Effect of Ambient Osmolarity and Calcium on Prolactin Cell Activity and Osmotic Water Permeability of the Gills in the Teleost Sarotherodon mossambicus

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In the presence of low ambient calcium levels, prolactin cell activity is directly related to the height of the osmotic gradient between blood plasma and external medium, and not to ambient osmolarity. Prolactin cell activity is minimal in fish adapted to iso-osmotic saline. The osmotic water permeability of the gills is inversely related to the height of the osmotic gradient and to prolactin cell activity. In gills of fish from iso-osmotic saline the osmotic water permeability is maximal. This high permeability is reduced after injection of ovine prolactin. It is concluded that the rate of prolactin secretion is related directly to the rate of the osmotic water fluxes—and, possibly, passive ion fluxes—the fish are facing, irrespective of the direction of these fluxes. In the presence of high calcium levels, however, prolactin cell activity as well as osmotic water permeability of the gills were low and independent of ambient osmolarity. Prolactin injections did not influence the osmotic water permeability of gills from high-calcium-adapted fish. High prolactin secretion in freshwater-adapted fish is likely due to the presence of low environmental calcium levels and a high osmotic gradient between blood and environment. Low prolactin secretion in seawater fish—fish that are facing an even higher osmotic gradient—is probably caused by the high ambient calcium and magnesium levels, which may make prolactin superfluous for the control of the osmotic water permeability.

This study deals with the environmental factors that control prolactin secretion and osmotic water permeability of the gills. Prolactin secretion in freshwater fish greatly surpasses that in seawater fish. For the stickleback Gasterosteus aculeatus and the cichlid Sarotherodon mossambicus we have shown that the difference in prolactin cell activity depends on the difference in the external concentration of calcium ions and, to a lesser extent, magnesium ions between fresh water and seawater. An inverse relationship was observed between prolactin cell activity of fish and ambient calcium concentration, in fresh water as well as seawater (Wendelaar Bonga, 1978a, b; Wendelaar Bonga and Van der Meij, 1980).

Both prolactin and external divalent cations have been implicated in the control of permeability for water and ions of the fish integument, especially the gills. External calcium and magnesium ions are known to reduce the passive sodium fluxes across the gills (Potts and Fleming, 1971) and the osmotic and diffusional permeability of the gills for water (Ogawa, 1974; Potts and Fleming, 1970; Oduleye, 1976). Mammalian prolactin is known to reduce passive sodium fluxes (Potts and Fleming, 1971; Dharmamba and Maetz, 1972) and the osmotic water permeability (Lam, 1969; Ogawa et al., 1973; Ogawa, 1974, 1977). We have suggested therefore that the enhanced prolactin secretion following reduction of the ambient calcium and magnesium levels is a hormonal response that counteracts the rise in the gills of the permeability for ions and the osmotic permeability for water caused by removal of these ions (Wendelaar Bonga et al., 1978; Wendelaar Bonga and Van der Meij, 1980). This view implicates that the relation between prolactin secretion and the external calcium and mag-
nesium concentration might be indirect and mediated by changes in integumental permeability.

To test this hypothesis we studied the effects of changes in external osmolarity on prolactin cell activity and on osmotic water permeability of the gills. The necessity to limit the branchial permeability for water and ions exists as long as there are differences in osmolarity and ion composition between the blood and the outer medium. Consequently, if the rate of prolactin secretion is primarily involved in the control of integumental permeability, one would expect that prolactin cell activity is minimal under iso-osmotic and iso-ionic conditions, irrespective of the presence of divalent cations. On the other hand, increased prolactin secretion will follow exposure to hypo- and hyperosmotic media, especially at low external calcium and magnesium concentrations, since high concentrations of these ions will reduce water and ion fluxes across the integument.

