Irvine, CA). Prolactin release was calculated as a percentage of hormone released into the culture media divided by the total hormone in the incubations (media + tissue).

Monitoring \([Ca^{2+}]_i\) by Dual Microspectrofluorometry with Fura-2

Prolactin cells, plated on poly-L-lysine coated cover slips and incubated in hyperosmotic medium, were loaded with 10 µM of the membrane permeable acetoxymethyl ester derivative of fura-2, fura-2/AM (Molecular Probes, Eugene, OR), for 90 min at 28 ± 1°C. The fura-2/AM was solubilized in anhydrous dimethyl sulfoxide (DMSO; Aldrich Chemical, Milwaukee, WI) to a concentration of 10 mM prior to its final dilution to 10 µM (< 0.10% DMSO v/v). During this loading period, fura-2/AM is cleaved within
the PRL cells by endogenous esterases to its Ca\textsuperscript{2+}-sensitive membrane impermeable form, fura-2. The cover slips were then mounted onto a perifusion chamber. This stainless steel chamber is a wafer with an oval slot machined in the middle. Two 22-gauge ports at opposing ends of the slot provide the input and output paths for the perifusate (Negulescu and Machen, 1990). A round 22 mm coverslip is attached in the recess on one side (i.e. top) of the perifusion chamber by vacuum grease. On the bottom side of the wafer, a coverslip with the plated PRL cells is attached using vacuum grease, such that the cells face the perifusate. The chamber is placed on a microscope stage and the PRL cells perifused with hyperosmotic medium for at least 30 min to allow the fura-2/AM to further deesterify (Gryniewcz et al., 1985; Lewis et al., 1988).

Different experimental media were maintained at 28 ± 1°C in hanging 60 ml plastic syringes connected to an eight-point manifold perifusate selector (Hamilton Co., Reno, NV) via one-way stop cocks and polyethylene tubing. The manifold output is connected to the input port of the perifusion chamber by another piece of polyethylene tubing. The rate of perifusion through the chamber was maintained at 360 μl/min by keeping the height of the
syringes and volume of all solutions in the syringes constant throughout the experiment.

Single cell fura-2 ratio measurements were made with a dual excitation spectrofluorometer (ARCM-MIC-N, Spex Industries, Edison, NJ) interfaced with a Diaphot-TMD inverted microscope (CF 40 X oil immersion fluorite objective, Nikon). The microscope was equipped with fluorescence optics, a 50 watt halogen illuminator, epifluorescence illumination, and a quartz nosepiece (for UV). Excitation light alternated between 340 and 380 nm (narrow bandpass filters, SPEX) by a computer-controlled chopper mirror. Fluorescent emission intensity was transduced every 2 sec by a photomultiplier tube focused on a single PRL cell after it had passed through a 500 nm emission filter. A pinhole (1 mm) placed in the epiillumination path restricted the UV illumination to only the cell of interest.

All data are 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 by 380 nm (free fura-2) from which background (autofluorescence) was subtracted. Shifts in this ratio (340/380) result directly from changes in [Ca^{2+}]_{i} which are independent of dye concentration, cell thickness, and absolute optical
efficiency of the instrument (Tsien et al., 1985; Grynkiewicz et al., 1985; cf. Poenie et al., 1986).

Linearity between the relative intensity of the fura-2 fluorescence and [Ca$^{2+}$] in my set-up was examined in a cell-free system in which fura-2 was added directly to hyperosmotic medium containing different [Ca$^{2+}$] (Fig. 14).

**Statistical Analysis.**

Statistical analyses were performed using PC-SAS (SAS Institute, Cary, NC). Prior to analysis, raw data were log$_{10}$ or log$_e$ transformed to decrease heteroscedasticity. Measurements of intracellular changes in Ca$^{2+}$ were analyzed in 10 sec increments by repeated measures ANOVA (PC-SAS; Huynh and Feldt, 1970). Regression analysis using the least-squares method was utilized to determine whether alterations in [Ca$^{2+}$] are linearly correlated with changes in relative fluorescent intensity (340/380)(Steele and Torrie, 1980).
RESULTS

Isolation of Single PRL Cells by Dissociation of RPD

Exposure to reduced osmotic pressure augmented PRL release from single PRL cells and from the RPD (Fig. 13). Likewise, during exposure to hyposmotic medium, SRIF, a potent inhibitor of PRL release in tilapia (Grau et al., 1982; Rivas et al., 1986), dramatically reduced release of PRL from dissociated PRL cells in a similar manner to that from intact tissue (Fig. 13).

