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Effects of Prolactin on Chloride Cells in Opercular Membrane of Seawater-Adapted Tilapia

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Effects of prolactin on morphology and numbers of chloride cells in the opercular membrane of seawater-adapted tilapia (*Oreochromis mossambicus*) have been examined. Following five daily injections of ovine prolactin at a dose of 10 μg · g body wt^{-1}, blood samples were taken and opercular membranes were removed and stained with a fluorescent mitochondrial dye (dimethylaminostyrylethylpyridiniumiodine), a fluorescent derivative of ouabain (anthroylouabain), and a histological stain specific for the extensive tubular system of chloride cells (zinc–osmium–iodine). Mean plasma osmolarity and sodium increased 23–24% following prolactin injection. An increase in the relative frequency of chloride cells between 20 and 180 μm^2 in cross-sectional area and a decrease in the relative frequency of chloride cells greater than 180 μm^2 were observed following prolactin injections. Average cell size decreased 46–70% and cell height decreased 26–38% following prolactin injections. There was no significant change in cell density. Anthroylouabain staining was observed in both prolactin- and saline-injected fish, and no significant effect on Na^+,K^+-adenosinetriphosphatase activity was seen in either opercular membrane or gill tissue. The results demonstrate an effect of prolactin on chloride cell size and provide a morphological correlate for decreased secretory activity of chloride cells following prolactin injections.

Prolactin is generally believed to be responsible for the freshwater adaptation of many euryhaline teleosts (Pickford and Phillips, 1959). Osmoregulation is considered the primary role of prolactin in teleost fish based on observations on ion regulation (Bern, 1975; Hirano, 1977, 1986; Nicoll, 1980; Loretz and Bern, 1982; Hirano *et al.*, 1987), in particular promoting the retention of plasma sodium in fresh water (Hirano, 1986). Following prolactin treatment of seawater-adapted tilapia, Foskett *et al.* (1982) observed a dose-dependent decrease in chloride secretion across the tilapia opercular membrane, a surface rich in chloride cells. These authors suggested that an increase in prolactin levels may cause a reduction in chloride cell numbers and inhibit the active transport pathway conductance of the remaining cells. However, the effects of prolactin on chloride cell morphology and numbers have not been examined in any teleost.

In our study, several morphological and biochemical characteristics of the chloride cell were examined following the injection of seawater-adapted tilapia with prolactin. Prolactin treatment of seawater-adapted tilapia is particularly useful in that it (partially) simulates the seawater-to-freshwater transition. The mitochondrial stain dimethylaminostyrylethylpyridiniumiodine (DASPEI) (Bereiter-Hahn, 1976) was used to identify chloride cells and measure their size and numbers. Anthroylouabain (Fortes, 1977; McCormick, 1990a), a fluorescent derivative of ouabain, was used to identify cells high in Na^+,K^+-adenosinetriphosphatase (ATPase) and to localize any changes in Na^+,K^+-ATPase. An os-

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mium-containing fixative, which stains the extensive tubular system of chloride cells, was employed to measure cell height. Finally, Na^+,K^+-ATPase activity was determined in both opercular membrane and gill tissue.

MATERIALS AND METHODS

Fish and maintenance. Tilapia, *Oreochromis mossambicus*, were raised in an outdoor freshwater pond in the courtyard of the Life Sciences Building at the University of California at Berkeley. Fish of both sexes weighing between 15 and 60 g were gradually adapted to artificial seawater (33 ± 1 ppt) in 20-liter indoor tanks over a period of 10 days. They were maintained at this salinity for at least 1 week. The water was changed daily, the temperature was maintained at 26–28°, and continuous charcoal filtering and aeration were provided. Animals were fed daily with Hikari Cichlid Gold. The photoperiod was a 12-hr light:12-hr dark cycle.