In the present study the effects of hypo-, iso-, and hyperosmotic media on prolactin secretion and osmotic water permeability of the gills were determined, in the presence of high or low calcium levels. Prolactin secretion rates were estimated by morphometrical techniques at light and electron microscopic levels. Osmotic water permeability was estimated by determination of the net rates of water inflow and outflow across gills freshly isolated from fish adapted for 4 weeks to experimental media.

MATERIALS AND METHODS

Sexually mature male *S. mossambicus* (*Tilapia mossambica*) of about 12 cm in body length and approximately 20 g body weight were obtained from our laboratory stock. The fish were kept in 100-liter freshwater aquaria at 25° and a 12-hr photoperiod. Fish were fed throughout the experiments with Tetramin tropical fish food and minced beef heart.

**Experiment 1.** Fish were exposed for 28 days to fresh water (a; composition in mmoles/liter: Na+, 3.0; K+, 0.06; Ca^{2+}, 0.8; Mg^{2+}, 0.2; Cl−, 4.2; SO₄^{2−}, 0.5) or to solutions of NaCl in fresh water (b–e) of the following osmolarities:

- a. 16 mosm/liter,
- b. 167 mosm/liter,
- c. 330 mosm/liter,
- d. 680 mosm/liter,
- e. 980 mosm/liter.

At the start of the experimental period of 28 days, the NaCl concentrations of solutions b–e were increased daily and their final concentration was reached at Day 6 of the experimental period. At Day 28 fish were anesthetized in MS-222 and blood collected from the caudal arteries in heparinized hematocrit tubes. Immediately afterwards the blood was centrifuged and plasma osmolarity determined in a Vogel Micro Osmometer. Pituitary glands and gills were excised and treated as described below.

**Experiment 2.** This was similar to experiment 1, but with 10.2 mmol Ca^{2+}/liter in all solutions. The osmolarity of solution 2a was 43 mosm/liter. The osmolarities of solutions 2b–2e were similar to those of solutions 1b–1e, which was accomplished by a reduction of the NaCl content for each solution by about 15 mmol/liter.

**Structure and morphometry of the prolactin cells.** For light and electron microscopy pituitary glands of all experimental groups were fixed as described elsewhere (Wendelaar Bonga and Van der Meij, 1980), dehydrated, and embedded in Spurr's resin. For light microscopy 1-μm-thick sections were stained with toluidine blue and the volumes of the cells determined as described earlier (Wendelaar Bonga, 1978a).

Prolactin cells of fish from groups a, c, and e (experiments 1 and 2) were examined with the electron microscope. Ultrathin sections were poststained with Reynolds' lead citrate and examined with a Philips EM 300 electron microscope. Randomly selected samples of the prolactin cells, totaling about 1000 μm² of cytoplasm per animal, were analyzed. Electron micrographs of these areas with a final magnification of 13,000× were scanned using Kontron Digiplan integration equipment with a magnetostriiction tablet. Magnification was calibrated with carbon replica grating. The fractional volumes of the granular endoplasmic reticulum and of the Golgi apparatus were determined by measuring the areas occupied by single strands or stacks of granular endoplasmic reticulum or by Golgi areas. A Golgi area was defined as the cytoplasmic area occupied by a stack of Golgi saccules and the associated vesicles and presecretory granules. A Golgi field was considered as "active" when electron-dense presecretory material was present within the Golgi sacculles. The fractional volumes of the granular endoplasmic reticulum and of the Golgi apparatus were determined by measuring the areas occupied by single strands or stacks of granular endoplasmic reticulum or by Golgi areas. A Golgi area was defined as the cytoplasmic area occupied by a stack of Golgi sacculles and the associated vesicles and presecretory granules. A Golgi field was considered as "active" when electron-dense presecretory material was present within the Golgi sacculles. The fractional volumes of mitochondria and secretory granules (including presecretory granules) were determined, as well as the number of presecretory granules per unit area of cytoplasmic surface. Differences between the experimental groups concerning the light and electron microscopic data were tested for significance by Wilcoxon's test.
Data on plasma osmolarity were analyzed by Student's t test. All tests were two sided, at the 5% level.

