Prolactin Cell Activity and Sodium Fluxes in Tilapia (Oreochromis mossambicus) after Long-Term Acclimation to Acid Water

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In tilapia exposed for 3 months to water of pH 4.5, prolactin cell activity, as estimated by ultrastructural morphometry and determination of prolactin synthesis in vitro, was significantly higher than in controls from neutral water. Sodium influx from the water was 50% lower than in the controls, indicating impaired branchial sodium uptake mechanisms. In contrast to predictions based on the results of short-term exposure to acid water—which is known to induce an increase of sodium efflux—the sodium efflux rate was reduced to 70% of the control value. It is concluded that tilapia are able to acclimate to acid water by successful control—probably via prolactin—of diffusional sodium losses across the integument, in particular the gill surface. This compensates for the impaired sodium uptake, and enables the fish to reestablish a positive sodium balance in acid water. © 1989 Academic Press, Inc.

For the last few decades, massive fish mortality has been observed in acid fresh waters. The primary cause of death in acid water seems to be related to a failure of body ionoregulation (Muniz and Leivestad, 1980). It has been suggested that mortality during acid stress is primarily caused by diffusional sodium loss, as indicated by diminished blood and whole body sodium contents (Packer and Dunson, 1970; Muniz and Leivestad, 1980; Krout and Dunson, 1985).

Some fish species survive in acid waters and, therefore, appear capable of acclimating to prolonged acid stress. The tilapia Oreochromis mossambicus, an African cichlid fish, is well known to tolerate strenuous conditions, including low pH waters (Wendelaar Bonga et al., 1984). Similar to other fish (McDonald, 1983), tilapia may experience a drop in plasma electrolytes, in particular sodium, after acute exposure to water of pH 4.0. A restoration of plasma electrolytes is observed within 10 days (Wendelaar Bonga et al., 1984).

The capacity of tilapia to tolerate acid water probably depends on hormonal action. During the first days of acid exposure the activity of prolactin, ACTH, MSH, and interrenal cells increases dramatically (Wendelaar Bonga et al., 1987; Balm et al., 1987; Wendelaar Bonga and Balm, 1989). The increase of prolactin cell activity is of interest because an important action of prolactin in teleost fish is the control of sodium permeability of several epithelia, in particular the passive Na⁺ efflux across the branchial epithelium (Hirano, 1986). Since the loss of sodium is considered a major cause of death in acid water, the activation of prolactin cells in acid-stressed tilapia seems an adequate response that should lead to restoration of its sodium balance. However, so far only increased sodium efflux and a reduced influx have been reported in acid-stressed fish (McWilliams, 1980; 1982; Mc-
Donald, 1983; Gonzalez and Dunson, 1987). Thus, these fish were suffering net Na\(^{+}\) losses. However, this may be connected with the short duration of the period of acid exposure, usually a few days or less. Here we present data on prolactin cell activity and sodium in- and effluxes in tilapia exposed for 3 months to acid water. These fish proved to have reestablished a positive sodium balance.

MATERIALS AND METHODS

Tilapia (O. mossambicus) of both sexes were obtained from local stock. The fish were held under two conditions, viz pH 7.0 or pH 4.5 in artificial fresh water; the ionic content of the water was (in mmol/liter): 0.5 NaCl, 0.2 CaCl\(_2\), 0.2 MgSO\(_4\), and 0.06 KCl. They were kept in well-aerated all-glass aquarium at 28\(^\circ\)C. The water was filtered through cotton-wool and maintained at pH 4.5 by the addition of H\(_2\)SO\(_4\), using pH-stat equipment (Radiometer PHM 83 + TTT 80 + ABU 80). The fish were fed fixed rations of Tetramin tropical fish food. All fish were acclimatized to these conditions for at least 3 months prior to the experiments. Experiments were carried out with actively feeding fish.