Prolactin injections. After the fish were maintained for at least 1 week in artificial seawater, prolactin was injected daily for 5 days. Owing to the scarcity of fish prolactins, teleost studies, with few exceptions, make use of mammalian prolactin (Hasegawa *et al.*, 1986; Young *et al.*, 1988). Ovine prolactin (NIH-NIADKD-PRL-18°) was dissolved in a 0.9% saline vehicle made slightly basic with 0.01 *N* NaOH, which was then adjusted to pH 7.5 with 0.01 *N* HCl. Fish were anesthetized with 0.02% 2-phenoxyethanol in seawater. A dose of 10 μg prolactin/g body wt was administered in injections of 2 μl/g body wt daily. A control group was injected with the vehicle.

Preparation of opercular membranes and gill tissue. Two hours after the injection on Day 5, fish were killed by a blow to the head and blood was collected from the dorsal vessel into heparinized syringes. The entire operculum was removed and placed in tilapia-Ringer (in m*M*: 140 NaCl, 15 $NaHCO_3$, 4 KCl, 1 $MgSO_4$, 1 KH_2PO_4, 2 $CaCl_2$, 5.6 glucose; pH 7.8). The membrane was removed from the opercular bone by a modification of the method of Foskett *et al.* (1981). With the severed end held stationary, the membrane was dissected by cutting the connective tissue with iridectomy scissors. In order to maintain *in vivo* orientation, the membranes were then transferred to a small piece of collagen-coated lens paper previously sterilized with ultraviolet light.

The first gill arch from each side was removed and placed in ice-cold buffer (SEI: 0.3 M sucrose, 0.02 M Na_2EDTA, 0.05 M imidazole; pH 7.3), frozen immediately on dry ice and stored at −80°C for later determination of Na^+,K^+-ATPase activity.

DASPEI and anthroylouabain. DASPEI is a vital stain that binds to mitochondria (Bereiter-Hahn, 1976). The mounted opercular membranes were incubated in 2 μ*M* DASPEI in tilapia-Ringer at room temperature for 1 hr. Each membrane was then rinsed in tilapia-Ringer. The lens paper backing was removed and the membrane mounted on a glass slide. A Zeiss standard RA microscope modified for epi-fluorescence imaging with a Ploemopak illuminator (100 W HBO) containing a 450–490 bandpass excitation filter, a 510 chromatic beam splitter, and a 520 longwave pass filter, was used to examine the tissue.

The method of anthroylouabain treatment follows that of McCormick (1990). After dissection of the membrane, the tissue was rinsed in 4 ml low-K^+ tilapia-Ringer (LKTR; tilapia-Ringer with 0.1 m*M* KCl) at room temperature for 1 min. The tissue was then placed in 4 ml 2 μ*M* anthroyouabain in LKTR aerated with 100% O_2 at room temperature for 1 hr. It was then rinsed twice in 2 ml ice-cold LKTR for 3 min, mounted on a glass slide, and examined as above with a 363 nm excitation filter, a 395 chromatic beam splitter, and a 420 longwave pass filter.

Cell density and size. The proportion of the membrane containing chloride cells was first estimated by determining the proportion of fields (0.46 mm^2) that contained one or more chloride cells (always greater than 80%). Ten fields containing chloride cells were selected at random and positively staining cells were counted. The average number of cells for these fields was calculated, then multiplied by the proportion of fields with chloride cells to yield a value for cell density of the entire membrane expressed as cells per cm^2.

Several representative micrographs (Ektachrome, ASA 400, 12s) were taken for each preparation. These were then projected onto a digitizing board and the outlines of the cells were traced to determine their cross-sectional areas (Sigmascan, Jandel Scientific, Sausalito, CA). All the cells in each micrograph were measured and an average area for each individual was calculated.

Osmium staining and sectioning. Zinc–osmium–iodine was used following the technique of Maillet (1959). This mixture stains the phospholipids of plasma membranes and the extensive tabular system of chloride cells. A 0.2% osmic acid solution saturated with zinc powder and 25 mg · ml^{-1} iodine was prepared just prior to use. Membranes were fixed in this solution for 16 hr. After rinsing with distilled water three times, the tissues were dehydrated and imbedded in paraffin using standard histological procedure. These were sectioned at 7 μm and mounted on glass slides. Cell height and the proportion of the membrane spanned by the cell were measured on cells chosen at random. Membrane thickness was estimated from the cells adjacent to the chloride cell being measured. Cell height is reported in μm and the proportion of the membrane spanned as a percentage.