Branchial osmotic water flow rates. These were determined in isolated gills after the method described by Ogawa et al. (1973). Only the first two pairs of gills were used for the measurements. To equilibrate the internal osmolarity the freshly dissected gill arches were preincubated in an aerated physiological saline solution (composition in g/liter: NaCl, 9.5; KCl, 0.18; CaCl$_2$·2H$_2$O, 0.26; NaHCO$_3$, 0.017; pH 7.6; 340 mosm/liter). For determination of the net water inflow (in fish from groups a, b, and c), four gill arches of every fish were incubated in demineralized water (2 mosm/liter). For determination of net water outflow (in fish from groups c, d, and e), four gill arches per fish were incubated in a NaCl solution in demineralized water of 680 mosm/liter. Each gill arch was weighed before incubation and every 15 min during incubation. Before weighing the gill arches were blotted carefully on both sides with moistened absorbing tissue. After incubation the gill arches were freeze-dried for 24 hr and their dry weight determined.

The water inflow or outflow rates per animal (in milliliters per 100 ml gill water, per milliosmole of the osmotic gradient between blood plasma and incubation medium, per minute) were determined on the basis of the weight increase or weight loss of the gill arches during the first 30 min of incubation. During this period the weight of the gills changed linearly. The data for water inflow and outflow rates were statistically analyzed with Student's t test (two sided).

Effect of prolactin and calcium on osmotic water permeability. The fish to be injected were adapted for 5 weeks to iso-osmotic (340 mosm/liter) NaCl solutions in fresh water with high (Ca$^{2+}$ $\approx$ 10.2 mmol/liter) or low (Ca$^{2+}$, 0.8 mmol/liter) calcium concentrations. Ovine prolactin (kindly supplied by the National Institutes of Health, Bethesda, Md.) was administered by two injections, given 24 and 11 hr before measurement, of 0.3 IU/g body wt, dissolved in 0.03 ml of a 0.6% NaCl solution. Controls were injected twice with the same volume of the saline solution. Water inflow and outflow rates of the gills were determined.

RESULTS

Prolactin Cell Activity

The general structure of the prolactin cells of S. mossambicus has been described recently (Wendelaar Bonga and Van der Meij, 1980).

Experiment 1 (low external Ca$^{2+}$). After adaptation to fresh water enriched with different concentrations of NaCl, the highest values for prolactin cell volume were found in fish from the most hypo-osmotic solution and from the most hyperosmotic solution. The cell volume was minimal in fish adapted to iso-osmotic saline (Fig. 1a) and differed significantly from the volumes for the fish of the other groups ($P < 0.001$). Thus prolactin cell volume was positively related to the height of the osmotic gradient between the ambient medium and the body fluids.

The prolactin cells of the fish adapted to the most hyposmotic solution (fresh water), the iso-osmotic solution, and the most hyperosmotic solution were analyzed quantitatively at the ultrastructural level.

In fish adapted to normal fresh water (16 mosm/liter) the prolactin cells were large and well developed. They contained large arrays of granular endoplasmic reticulum and extensive Golgi zones (Fig. 2). The Golgi zones frequently showed signs of active secretory activity, such as the presence of electron-dense presecretory material confined within the Golgi saccules and of presecretory granules (Fig. 2). Such granules were distinguished from mature secretory granules by their irregular outlines or by the presence of a wide electron-transparent halo around the dense core. Presecretory granules occurred mainly in the Golgi regions. Golgi zones with saccules containing electron-dense material were considered as "active" in the quantitative analysis of the cells (Table 1). Indications for release of the contents of the granules by exocytosis were occasionally observed.