Effect of [Ca$^{2+}$] on the Relative Intensity (340/380) of Fura-2 Fluorescence

The linearity of the relation between variations in [Ca$^{2+}$] and shifts in fura-2 fluorescence (e.g., [Ca$^{2+}$]$_i$) was confirmed in my set-up by measuring the relative fluorescence of fura-2 in media containing different [Ca$^{2+}$]. Increasing log$_e$[Ca$^{2+}$] ranging from 1-100 μM correlated linearly with increases in the fluorescence of fura-2 ($R^2$=0.99, Fig. 14).
Figure 13. Effect of hyperosmotic (355 mOsm), hyposmotic (300 mOsm), and hyposmotic medium containing 300 nM somatostatin (300/SRIF) on PRL release from dispersed PRL cells and from the rostral pars distalis (RPD; tissue). Static incubations were 18-20 hr. (mean ± SEM; n = 9 RPD).
\[ f(x) = 4.19 \cdot \ln(x) + 5.67 \]

\[ R^2 = 0.99 \]
Figure 14. Effect of Ca$^{2+}$ concentrations (expressed as log$_e$) on the relative intensity of fura-2 fluorescence expressed as the ratio of fura-2 bound to Ca$^{2+}$ to free fura-2 (340/380).
Effects of Hyposmolarity and Hyperosmolarity on $[Ca^{2+}]_i$ in Single PRL Cells

Previous studies have shown that reductions in medium osmotic pressure rapidly stimulate PRL release from the tilapia RPD within 10-20 min (Grau et al., 1987). In order to determine whether hyposmotic pressure alters $[Ca^{2+}]_i$ in PRL cells within a similar time course over which it stimulates PRL release we continuously measured changes in $[Ca^{2+}]_i$ in dispersed PRL cells during exposure to hyposmotic medium. These measurements show two distinct types of PRL cells: silent and spontaneously active (Fig. 15). During exposure to hyperosmotic medium there is approximately a 2-fold change between minimum and maximum $[Ca^{2+}]_i$ for each oscillation in the active cell. These fluctuations occur repeatedly every 45-60 sec (Fig. 15B). During exposure to hyperosmotic medium the silent cells showed relatively constant levels of $[Ca^{2+}]_i$ (Fig. 15A). Of the cells we have measured, 75% were silent and 25% were spontaneously active. Fura-2 measurements of relative changes in $[Ca^{2+}]_i$ in both types of cell reveal that $[Ca^{2+}]_i$ rapidly rises during exposure to hyposmotic medium (Figs. 15, 16). In the silent PRL cell, exposure to hyposmotic medium causes a rapid increase in $[Ca^{2+}]_i$. 

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Figure 15. Effect of hyposmotic medium on the relative intensity of fura-2 fluorescence (340/380) in a typical silent (A) and spontaneously active (B) PRL cell during perifusion. Prolactin cells were perifused with hyperosmotic medium (355 mOsm) and then were exposed to hyposmotic medium (300 mOsm). Increases in the relative intensity of fura-2 fluorescence (340/380) indicate increases in $[\text{Ca}^{2+}]_i$. 
Figure 16. Effect of hyposmotic medium on \([\text{Ca}^{2+}]_i\) expressed as the relative intensity of fura-2 fluorescence (340/380, log transformed). The last minute of the initial 5-10 min perifusion in hyperosmotic medium (355 mOsm) are shown between -60 and -10 sec. Hyposmotic medium (300 mOsm; closed squares) or hyperosmotic medium (open circles) was introduced to half of the cells immediately after the -10 sec point marked by the arrow. Asterisks denote differences between effects of hyposmotic and hyperosmotic medium (**, p < 0.01). Each point represents the mean ± SEM of 9 cells.
that peaks to almost 2 X over levels observed during incubations in hyperosmotic medium (Figs. 15A, 17A, 18). Upon exposure of spontaneously active cells to hyposmotic medium, the amplitudes of the oscillations in \([\text{Ca}^{2+}]_i\) seen during exposure to hyperosmotic medium are greatly reduced (Fig. 15A). Compared with exposure to hyperosmotic medium, however, the average \([\text{Ca}^{2+}]_i\) is higher after exposure of active cells to hyposmotic medium. On average, the rise in \([\text{Ca}^{2+}]_i\) induced by hyposmotic medium in both types of PRL cells becomes statistically significant within 30 sec (Fig. 16).