Measurement of Na^+,K^+-ATPase activity. Na^+,K^+-ATPase activity was measured following the method of McCormick and Bern (1989). Opercular membrane and gill tissue that had been stored at −80°

were thawed 5 min prior to assay and homogenized in 85 μl SEI buffer with 0.1% Na deoxycholate in a 200-μl capacity ground glass homogenizer (Wheaton No. 357848). The homogenate was centrifuged at 5000*g* for 30 sec; the supernatant was removed and assayed for Na^+,K^+-ATPase activity and protein content.

The homogenate (15–25 μl) was added to the assay mixture (50 m*M* imidazole, 1 U/ml lactic dehydrogenase, 2.5 U/ml pyruvate kinase, 2 m*M* phosphoenolpyruvate, 0.05 m*M* nicotinamide adenine dinucleotide (reduced form = NADH), 0.5 m*M* ATP, 0.4 m*M* KCN, 45 m*M* NaCl, 2.5 m*M* $MgCl_2$, 10 m*M* KCl; pH 7.5) in a final volume of 1 ml. A duplicate cuvette was run simultaneously containing 0.5 m*M* ouabain. The assay mixture was mixed and the change in absorbance (340 nm) was recorded in a Perkin–Elmer spectrophotometer at 25°. The rate of NADH oxidation was calculated from the linear rate between 6 and 10 min after initiation of the reaction. Protein content was determined by the Lowry method. Na^+,K^+-ATPase activity was determined as the difference in ouabain-sensitive and ouabain-insensitive ATP hydrolysis and expressed as μmole ADP · mg protein^{-1} · hr^{-1}.

Plasma osmolarity and sodium levels. Following the collection, blood was centrifuged at 5000*g* for 2 min and plasma stored at −80°. Osmolarity was determined after thawing with a vapor pressure osmometer and expressed as mosm · liter^{-1}. Plasma sodium levels were determined with an atomic absorption spectrophotometer and reported in m*M*.

Statistics. Nonparametric Mann–Whitney test (CRISP statistical package) was used to determine statistical differences between treatments ($P < 0.05$).

RESULTS

Plasma osmolarity in the prolactin-injected group was 407 ± 9 mosm · liter^{-1} compared with 330 ± 4 mosm · liter^{-1} in the control group (Table 1). Plasma sodium levels were also elevated in the prolactin-injected group (217 ± 6 m*M*) compared with the control group (175 ± 2 m*M*). These were significant changes ($P < 0.05$).

Representative micrographs of DASPEI staining (Fig. 1) show a clear shift in the distribution of cell size (cross-sectional area) of chloride cells following prolactin injection. There was an increase in the relative frequency of chloride cells between 20 and 180 μm^2, a decrease in the relative frequency of chloride cells larger than 180 μm^2, and no chloride cells greater than 400 μm^2 following prolactin injection (Fig. 2). As a result of this shift in cell size distribution there was a significant decrease in average cell size (Table 1): mean cross-sectional area of chloride cells from controls were 2.5-fold greater than cells from prolactin-injected tilapia. There was no significant change in the cell density (number of DASPEI-positive cells per area of opercular membrane) between control and prolactin-injected groups.

Osmium staining occurred in chloride cells from both prolactin- and saline-injected groups. The mean cell height of chloride cells in the prolactin-injected group (12.1 ± 0.7 μm) was significantly lower than in the saline-injected group (17.9 ± 0.5 μm, $P < 0.05$) (Table 3). The proportion of the opercular membrane spanned by

TABLE 1
PHYSIOLOGICAL EFFECTS OF PROLACTIN (10 μG · G BODY WT^{-1} PER DAY FOR 5 DAYS) IN SEAWATER-ADAPTED TILAPIA

	Saline-injected ($N = 6$)	Prolactin-injected ($N = 6$)
Plasma osmolarity (mosm · l^{-1})	330 ± 4	407[a] ± 9
Plasma sodium (m*M*)	175 ± 2	217[a] ± 6
Opercular membrane Na^+,K^+-ATPase activity (μmol ADP · mg protein^{-1} · hr^{-1})	2.4 ± 0.5	2.4 ± 0.3
Gill Na^+,K^+-ATPase activity (μmol ADP · mg protein^{-1} · hr^{-1})	9.9 ± 1.4	11.0 ± 1.8

Note. Values are mean ± SE.
[a] Significantly different from control values ($P < 0.05$).