In fish adapted to iso-osmotic saline (340 mosm/liter) prolactin cell activity was obviously reduced in comparison to that of normal freshwater fish (Fig. 3). Cell volume ($P < 0.001$) and nuclear volume ($P < 0.01$) were significantly smaller (Table 1). Volumes per cell comprised by granular endoplasmic reticulum, Golgi areas, and mitochondria were also reduced considerably ($P < 0.001$). The morphology of the Golgi zones reflected a low rate of granule formation. The volume of active Golgi zones was only 25% of that for the controls ($P$
< 0.01). The number of presecretory granules per unit area of cytoplasm was only 45% of that for the former group (P < 0.001). Indications of exocytosis were not found.

The structure of prolactin cells in fish adapted to hypertonic saline (980 mosm/liter) was generally similar to that of fish from normal fresh water (Fig. 4), as is evident from the quantitative data (Table 1). However, data concerning the Golgi apparatus suggest that the secretory activity was even higher than that of the fish from normal fresh water. The percentage of active Golgi areas was significantly higher (P < 0.01).

Experiment 2 (high external Ca²⁺). In the presence of calcium concentrations typical for seawater, prolactin cell volume was low and no longer influenced by ambient osmolarity (Fig. 6a). Electron microscopic examination of prolactin cells of fish exposed to solutions of 43, 340, or 980 mosm/liter showed that these were structurally similar to the inactive cells of fish adapted to low-calcium iso-osmotic fresh water (Figs. 5, 7, 8).

Relation of Internal and External Osmolarity

Experiment 1 (low external Ca²⁺). Plasma osmolarity was not noticeably affected by a rise of the ambient osmolarity up to 680 mosm. In the most hypertonic solution, however, plasma osmolarity was significantly increased in comparison to that of fish exposed to the hypotonic or isotonic solutions (Fig. 1a; P < 0.001).

Experiment 2 (high external Ca²⁺). In the presence of high calcium levels plasma osmolarity was not influenced by the height of the external osmolarity (Fig. 6a).

Osmotic Water Permeability of the Gills

The osmotic water permeability of the gills was estimated by measuring the net osmotic water inflow rates in fish from the hypo-osmotic and iso-osmotic media, and the net osmotic water outflow rates in fish from the iso-osmotic and hyperosmotic media.

Experiment 1 (low external Ca²⁺). Net osmotic water flow rate was low in gills from freshwater fish. It increased with increasing osmolarity of the ambient medium and was maximal in gills of fish from iso-osmotic saline (Fig. 1b). However, in gills of fish from hyperosmotic media, water flow rates decreased with increasing osmolarity (Fig. 1b).

Experiment 2 (high external Ca²⁺). The net osmotic water flow rates through the gills of fish from the high-calcium solutions...
Fig. 2. Prolactin cell of fish from normal fresh water; granular endoplasmic reticulum (ger) and Golgi area (Ga) are well developed; arrows, presecretory granules; arrowheads, presecretory material within Golgi membranes; sc, stellate cells.

Fig. 3. Prolactin cell of fish from iso-osmotic low-calcium saline; granular endoplasmic reticulum is scarce; sc, stellate cells.
TABLE I
Morphometrical Analysis of Prolactin Cells of Fish Exposed to Fresh Water (16 mosm/liter) or Fresh Water Containing NaCl with an Osmolarity of 340 or 980 mosm/liter for 4 Weeks

