The Relationship between Prolactin Cell Activity, Environmental Calcium, and Plasma Calcium in the Teleost Gasterosteus aculeatus. Observations on Stanniectomized Fish

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The relationship between prolactin cell activity and plasma calcium, sodium, and osmolarity was studied in stanniectomized sticklebacks. In freshwater fish removal of the Stannius bodies led to a threefold increase of plasma calcium and a slight reduction of plasma sodium, while osmolarity was unaffected. Under these conditions the prolactin cells atrophied. It is concluded that plasma calcium, and not sodium or osmolarity as is generally assumed, is inversely related to prolactin cell activity. In seawater fish, normally characterized by low prolactin secretion, stanniectomy induced the same changes in plasma composition, but the prolactin cells remained unaltered. If seawater fish were transferred to fresh water, the expected activation of the cells occurred in the controls only. The lack of response in the stanniectomized fish is attributed to the high internal calcium level. Adaptation of freshwater fish to low-calcium or calcium-enriched fresh water showed that in the controls prolactin cell activity was inversely related to internal as well as external calcium concentration. In the stanniectomized fish in low-calcium fresh water prolactin cell activity was higher than expected on the basis of their high internal calcium concentration. This discrepancy indicates that internal and external calcium concentrations influence prolactin secretion by different mechanisms.

In teleosts, prolactin is of major importance for maintenance of water and ion homeostasis, especially in a freshwater environment. In euryhaline species like the three-spined stickleback, prolactin cells are highly active in fresh water, while the secretory activity is reduced upon migration or transfer to seawater (Leatherland, 1970; Schreibman et al., 1973; Wendelaar Bonga, 1978). The high sodium content and the high osmolarity of seawater have been considered as the main environmental factors accounting for this reduced prolactin secretion. The results of studies on pituitary transplants and on pituitary glands in vitro have led to the hypothesis that the environmental influence on prolactin secretion in vivo is mediated by plasma sodium and osmolarity (Nagahama et al., 1974, 1975; Wigham and Ball, 1977). But in a recent study (Wendelaar Bonga, 1978) we presented evidence that in sticklebacks the concentrations of calcium and, to a lesser extent, magnesium ions are the main environmental factors influencing prolactin secretion. In these experiments prolactin cell activity was inversely correlated with plasma calcium and sodium concentration. No correlations were found with internal or external osmolarity.

Two questions arising from these investigations are the subject of this report. The first concerns the problem whether prolactin cell activity is related to plasma calcium or to plasma sodium. The second question deals with the relationship between external calcium and prolactin secretion. Whether or not the effects of external calcium ions on prolactin cell activity are mediated by changes in plasma electrolyte levels is investigated.

It is difficult to establish whether prolac-
tinct cells in situ respond to plasma sodium or plasma calcium, since internal sodium and calcium levels are usually closely linked. Thus, addition of ionic calcium to the ambient medium is followed by a reduction of the sodium efflux and an increase of the plasma concentrations of both sodium and calcium. Injection of prolactin exerts the same effects (Potts and Fleming, 1971; Pang et al., 1973b; Wendelaar Bonga et al., 1978). However, removal of the Stannius bodies has been found to disturb the relationship between plasma levels of sodium and calcium (for a review see Krishnamurthy, 1976). The Stannius bodies are small endocrine glands that likely produce a hypocalcemic hormone (Pang and Pang, 1974). Exirpation of the glands leads to a marked rise in plasma calcium concentration. Plasma sodium remains unaltered or becomes slightly reduced (Krishnamurthy, 1976). These observations prompted us to study the effect of removal of the Stannius bodies of plasma sodium and calcium in sticklebacks, with a view toward establishing whether prolactin cell activity is primarily related to plasma sodium or to plasma calcium concentration.

The question whether external calcium influences the prolactin cells by changes in plasma electrolytes was studied in stannietomized fish exposed to changes in osmolarity and calcium content of the ambient medium. These experiments were aimed at determining whether prolactin cell activity is still inversely related to the external calcium concentration when internal calcium levels are enhanced by the removal of the Stannius bodies.

The secretary activity of the prolactin cells was assessed by morphometrical analysis using light and electron microscopy.

**MATERIALS AND METHODS**

Adult immature female sticklebacks of the migrating form (*Gasterosteus aculeatus trachurus*), with body lengths between 60 and 70 mm, were collected in winter in freshwater streams and along the coast of the Wadden Sea. They were kept in containers with running tap water or Wimex artificial seawater, at 15° and a daily light period of 8 hr, for at least 5 weeks. Concentrations of the main electrolytes in these media have been presented earlier (Wendelaar Bonga et al., 1976).