The rise in \([\text{Ca}^{2+}]_i\) observed in PRL cells exposed to hyposmotic medium can last up to at least 20 min (Fig. 17). This elevation in \([\text{Ca}^{2+}]_i\) seen during exposure of PRL cells to hyposmotic medium can be reversed by elevating the osmotic pressure of the incubation medium (Fig. 17). Exposure of PRL cells to hyperosmotic medium significantly reduced \([\text{Ca}^{2+}]_i\), within 10 sec, from levels observed in hyposmotic medium (Figs. 17, 18). These changes in \([\text{Ca}^{2+}]_i\) in PRL cells exposed to alterations in osmotic pressure can be replicated in a single recording of a cell (Fig. 18).
Figure 17. Effect of hyperosmotic medium on the relative intensity of fura-2 fluorescence (340/380; log transformed) in single PRL cells. Graph A shows a typical response of a PRL cell to hyperosmotic (355 mOsm) and hyposmotic medium (300 mOsm). Two cells were initially exposed to hyperosmotic medium and then to hyposmotic medium. Then one cell was exposed to hyperosmotic medium (A, open arrow) and the other to hyposmotic medium (A, closed arrow). Decreases in the fura-2 fluorescence (340/380) indicate decreases in [Ca$^{2+}$]$_{i}$. Graph B shows the average response to hyperosmotic medium of 9 separate measurements of single cells. The last minute of the initial 5-10 min perifusion in hyposmotic medium are shown between -60 and -10 sec. Hyperosmotic medium (open circles) or hyposmotic medium (closed squares) was introduced to half of the cells immediately after the -10 sec point marked by the arrow. Asterisks denote differences between effects of hyposmotic and hyperosmotic medium (** ,p < 0.01). Each point represents the mean ± SEM of 9 cells.
Figure 18. Effect of depleting Ca$^{2+}$ from the medium on the hyposmolar-induced increase in relative intensity of fura-2 fluorescence (340/380) in a typical cell. The cell was initially perfused with hyperosmotic (355 mOsm) medium and then was introduced to hyposmotic (300 mOsm), then to hyperosmotic, then to hyposmotic, and finally to hyposmotic medium absent of Ca$^{2+}$ and containing 10 mM EGTA (arrow).
Effects of the absence of extracellular Ca\textsuperscript{2+} on [Ca\textsuperscript{2+}]_i

In order to determine whether the sustained hyposmolar-induced elevation in [Ca\textsuperscript{2+}]_i might be dependent on extracellular Ca\textsuperscript{2+} we measured changes in [Ca\textsuperscript{2+}]_i in PRL cells first exposed to normal hyposmotic medium for at least 5 min and then to Ca\textsuperscript{2+}-depleted medium.

Extracellular calcium was depleted by omitting CaCl\textsubscript{2} from the incubation medium and by adding the Ca\textsuperscript{2+}-chelating agent, EGTA (10 mM). Exposure of PRL cells to Ca\textsuperscript{2+}-depleted hyposmotic medium resulted in a dramatic and rapid decline in [Ca\textsuperscript{2+}]_i compared with levels observed in cells incubated in normal hyposmotic medium (Fig. 18).