Prolactin Cell Activity

Prolactin cell activity was estimated by morphometrical analysis of cell size, nuclear size, and fractional volume of mitochondria, Golgi area, and granular endoplasmic reticulum in electron micrographs. For technical details on fixation and morphometrical procedures see Wendelaar Bonga et al. (1980). For determination of the cell area, only cell profiles containing a nucleus were taken into account. Another parameter used for prolactin cell activity was the rate of uptake and incorporation into prolactin of tritiated amino acids by freshly dissected pituitary glands in vitro, as described recently (Wendelaar Bonga et al., 1988). Briefly, the rostral pars distalis was separated from the pituitary gland and incubated in the presence of labeled leucine for 4 hr. Subsequently the tissue was homogenized and the homogenate as well as the incubation medium were subjected to sodium dodecyl sulfate gel electrophoresis. The total amount of labeled (newly synthesized) prolactin present in tissue and medium was determined by densitometry of the prolactin bands in autoradiographs of the gels. The two bands characteristic for tilapia prolactin in our system, with apparent molecular radii of 20.5 and 22 kDa, respectively, were added. The fish used for determination of prolactin cell activity varied in body weight from 10 to 15 g.

Analytical Procedures

Sodium determination. Total Na\(^{+}\) concentrations in water and plasma samples were determined by means of flame spectrophotometry. For determination of plasma Na, the tail of the fish (body weight 10–20 g) was severed and blood was collected from the caudal peduncle into NH\(_4\)-heparinized capillaries; the latter were centrifuged at 9000g for 5 min to separate plasma and cells. The Na\(^{+}\) content of whole fish was determined by instrumental neutron activation analysis (\(^{24}\)Na\([\text{n,}\gamma]\)\(^{25}\)Na), according to the method described by Korthoven and De Bruin (1977).

Determination of sodium fluxes. Na\(^{+}\) fluxes were followed using \(^{24}\)Na as radiotracers. \(^{24}\)Na was produced by means of neutron activation of Na\(_2\)CO\(_3\) dissolved in water in the "Hoger Onderwijs Reactor” at the University University Reactor Institute, Delft. Typically, the product specific activity was 70 GBq/mol. The \(^{24}\)Na radioactivity was measured in a well-type 3 × 3" NaI(Tl) scintillation detector (Harshaw 125SW12) equipped with a single- or multichannel \(\gamma\)-ray spectrometer (ND60, Nuclear Data Inc. or Elscint INS-11). \(^{24}\)Na \(\gamma\)-emission was measured with a window set for energies ranging from 1.2 to 3.2 MeV.

Sodium influx was determined on the basis of \(^{24}\)Na uptake from the water. Body weights of the fish were 6.41 ± 1.94 (pH 7.0) and 6.46 ± 2.22 g (pH 4.5). Flux experiments were performed in a thermostatted fish container connected via silicon tubing with a 18-ml counting vial installed in the well of the scintillation detector. Tracer was injected via a valve in the silicone tubing. Water in the system was circulated at a rate of 11 liters/hr. Evaporation of water due to aeration was prevented by an air vent on top of the fish container. Radioactive homogeneity of the water was reached 30 sec after injection of \(^{24}\)Na into the system.

To minimize stress, fish were placed in the experimental containers 24 hr (Dharmamba and Maetz, 1972) before addition of \(^{24}\)Na to the exposure system; during this period of acclimation, water was recycled from a 200-liter volume. After closing the system the experiment was started by the injection of \(^{24}\)Na into the water; a volume of 400 ml water was recirculated. A volume of 18 ml water was analyzed continuously for radioactivity. At the start of an influx experiment, 0.185 MBq \(^{24}\)Na was injected into the system and the water radioactivity was monitored by registration of \(\gamma\)-emissions over 30-sec periods for 2 hr. The water \(^{24}\)Na activity at the end of the experiment was determined in 5-ml samples that were counted in the well of the detector. Also, the \(^{24}\)Na activity in the fish was measured upon completion of flux experiments. For this purpose, the fish were rinsed in water, weighed, killed by an overdose of MS-222, and counted in the well of the detector. Corrections were made for apparent volume dependency of the counting efficiency.
The volume of the fish was estimated on the basis of body weight, assuming a density of 1 g/ml.