<table>
<thead>
<tr>
<th></th>
<th>16 mosm</th>
<th>340 mosm</th>
<th>980 mosm</th>
</tr>
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<tbody>
<tr>
<td>Cell volume (µm³)</td>
<td>499 ± 35</td>
<td>250 ± 31</td>
<td>560 ± 48</td>
</tr>
<tr>
<td>Nuclear volume (µm³)</td>
<td>80 ± 8</td>
<td>61 ± 9</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>Mitochondria (µm³ per cell)</td>
<td>7.6 ± 1.8</td>
<td>3.1 ± 1.3</td>
<td>9.0 ± 2.1</td>
</tr>
<tr>
<td>GER* (µm³ per cell)</td>
<td>110 ± 18</td>
<td>38 ± 13</td>
<td>88 ± 19</td>
</tr>
<tr>
<td>Total Golgi area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µm³ per cell)</td>
<td>25.5 ± 6.4</td>
<td>11.2 ± 4.2</td>
<td>26.8 ± 6.1</td>
</tr>
<tr>
<td>Active Golgi area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µm³ per cell)</td>
<td>11.3 ± 4.6</td>
<td>2.8 ± 2.2</td>
<td>22.6 ± 4.3</td>
</tr>
<tr>
<td>Presecretory granules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n per 100 µm³)</td>
<td>22.6 ± 4.0</td>
<td>10.4 ± 3.8</td>
<td>26.0 ± 5.9</td>
</tr>
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* Means ± SD of six fish per group.
* GER, granular endoplasmic reticulum.

were low, and not influenced by ambient osmolarity (Fig. 6b).

Experiment 3 (effect of prolactin injection). Two injections of ovine prolactin in fish adapted to low-calcium iso-osmotic saline led to a significant reduction of the net osmotic water in- and outflow rates of the gills (P < 0.01; Fig. 9a). Prolactin injection in fish from high-calcium iso-osmotic saline did not influence the osmotic water in- and outflow rates (Fig. 9b).

DISCUSSION

Prolactin Cells, Ambient Calcium, and Osmolarity

In contrast to inferences made in earlier reports (Wendelaar Bonga, 1978a, b), the present data demonstrate that prolactin secretion is dependent on ambient osmolarity, at least under low-calcium conditions. However, the relation between prolactin cell activity and ambient osmolarity is indirect. The secretory activity of the cells is minimal in fish adapted to iso-osmotic saline, whereas it is enhanced in fish from hypo-osmotic as well as hyperosmotic media. Thus, not the external osmolarity, but the height of the osmotic gradient between the environment and the body fluids determines prolactin cell activity in S. mossambicus. Preliminary experiments on sticklebacks led to the same conclusion (unpublished observations).

Suggestions that prolactin cell activity is controlled by environmental osmolarity have been made to explain why prolactin secretion is high in freshwater and low in seawater fish (Ensor and Ball, 1972; Batten and Ball, 1977). However, we have demonstrated for sticklebacks (Wendelaar Bonga, 1978a, b) and S. mossambicus (Wendelaar Bonga and Van der Meij, 1980) that the high calcium and magnesium concentrations in seawater are responsible for the low prolactin cell activity in seawater fish. The present results show that the high prolactin secretion in freshwater fish is due to the combination of the high osmotic gradient between blood and outer medium, and the low calcium and magnesium levels of fresh water.

Studies of teleost prolactin cells in vitro (Nagahama et al., 1973, 1975; Baker and Ingleton, 1975) led to the hypothesis that plasma osmolarity plays a role in the control of prolactin secretion in vivo. This hypothesis was based on the observation that reduction of the osmolarity of the culture fluid leads to an increase in the incorporation of amino acids in the prolactin cells, and to an enhanced release of prolactin. An inverse relationship between plasma osmolarity and prolactin cell activity has been
Fig. 4. Prolactin cell of fish from hyperosmotic low-calcium saline (980 mosm/liter): granular endoplasmic reticulum (ger) and Golgi areas (Ga) are well developed; arrows, presecretory material within Golgi membranes.

Fig. 5. Prolactin cell of fish from high-calcium fresh water; Golgi areas (Ga) are small and granular endoplasmic reticulum is scarce; arrow, presecretory granule; sc, stellate cells.
suggested (Nagahama et al., 1975). We have questioned before the value of these in vitro results for prolactin cells in situ, as in a variety of experiments on sticklebacks and S. mossambicus we never found a consistent relation between the activity of the prolactin cells and plasma osmolarity (Wendelaar Bonga, 1978a, b; Wendelaar Bonga and Van der Meij, 1980). An inverse relationship was neither found in the present experiments. In fish exposed to hyperosmotic media in low-calcium conditions, there was even a positive relationship between prolactin cell activity and plasma osmolarity. Thus, it is likely that in the presence of intact connections between the brain and the pituitary gland, plasma osmolarity is of minor importance for the control of prolactin secretion.