DISCUSSION

This is the first report of direct measurements of changes in intracellular free Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]_i) in osmosensitive endocrine cells of known osmoregulatory output. Small reductions in medium osmotic pressure, well within the physiological range of the tilapia, increased [Ca\textsuperscript{2+}]_i in single PRL cells (Hwang, 1989; Gardner, 1989). This rise commenced immediately (< 10 sec) and reached statistical significance within 30
sec (Figs. 15, 16). These $[\text{Ca}^{2+}]_i$ levels remained elevated for as long as the cells were exposed to hyposmotic medium (30 min; data not shown), but promptly returned to prestimulated levels once medium osmotic pressure was increased (Figs. 17, 18). These changes reproduced repeatedly during alternate, 5-10 min exposures to hyposmotic and hyperosmotic (see Fig. 18).

The rise in $[\text{Ca}^{2+}]_i$ which I observed in PRL cells exposed to hyposmotic medium occurs well within the time resolution with which others have been able to measure changes in PRL release (10-20 min) from the tilapia RPD after exposure to hyposmotic medium (Grau et al., 1987). It is likely, then, that the elevation in $[\text{Ca}^{2+}]_i$ reported here (< 30 sec) occurs prior to the induction of PRL release by reduced osmotic pressure. This order of events suggests that a rise in $[\text{Ca}^{2+}]_i$ in the tilapia PRL cell couples the osmotic stimulus to elevated secretion in the same way in which it acts in mediating receptor-ligand interactions in neurons, and other endocrine and neuroendocrine cells (cf. Rubin, 1982; Rasmussen and Barrett, 1984; Rasmussen et al., 1984, Berridge, 1985).

A problem inherent in physiological studies of disaggregated tissue is the potential for cell damage during enzymatic dissociation. This may lead to uncertainty
about the extent to which the results obtained from isolated cells actually represent their behavior in vivo.

Several lines of evidence indicate that the dispersed PRL cells used in these studies behave like the aggregated PRL cells of the RPD. First, the trypan blue exclusion test indicated that the viability of individual disaggregated cells exceeded 90% of the population. Second, these dispersed PRL cells respond in the same manner as the intact RPD to osmotic pressure and SRIF (Fig. 13, Grau et al., 1981; Grau et al., 1982; Rivas et al., 1986). This similarity suggests that receptors and other membrane components that mediate the regulation of cell output remain functional. Finally, our laboratory has successfully characterised the Na⁺ and K⁺ channels of these tilapia PRL cells using patch-clamp technology. These studies showed these channels to be voltage-gated and sensitive to tetrodotoxin and tetraethylammonium, respectively (Helms, et al., in preparation). Taken together, these three approaches have furnished strong evidence that the method used here produces PRL-secreting cells that are functionally equivalent to those of the intact RPD.

By now, it is clear that a stimulus-invoked rise in [Ca²⁺]ᵢ can develop from two sources, the release of Ca²⁺ from intracellular Ca²⁺-sequestering pools or the influx
of extracellular Ca\textsuperscript{2+} (Gershengorn, 1986). Depending on the cell and circumstance, these two sources may operate alone or in sequence. Typically, the release of Ca\textsuperscript{2+} from intracellular stores is believed to initiate the events leading to hormone secretion, while the influx of extracellular Ca\textsuperscript{2+} extends the response (e.g., PRL release). Calcium\textsuperscript{2+} entry occurs through channels that are governed by voltage-, receptor-, stretch-, and/or messenger-operated channels.

Earlier findings have pointed to an important role for extracellular Ca\textsuperscript{2+} in mediating the stimulatory actions of osmotic pressure on PRL release from the tilapia RPD. First, the depletion of Ca\textsuperscript{2+} from the incubation medium blocks prolactin release both chronically and acutely (Grau et al., 1981; 1986; Richman et al., 1991). Here, I have shown that the depletion of medium Ca\textsuperscript{2+} blocked the hyposmolar-induced sustained rise in [Ca\textsuperscript{2+}].