$Na^+$ flux from fish to water ($Na^+$ efflux) was estimated by determining $^{24}Na$ appearance in the water from fish that had been loaded with $^{24}Na$ via the water. Body weights of the fish were 4.38 ± 1.92 (pH 7.0) and 5.92 ± 2.14 g (pH 4.5). For $^{24}Na$ loading, the fish were kept for a period of 24 hr in 700 ml water, to which 1.85 MBq $^{24}Na$ had been added. The water was recirculated as described above for influx experiments. A 24-hr period of exposure to the tracer proved sufficiently long to obtain $^{24}Na$ specific activity in blood plasma and in the whole fish. After the tracer loading period, the fish were rinsed with water without handling, to prevent skin damage and to minimize stress. Next, the water volume was brought to 400 ml, which was recirculated via a counting vessel as described for influx experiments. Measurements of water $^{24}Na$ radioactivity lasted for 2 hr. Upon completion of the experiment, the fish were anesthetized in Tris-adjusted MS-222 (0.5 g/liter; pH 7.0 or 4.5) for blood sampling. Plasma samples were diluted to a 5 ml volume with demineralized water. Fish, plasma, and 5-ml water samples were counted in the well of the detector to determine $^{24}Na$ activity. After decay of the $^{24}Na$ (10 half-lives), the sodium content of the samples was determined.

**Notations, Calculations, and Statistics**

The symbols, definitions of symbols, and units used were taken from Shipley and Clark (1972): $q_w$, quantity of tracer in compartment (counts per second, cps); $SA_w$, specific activity in compartment ($a$ (cps/mol); $F$, flux (mol/hr); $W$, wet weight (g). Subscripts: f, fish; w, water; $t$, zero time; t, time.

The whole body $Na^+$ influx was calculated on the basis of whole body tracer content at the end of the experiment. This approach was validated by the fact that tracer disappearance from the water was apparently linear over the 2-hr period and that the amount of tracer accumulated in the animal equaled the amount of tracer that was calculated to disappear from the water over the same period. Calculation of $Na^+$ influx ($F_{in}$) on the basis of whole body tracer content was carried out according to:

$$F_{in} = \frac{q_w}{2}SA_w \text{ (nmol/hr)},$$

where $q_w = \text{whole body }$ $^{24}Na$ after a 2-hr exposure and $SA_w = \text{the water }^{24}Na$ specific activity over this period.

$Na^+$ efflux ($F_{out}$) was calculated on the basis of tracer appearance in the water ($q_w$) over a 2-hr period and the $^{24}Na$ specific activity of the fish at zero time ($SA_0 = q_0/Q_i$; $q_0 = q_{t2} + q_{w2}$), according to:

$$F_{out} = \frac{q_{w2}}{2}SA_0 \text{ (nmol/hr),}$$

where $q_{w2} = \text{the increase in water }^{24}Na$ activity of a 2-hr period and $SA_0 = \text{the specific activity of the fish at zero time.}$ Zero time is the moment of exposure of the fish containing $^{24}Na$ to the volume of 400 ml tracer-free water. It was ascertained that the decrease in $^{24}Na$ specific activity during the flux period allowed the assumption of an apparently linear tracer loss from the plasma compartment; this was indicated by an apparently linear increase in water radioactivity. In order to compare our data with those reported in the literature, fluxes were normalized according to body weight by linear extrapolation and are expressed as nmol/hr/g. The fish used for the flux experiments varied in weight from 2 to 12 g.

Results are presented as means ± 1 SD, unless otherwise stated. Data were statistically analyzed by the Mann-Whitney U test. Statistical significance was accepted at the 5% level. The linear regression analysis was based on the least-squares method.

**RESULTS**

**Prolactin Cell Activity**

Morphometrical analysis (Table 1) showed that the mean cell area of the prolactin cells was 30% higher in the acid-
stressed fish, which corresponds with a 70% higher cell volume than in the controls. Nuclear areas were slightly but not significantly higher. The percentage area represented by Golgi zones and mitochondria was similar in both groups, which implies that the total volume per cell of mitochondria and Golgi apparatus was about 70% higher in the acid exposed fish. The mean percentage area of the granular endoplasmic reticulum was about 50% higher in the acid-exposed fish, which corresponds with a more than twofold increase of the volume per cell.