Prolactin, Ambient Calcium, and Branchial Osmotic Water Permeability

To estimate osmotic water permeability of gills, the osmotic water inflow was used as a parameter for fish from hypo- and iso-osmotic media, and osmotic water outflow for fish from iso- and hyperosmotic media. In fish experiencing an osmotic gradient between blood and ambient medium the osmotic permeability for water of the gills in the inward direction differs from the permeability in the outward direction (Isaia and Hirano, 1975; Gallis et al., 1979). This was confirmed during the present study (unpublished results). In a hypo-osmotic environment fish are experiencing water uptake, whereas in a hyperosmotic environment they are facing water loss. Thus, for fish adapted to hypo-osmotic media only the water permeability in the inward direction is of physiological significance, and for fish in hyperosmotic media only the water permeability in the outward direction.

Our data show that in fish from high-calcium media the osmotic water permeability of the gills is low and independent of ambient osmolarity. In fish from low-calcium media, however, the osmotic water permeability of the gills was inversely related to the height of the osmotic gradient between blood plasma and ambient medium. Osmotic water permeability was highest in fish from the iso-osmotic medium, equally high in inward as outward directions. Thus, if osmotic stress is minimal, the activity of internal mechanisms controlling osmotic water permeability is likely to be very low. It is significant, therefore, that prolactin cell activity is minimal in fish under iso-osmotic conditions.

Prolactin likely participates in the control of water permeability of gills. Administra-
Fig. 7. Prolactin cell of fish from iso-osmotic high-calcium saline; granular endoplasmic reticulum is scarce; sc, stellate cells.

Fig. 8. Prolactin cell of fish from hyperosmotic high-calcium saline (980 mosm/liter); granular endoplasmic reticulum is scarce and Golgi areas (Ga) are small; sc, stellate cells.
Calcium ions, in concentrations as high as in seawater (±10 mmol/liter), also effectively reduce osmotic water permeability of the gills of *S. mossambicus*, in both inward and outward directions. A reduction of the integumental permeability for water by calcium ions has been described for several other teleost species (Potts and Fleming, 1970; Isaia and Masoni, 1976; Ogawa, 1974; Gallis *et al.*, 1979). In the experiments reported here, the osmotic water flow rates across the gills of fish from high-calcium media were the lowest observed, which indicates that high external calcium concentrations are more effective in reducing osmotic water permeability than high rates of prolactin secretion. We further found that prolactin injections reduced the high osmotic water permeability of gills of fish from low-calcium iso-osmotic saline, but not the low permeability of gills of fish from high-calcium iso-osmotic saline. Thus, the effects of external calcium ions and of prolactin are not supplementary. These observations may explain the low rate of prolactin secretion characteristic for seawater fish (Ensor and Ball, 1972; Wendelaar Bonga and Van der Meij, 1980). Although the osmotic water permeability of gills from seawater fish is relatively low, due to the high calcium and magnesium concentrations in seawater, seawater fish are still suffering considerable water loss (Maetz, 1974). If the effects of external calcium ions and of prolactin on branchial osmotic water permeability were supplementary, a high rate of prolactin secretion might have been expected in seawater fish. The possibility needs consideration that the mechanisms of action of calcium ions and prolactin on the osmotic water permeability of the gills are similar. In this connection it is of interest that prolactin has hypercalcemic effects in several fish species (Pang *et al.*, 1978; Wendelaar Bonga *et al.*, 1978), including *S. mossambicus* (our unpublished observations).
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