Earlier studies have also shown that PRL release is suppressed by the divalent antagonist of Ca\textsuperscript{2+}-mediated processes, Co\textsuperscript{2+} (Grau et al., 1986; Richman et al., 1991). Conversely, forced increases in intracellular Ca\textsuperscript{2+}, induced by exposure to the Ca\textsuperscript{2+} ionophore, A23187, provoked a large and rapid increase in PRL release (Grau et al., 1982; Helms, 1988).
The participation of extracellular Ca\textsuperscript{++} in augmenting intracellular Ca\textsuperscript{2+} during the osmotic response of the tilapia PRL cell was indicated when the accumulation of extracellular \(^{45}\)Ca\textsuperscript{2+} into the La\textsuperscript{3+}-resistant (presumed intracellular) pool of the tilapia RPD was observed to increase strongly after medium osmotic pressure was reduced (Richman et al., 1990). The present findings confirm this conclusion and establish the rise in intracellular Ca\textsuperscript{++} as an early event in the response of the tilapia PRL cell to osmotic pressure.

My spectrophotometric recordings of changes in [Ca\textsuperscript{2+}]\textsubscript{i} indicate that there are at least two types of tilapia PRL cells, those that show relatively constant [Ca\textsuperscript{2+}]\textsubscript{i} levels in hyperosmotic medium. I will refer to these as silent. Other PRL cells show spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (e.g., Fig. 15). Spontaneous and agonist-induced fluctuations in [Ca\textsuperscript{2+}]\textsubscript{i} have been reported in a variety of mammalian "normal" pituitary cell types as well as in neoplastic cell lines (for review see Stojilkovic and Catt, 1992). These include but are not restricted to rat PRL cells and PRL-secreting GH\textsubscript{3} cells (Biales et al., 1977; Ingram et al., 1986; Schlegel et al., 1987; Margaroli et al., 1987; Lewis et al., 1988; Law et al., 1989), somatotrophs (Lewis et al., 1988; Holl et al., 1989), and gonadotrophs (Croxton et al., 1988; Stojilkovic 107
et al., 1992). Fura-2 measurements of Ca$^{2+}$ in 16 tilapia PRL cells indicate that 75% are silent and 25% show spontaneously active [Ca$^{2+}$]$_i$ oscillations. The proportion of quiescent and active PRL cells that I find in tilapia is remarkably similar to the percentages reported in acutely dispersed rat PRL cells; 78% display silent activity, while 22% exhibit spontaneous fluctuations in [Ca$^{2+}$]$_i$ (Lewis et al., 1988). By examination of these mammalian cells with patch clamp and fura-2 technologies, Lewis et al. (1988) found that these oscillations in [Ca$^{2+}$]$_i$ were due to the influx of extracellular Ca$^{2+}$ through Ca$^{2+}$ channels activated by spontaneous membrane action potentials. Addition of Co$^{2+}$ was able to reversibly block the voltage-dependent inward current of Ca$^{2+}$. The silent group of PRL cells did not display spontaneous depolarizations. Preliminary investigations using the patch clamp technique on dispersed tilapia PRL cells indicate that tilapia PRL cells fire slow, regenerative action potentials (Helms et al., in preparation). Sodium and K$^+$ voltage-dependent currents have been measured with the latter containing a Ca$^{2+}$-sensitive component. Thus, tilapia PRL cells, like mammalian PRL cells, may to possess Ca$^{2+}$ channels which allow entry of Ca$^{2+}$ from extracellular pools.
Exposure to reduced osmotic pressure elicits a biphasic increase in $[\text{Ca}^{2+}]_i$ in PRL cells from the tilapia. Hyposmolar stimulation causes an immediate steep rise in $[\text{Ca}^{2+}]_i$ in tilapia PRL cells which reaches a peak in 1-2 min (see Figs. 15, 17, 18). This peak in $[\text{Ca}^{2+}]_i$ is typically followed by a decrease to levels that are intermediate to the peak and initial baseline $[\text{Ca}^{2+}]_i$. This plateau in $[\text{Ca}^{2+}]_i$ (Figs. 15, 17A, 18) lasts as long as exposure to reduced osmotic pressure is maintained (30 min was the longest time tested; data not shown). The biphasic pattern in $[\text{Ca}^{2+}]_i$ I observe in tilapia PRL cells during hyposmolar stimulation is similar to that reported in other systems. Thyrotropin-releasing hormone stimulates a biphasic increase of $[\text{Ca}^{2+}]_i$ in rat PRL-secreting tumor cells (GH$_3$; Gershengorn and Thaw, 1985; Gershengorn, 1986; Winiger and Schlegel, 1988). Exposure of nonendocrine epithelial cells to hypotonic medium stimulates cell swelling which is transduced by both a spike and sustained elevation in $[\text{Ca}^{2+}]_i$ (Hazama and Okada, 1990a,b; Foskett et al., 1989; Foskett and Melvin, 1989).