The rate of prolactin synthesis, as determined by incubation of the freshly dissected rostral part of the pituitary glands in the presence of labeled amino acids, was 68% higher in the fish from low pH (Table 1).

Growth and Sodium Accumulation

The fish grew under both conditions (Table 2). No mortality was recorded. The growth rate at pH 4.5 was only one-third of that at neutral pH ($P < 0.01$). No significant difference was found between the plasma sodium concentrations of both groups of fish (Table 2). Relationships are given for total body sodium and body weight, and highly significant correlations were found (Table 2; $r_0 > 0.84; P < 0.001$ under both conditions). No significant difference between the total body sodium content of the fish acclimatized to either pH 7.0 or pH 4.5 was observed, indicating a growth-related net uptake of sodium for both groups.

$Na^+$ Fluxes

As shown in Fig. 1, $Na^+$ influx at pH 4.5 was reduced to 55% of the control value. $Na^+$ efflux at pH 4.5 was reduced to about 70%. Net $Na^+$ fluxes between fish and water, calculated as the difference in mean influx and efflux values, were 93 and 0 nmol/hr$^{-1}$g$^{-1}$ at pH 7.0 and pH 4.5, respectively.

**DISCUSSION**

Prolactin Cell Activity

We have shown before, using similar techniques as in this study, that the prolactin cells of tilapia become highly activated immediately after acidification of the water (Wendelaar Bonga et al., 1984, 1987). The present results show that the prolactin cells

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<td><strong>Eff ects of Exposu re for 3 Months of Tila pia to pH 4.5 on Plasma Sodium (Means ± SD), on Body Weight ($\Delta W_r$: Relative Increase in Weight during the Exposure Period) and on Whole Body Sodium (Expressed as the Relationship between Whole Body Sodium, $Q_f$, and Body Weight, $W$; Equations Were Obtained by Linear Regression Analysis of Plots of Whole Body Sodium Versus $W$)</strong></td>
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*Note. Numbers of fish are indicated in parentheses.*
remain highly active for at least 3 months in acid water. This indicates that acid water represents a continuous challenge for the fish. In this respect the effects of water acidity are different from the effects of another form of water pollution, viz. the presence of cadmium. Similar to water acidification, exposure of tilapia to cadmium induces the transient reduction of plasma electrolytes (Pratap et al., 1989; Fu et al., 1989). Prolactin cell activity also increases, but this increase is only transient and disappears when metallothioneins become demonstrable in liver and gills. These detoxifying proteins apparently effectively protect the osmoregulatory mechanisms against cadmium (Fu et al., 1989). Obviously such nonhormonal mechanisms are not involved in the acclimation of tilapia to acid stress.

**Growth and Na Content**

Tilapia grow steadily at pH 4.5, although the growth rate during the 3-month experimental period is slow and represents only about 35% of the control value. This is in line with earlier observations on this species showing that growth is almost inhibited in the first weeks in acid water, but is resumed later at a reduced rate (Wendelaar Bonga and Balm, 1989). Reduced growth rates have been reported for other species that were chronically exposed to low pH, e.g., brook trout (Kwain and Rose, 1985; Tam and Payson, 1986). In our experiments, food uptake was similar to the control group, indicating that the persistent osmoregulatory problems experienced in acid water may reduce the energy available for growth. After 3 months in acid water the total body sodium concentration is similar to that of the controls. This implies that the fish established a positive sodium balance, although the calculated sodium accumulation rate was only one-third of that of the controls (4.1 and 1.3 nmol/hr/g, respectively). The reduction of total body sodium is one of the most serious effects of severe water acidification (Packer and Dunson, 1970; Gonzalez and Dunson, 1987). McWilliams (1980) has reported a 20% reduction in brown trout exposed for 6 weeks to mildly acid water (pH 6). The maintenance of total body sodium content for 3 months at pH 4.5 qualifies tilapia as an acid resistant species. Since Na\(^+\) influx from the water equated Na\(^+\) efflux in the acid-stressed tilapia, the net sodium gain recorded on the basis of total body sodium content may be of dietary origin.