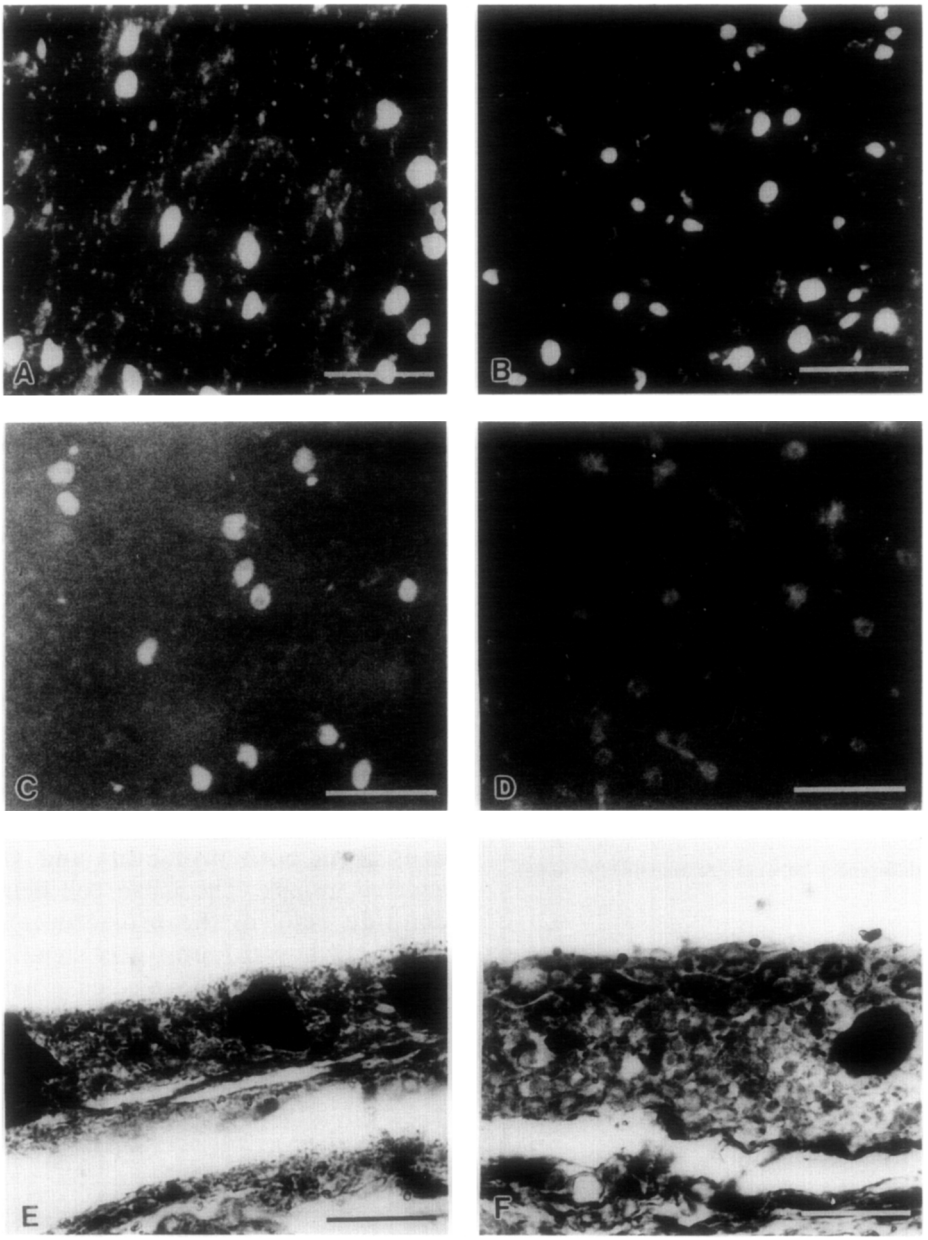

FIG. 1. (A) Chloride cells in opercular membrane of saline-injected, seawater-adapted tilapia following DASPEI staining. (B) Chloride cells in opercular membrane of prolactin-injected, seawater-adapted tilapia following DASPEI staining. (C) Chloride cells in opercular membrane of vehicle-injected, seawater-adapted tilapia following staining with anthroylouabain. (D) Chloride cells in opercular membrane of prolactin-injected, seawater-adapted tilapia following staining with anthroylouabain. (E) Chloride cells in opercular membrane of saline-injected, seawater-adapted tilapia following staining with zinc–osmium–iodine. (F) Chloride cells in opercular membrane of prolactin-injected, seawater-adapted tilapia following staining with zinc–osmium–iodine. (A–D) Bar length = 100 μm. (E, F) Bar length = 25 μm.

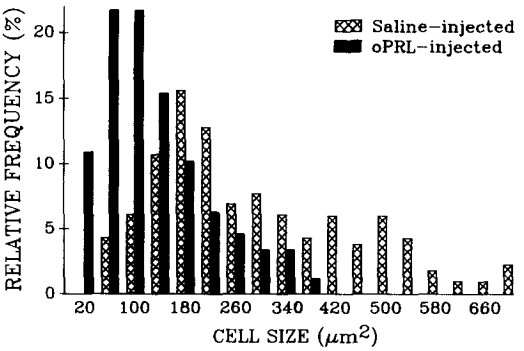

FIG. 2. Effect of prolactin on chloride cell size (cross-sectional area) in opercular membrane of seawater-adapted tilapia. Distribution from seven control and seven prolactin-injected fish with at least 50 cells measured per fish (N = 391 controls, 779 prolactin-injected).

the chloride cell is less in the prolactin-injected group (44.9 ± 3.6%) than in the control group (67.6 ± 1.6%; Table 2).