Calcium from intracellular pools is thought to be responsible for the initial rise in $[\text{Ca}^{2+}]_i$ and subsequent cellular response whereas the sustained increase in $[\text{Ca}^{2+}]_i$ and cellular response (e.g., PRL secretion)
appears to be derived from extracellular Ca\textsuperscript{2+} (Gershengorn, 1986; cf. Foskett et al., 1989). Delineation of the role of these two Ca\textsuperscript{2+} pools to the biphasic hyposmolar-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} and its subsequent mediation of PRL secretion in the tilapia remains to be investigated.

My finding that hyposmolar medium stimulates an increase in cytosolic free Ca\textsuperscript{2+} in tilapia PRL cells is supported by mammalian studies. Sato and colleagues (1990 a, b) found that medium hyposmolarity stimulates PRL release from rat clonal cell lines (GH\textsubscript{4}C\textsubscript{1}), an elevation that is mediated by increases in [Ca\textsuperscript{2+}]\textsubscript{i}. The physiological relevance of this osmotically-induced increase of PRL release to mammals is unknown, however (cf. Sato et al., 1990a). By contrast, the central role and actions of PRL in the FW osmoregulation of the tilapia are thoroughly characterized and understood (for review see Clarke and Bern, 1980; Loretz and Bern, 1982). The response of the tilapia PRL cell \textit{in vitro} to medium osmotic pressure fits elegantly with its effects in conserving blood osmotic pressure in a diluting environment. The direct response of the tilapia PRL cell to the factor it regulates, together with its simple shape and hardiness in primary culture make it an especially useful model. Seldom is one afforded the opportunity to clarify the cellular and molecular bases
upon which the adaptive mechanisms of physiology are mediated. The importance of the osmosensitivity of the tilapia PRL cell also leads one to question whether a similarly fundamental relation may exist in other vertebrates. Certainly, there are few processes outside diuresis that are more osmotically challenging than lactation.

In summary, I have developed a technique for dispersing tilapia RPD which yields a high percentage of viable single PRL cells. These dispersed cells respond in a similar manner as cells of the intact tissue. Using the Ca\(^{2+}\)-sensitive dye, fura-2, I show that reductions in medium osmotic pressure rapidly stimulate a biphasic increase in intracellular free Ca\(^{2+}\) in optically isolated PRL cells. This elevation in [Ca\(^{2+}\)]\(_i\) requires, at least in part, Ca\(^{2+}\) from extracellular sources. This is the first demonstration that a reduction in osmotic pressure can evoke a rise in [Ca\(^{2+}\)]\(_i\) in a cell of known osmoregulatory output. Accumulated evidence argues strongly that a rise in [Ca\(^{2+}\)]\(_i\) mediates the stimulatory actions of hyposmolarity on PRL release from the tilapia pituitary.
Consistent with PRL's role in FW osmoregulation, I have clearly shown that the RPD of FW tilapia contain and spontaneously release more tPRL\textsubscript{188} and tPRL\textsubscript{177} than the RPD of SW fish. During 18-20 hr incubations, exposure to hyposmotic medium significantly augments the release of both PRLs over levels observed in hyperosmotic medium. Moreover, the relative content or ratio (tPRL\textsubscript{188}/tPRL\textsubscript{177}) of tPRL\textsubscript{188} to tPRL\textsubscript{177} shifts with rearing or acclimating salinity. This ratio was significantly higher (1.3:1) in the RPD of FW tilapia than that seen SW fish (0.75:1). This disparate processing of the two tilapia PRLs in relation to environmental salinity suggests that these two molecules may possess distinct osmoregulatory actions in the tilapia. However, to date there is no study showing that the two tilapia PRLs act differently in the osmoregulatory physiology of this animal. It is possible that the existence of two PRLs might be advantageous for regulating other physiological processes in the tilapia. If this is true, then alterations in the proportions of the two PRLs may reflect a stage of readiness for the preferential modulation of release of one of the PRLs by
factors which regulate processes other than osmoregulation, but which are somehow tied to osmoregulation, such as reproduction or metabolism.