**Na\(^+\) Influx**

In tilapia exposed for 3 months to pH 4.5, Na\(^+\) influx is reduced to 55% of the control value. In general, Na\(^+\) influx is severely inhibited in fish at low pH, as has been established for several species on the first day of acid exposure, e.g., goldfish (Maetz, 1973), brook trout (Packer and Dunson, 1970), and brown trout (McWilliams and Potts, 1978). McDonald et al. (1983) reported a reduction with more than 90% for rainbow trout a few hours after exposure to pH 4.0. A slight restoration occurred after 40 hr. In our fish, Na\(^+\) influx was still significantly below control levels after an exposure period of 3 months, indicating that sodium uptake is strongly dependent on water pH, irrespective of the exposure period. This conclusion is in line with data on fish from natural acid waters. McWilliams (1980) has compared the Na\(^+\) uptake rates in Norwegian trout from an acid river and that of brown trout raised in a hatchery at pH 6.5. When tested at pH 4.5—5.4, the Na\(^+\) influx was much higher in the fish from the acid river, indicating acclimation of the Na\(^+\) uptake mechanisms. However, the values observed for these fish varied from about 15% at pH 4.5 to 50% at pH 5.4 of the levels measured at pH 6.5 in the hatchery-raised fish, showing a reduced Na\(^+\) uptake capacity in the long-term acid-acclimated fish. According to current con-
cepts (Maetz, 1973; Evans, 1984), Na\(^+\) uptake in freshwater fish takes place mainly in the gills and is a transcellular process involving entrance of the chloride cells, via a Na\(^+\)/H\(^+\) exchange mechanism, and active transport across the basolateral membrane, with Na\(^+\)/K\(^+\)-ATPase and probably Na\(^+\)/H\(^+\)- or Na\(^+\)/NH\(_4\)+-ATPase activities (Balm et al., 1988) as the driving force. It is possible that the observed reduction of Na\(^+\) uptake in acid water is a direct consequence of the steep hydrogen gradient at low pH, which will counteract the apical Na\(^+\)/H\(^+\) exchange (McWilliams and Potts, 1978).

**Na\(^+\) Efflux**

The Na\(^+\) efflux in the acid-stressed tilapia was reduced to about 70% of the control value. This is a surprising observation because acid stress is usually associated with increased Na\(^+\) efflux, as a result of increased permeability of the integument, in particular the gill epithelium, to water and ions (McDonald, 1983). In salmonids (Packer and Dunson, 1970; McWilliams and Potts, 1978; McDonald et al., 1983), goldfish (Maetz, 1973), mud minnows, garpike (Krout and Dunson, 1985), and sunfish (Gonzalez and Dunson, 1987), Na\(^+\) efflux rapidly increases after a sudden and severe drop in water pH. In tilapia we also have reported evidence for increased Na\(^+\) loss across the gills after acute water acidification (Wendelaar Bonga et al., 1984). In that study we showed that the permeability of the gills to water, which also increased dramatically, was restored to control levels after about 10 days, indicating restoration of branchial integrity. In rainbow trout, McDonald et al. (1983) showed a slight decline of the stimulated Na\(^+\) efflux already 40 hr after the start of acid exposure. Thus, restoration of Na\(^+\) efflux after prolonged exposure was not unexpected. However, the present observation shows a reduction of Na\(^+\) efflux below control levels, which to our knowledge has not been reported before. It seems a requisite for survival of fish in acid water.

**Prolactin and Na\(^+\) Efflux**

Given the low Na\(^+\) influx by impairment of the branchial Na\(^+\) uptake mechanisms, it is the effective reduction of the Na\(^+\) efflux which enables tilapia to maintain a positive sodium balance in acid water. Na\(^+\) efflux mainly consists of diffusional Na\(^+\) losses across the branchial epithelium (e.g., McDonald and Wood, 1981). In many teleost species these losses are controlled by prolactin (Hirano, 1986). The hormone reduces the branchial permeability to water and ions, including Na\(^+\), as has been demonstrated in killifish and tilapia (Potts and Fleming, 1971; Dharmamba and Maetz, 1972). We conclude therefore that the high prolactin secretion rate observed in chronically acid-stressed tilapia is causally connected with the effective reduction of the branchial Na\(^+\) efflux in these fish.

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