There was a decrease in the size of anthroylouabain-positive cells following prolactin injections (Fig. 1). This decrease was similar to that observed after DASPEI staining. Although some cells appeared less bright than in controls, there were still many cells that stained brightly. Anthroylouabain fluorescence in some cells was discontinuous, suggesting a patchy distribution of Na^+,K^+-ATPase. There were no significant effect of PRL injection on

TABLE 2
EFFECT OF PROLACTIN (10 μG · G BODY WT^{-1} PER DAY FOR 5 DAYS) ON DASPEI-POSITIVE CHLORIDE CELL SIZE AND DENSITY IN SEAWATER-ADAPTED TILAPIA

	Saline-injected (N = 6)	Prolactin-injected (N = 6)
Cell size (μm^2)	325 ± 55	130[a] ± 17
Cell density (cells · cm^2)	6979 ± 1825	7499 ± 1258

Note. Values are mean ± SE.
[a] Significantly different from control values ($P < 0.05$).

TABLE 3
EFFECT OF PROLACTIN (10 μG · G BODY WT^{-1} PER DAY FOR 5 DAYS) ON CHLORIDE CELL HEIGHT AND PROPORTION OF MEMBRANE SPANNED IN SEAWATER-ADAPTED TILAPIA

	Saline-injected	Prolactin-injected
Cell height (μm)	17.9 ± 0.5	12.1[a] ± 0.7
Proportion of membrane spanned (%)	67.6 ± 1.6	44.9[a] ± 3.6

Note. Values are mean ± SE. Chloride cells were stained with zinc–osmium–iodine.
[a] Significantly different from control values ($P < 0.05$).

Na^+,K^+-ATPase activity of the opercular membrane or gill (Table 1).