Accumulated evidence suggests that increases in the pituitary PRL content, PRL cell activity, and release of PRL in FW fish compared with SW fish may be derived, at least in part, by the direct actions of blood osmotic pressure (Dharmamba and Nishioka, 1968; Nagahama et al., 1973, 1975; Clarke and Bern, 1980; Nicoll et al., 1981; Nishioka et al., 1988). Reductions in medium osmotic pressure, which reflects the blood osmotic pressure of a fish adapting to FW, directly stimulate PRL release from the tilapia RPD, in vitro (Nagahama et al., 1975; Grau et al., 1981, 1982, 1986, 1987). To investigate this mechanism, I have developed a technique for dispersing the tilapia RPD which yields viable single PRL cells that respond in a manner similar to that of the intact tissue. Using the Ca$^{2+}$-sensitive fluorescent dye, fura-2, I show that reductions in medium osmotic pressure stimulate a biphasic increase in [Ca$^{2+}$]$_i$. Moreover, the sustained rise in [Ca$^{2+}$]$_i$ induced by hyposmotic medium is partly derived from extracellular Ca$^{2+}$ sources. In light of previous studies and the results presented here, rapid increases in [Ca$^{2+}$]$_i$ mediate, in part, the stimulatory
action of reduced osmotic pressure on PRL secretion in the tilapia.

By contrast with reduced osmotic pressure, elevations in osmotic pressure and cortisol, two events that occur when tilapia are transferred to SW, inhibit PRL release from RPD of tilapia (Wigham et al., 1977; Assem and Hanke, 1981; cf. Grau and Helms, 1989). During in vitro incubations I showed that cortisol inhibits PRL release in a dose-related manner. This action is mimicked by the synthetic glucocorticoid agonist, dexamethasone, but not by any other class of steroids tested. Cortisol rapidly reduces PRL release (≤ 20 min) during perifusion incubations. This rapid inhibition was accompanied by similarly rapid reductions in cAMP and $^{45}$Ca$^{2+}$ accumulation in the tilapia RPD (15 min). My results suggest that cortisol's rapid inhibitory effect on PRL release is transduced, in part, by the cAMP and Ca$^{2+}$ second messengers. This is one of few studies that has demonstrated a steroid hormone effect that is mediated via membrane-associated mechanisms rather than the classical genomic channels that are normally affiliated with transducing steroid hormone actions.

In nature, tilapia are confronted with unpredictable changes in their environmental salinity. It is essential, therefore, that the tilapia, when challenged with rapid
elevations in salinity, possess regulatory factors that are capable of rapidly inhibiting PRL secretion while simultaneously promoting tolerance to SW. Cortisol is one such factor. As previously discussed, the content of PRL in the pituitaries of SW tilapia is markedly reduced compared with the content seen in pituitaries of FW fish. This reduction of PRL cell activity may result, in part, from the actions of cortisol and elevated osmotic pressure.

To conclude, I have described the interactions between environmental salinity, osmotic pressure, and cortisol and their abilities to alter PRL cell function in the tilapia. During this process I have reported: 1) a novel mechanism of steroid hormone action, 2) the differential processing of the two tilapia PRLs, and 3) the first direct measurements of changes in [Ca^{2+}]_i in osmosensitive endocrine cells of known osmoregulatory output. These studies have laid the groundwork for future research in the mechanisms that mediate osmotic and cortisol induced changes in PRL secretion.
REFERENCES


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