DISCUSSION

In the present study, prolactin induced the expected increases in plasma osmolality and Na^+ concentration. Prolactin treatment also resulted in a decrease in the average cross-sectional area and height of chloride cells. The distribution of cell sizes (Fig. 2) indicates that the change in average cell size was the result of a decrease in the number of large cells and an increase in the number of small cells. The density of chloride cells was not altered by prolactin treatment. We could not determine whether the observed changes were due to modifications of existing chloride cells or of developing chloride cells or of both. Chrétien and Pisam (1986) observed that 25% of nuclei of gill cells of *Lebistes reticulatus* adapted to 18 ppt seawater were labeled 24 hr after injection of [^{3}H]thymidine. If this time course is applicable to tilapia, the 6 days of treatment in the present study would have been sufficient for more than a 50% turnover of gill cells. It therefore seems likely that the effect of prolactin is on recently formed chloride cells, possibly inhibiting their hypertrophy and differentiation.

The observed decrease in chloride cell height and in the proportion of the membrane spanned by chloride cells indicates that fewer chloride cells of prolactin-treated tilapia have both apical and basal exposure. Contact with both external medium and serosa is an important characteristic of secretory cells (Rodriguez-Boulan and Nelson, 1989); prolactin treatment apparently decreases the number of cells with both external and serosal contact, which in turn may preclude secretory activity of chloride cells. On the basis of decreases in short-circuit current (equivalent to chloride secretion) and conductance of the opercular membrane following *in vivo* prolactin treatment, Foskett *et al.* (1982) hypothesized that prolactin "effectively removes" chloride cells and inhibits the active transport pathway in remaining cells. Our results suggest that rather than decreasing the number of mitochondrion-rich cells per se, prolactin treatment results in the effective removal of actively secreting chloride cells by preventing (or reversing) cell hypertrophy and subsequent contact with both external medium and serosa.

The chloride cells are the primary transport sites of chloride secretion in the gill and opercular membrane (Foskett and Scheffey, 1982) of fishes. A widely accepted model for the movement of Na^+ and Cl^- out of the chloride cell utilizes Na^+,K^+-ATPase to generate a Na^+ gradient across an extensive tubular membrane system throughout the chloride cell. The large concentration of Na^+ in the lumen drives a secondary NaCl carrier that brings Cl^- into the cell where it exits via chloride channels at the apical crypt (Silva *et al.*, 1977; Karnaky, 1986).

In the present study, prolactin treatment did not affect Na^+,K^+-ATPase activity of either gill or opercular membrane. Young *et al.* (1988) also found no effect of prolactin treatment on gill Na^+,K^+-ATPase activity of hypophysectomized tilapia in 11 ppt seawater. In other species, however, prolactin does exert effects on gill Na^+,K^+-ATPase: enzyme activity of seawater *Chelon labrosus* (Gallis *et al.*, 1979) and of hypophysectomized, freshwater-adapted *Fundulus heteroclitus* (Pickford, 1970) decreases following prolactin treatment.

Anthroylouabain, a fluorescent analog of ouabain, binds specifically to Na^+,K^+-ATPase and increases in fluorescence upon binding (Fortes, 1977). McCormick (1990a) found that anthroylouabain-positive cells were detectable in the opercular membrane of seawater-adapted tilapia, but not freshwater tilapia. In the present study, anthroylouabain-positive cells were present in large numbers following prolactin treatment. Along with the absence of any effect of prolactin on Na^+,K^+-ATPase activity, this observation suggests that the amount of Na^+,K^+-ATPase in individual chloride cells is not affected by prolactin treatment. This observation may at first seem paradoxical, since Na^+,K^+-ATPase activity normally decreases during adaptation to fresh water, a process which is brought about, at least in part, by the actions of prolactin (Bern, 1975; Hirano, 1986). It seems likely that endocrine mechanisms aiding seawater adaptation will be compensatorily stimulated by the abnormally high plasma ion levels of prolactin-injected fish. Cortisol, which has been shown to increase gill Na^+,K^+-ATPase activity in tilapia *in vivo* (Dange, 1985) and *in vitro* (McCormick, 1990b), may be more active under these conditions. Whereas prolactin seems to have a major impact on the size of chloride cells in tilapia, it is apparently less potent in its effect on their Na^+,K^+-ATPase content.

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