**Surgical procedure.** Animals were anesthetized in MS-222 (80 mg/liter). The perivisceral cavity was opened by a 4-mm-long incision in the lateral body wall. The kidneys were gently tilted, and the Stannius bodies were exposed. In sham-operated fish the body wall was closed at this stage of the operation. For stannietomy the glands were removed by forceps. The connective tissue layer separating the glands from the kidneys and from the juxtaglomerular ganglion was left intact, to avoid damage of the main blood supply to the kidneys and of the innervation of the blood vessels and major renal ducts. The wounds were sutured carefully (Perma-Hand-Seide, Ethicon G-6), and it was unnecessary to place freshwater animals in dilute saline after the operation. After a few days 10 to 20% of the stannietomized fish stopped eating. Their skin turned dark, swimming movements became irregular, and plasma calcium concentrations were often extremely high (more than 20 meq/liter). Histological examination usually revealed renal necrosis in the caudal parts of the kidneys, and the animals concerned were discarded. The other fish surviving the operation remained in an apparently healthy condition. Freshwater as well as seawater-adapted fish were operated. After a recovery period of 4 days six groups were formed, each consisting of stannietomized and sham-operated fish, that were treated in one of the following ways for a 12-day period:

**F1. Freshwater controls.** Freshwater fish were kept for another 12 days in tap water; Ca²⁺ content, 0.1 mmol/liter.

**F2. Low-calcium artificial fresh water.** Four days after the operation freshwater fish were exposed to daily decreasing concentrations of CaCl₂·2H₂O (0.075, 0.050, 0.025, and 0.010 mmol/liter) in de-mineralized water with the same concentrations of Na⁺ and K⁺ and the same pH (7.4) as the tap water used, by addition of NaCl (2.1 mmol/liter), KCl (0.06 mmol/liter), and traces of NaHCO₃; from Day 8 until Day 16 after operation Ca²⁺ was kept at 0.01 mmol/liter.

**F3. Calcium-enriched fresh water.** Four days after the operation freshwater fish were exposed to daily increasing concentrations of CaCl₂·2H₂O (2.5, 5.0, 7.5, and 10.0 mmol/liter) in tap water; from Day 8 until Day 16 after the operation the fish were kept at 10.0 mmol/liter.

**F4. Transfer to seawater.** Four days after the operation freshwater fish were exposed to daily increasing concentrations of CaCl₂·2H₂O (2.5, 5.0, 7.5, and 10.0 mmol/liter) in tap water; from Day 8 until Day 16 after the operation the fish were kept at 10.0 mmol/liter.
S. Seawater controls. After recovery seawater fish were kept for another 12-day period in full-strength Wimex artificial seawater.

S/F. Transfer to freshwater. Four days after the operation seawater fish were adapted to fresh water by exposure to daily decreasing concentrations of seawater (75, 50, and 25% seawater in tap water) and kept in tap water from Day 8 until Day 16.

Before and during the experiments the animals were fed daily small amounts of Tubifex. At Day 16 after the operation the animals were decapitated, and the kidneys were excised for histological examination to ascertain that the operation had been successful and that the kidneys had not been affected by necrosis. The pituitary glands of seven operated and seven sham-operated fish (except for group S, which consisted of five operated and five sham-operated fish) were prepared for light microscopy. In addition the pituitary glands of five operated and five sham-operated fish of group F1 were prepared for ultrastructural examination.

The procedures for blood sampling, determination of plasma sodium, calcium, and osmolarity, as well as the preparation techniques for light and electron microscopy, and the method for light microscopical determination of cell and nuclear volumes have been described earlier (Wendelaar Bonga, 1978). Per animal 25 measurements were made for cell as well as for nuclear volumes, and the means per animal and per group were calculated.

For quantitative evaluation of prolactin cell activity at the ultrastructural level of the F1 group, randomly photographed samples totalling 500 μm² of cytoplasm per animal were analyzed (final magnification of the electron micrographs, 15,000 ×) by Kontron MOP AM 01 integration equipment with magnetostriction tablet. Magnification was calibrated with carbon replica grating. The lengths of the membranes of the granular endoplasmic reticulum per unit area of cytoplasm, as well as the volume fractions of Golgi areas, mitochondria, secretory granules (excluding presecretory granules), lysosome-like bodies (including autophagic vacuoles), and multivesicular bodies were determined. In addition of 10 lysosome-like bodies and of 10 multivesicular bodies per animal, the diameters were measured, and the number of presecretory granules per unit area of cytoplasm was determined. A Golgi area was defined as the cytoplasmic area occupied by a stack of Golgi saccules, the associated Golgi vesicles, and the presecretory granules. The prolactin cells of 10 animals of the F1 group (five sham-operated and five stanniectomized fish) were analyzed in this way, and the means (± SEM) were calculated.

Differences between the groups concerning the ultrastructural data and the data on plasma composition were tested for statistical significance by Student's t test. The values for cell and nuclear volumes were analyzed by Wilcoxon's test. All tests were two-sided, at the 5% level.

RESULTS

Plasma Calcium, Sodium, and Osmolarity

In the stanniectomized fish of all groups plasma calcium levels reached excessively high values in comparison with the levels in sham-operated fish of the same group (P < 0.001; Table 1). Significant differences between the values of the stanniectomized fish of the various groups were not observed. Between the sham-operated groups small differences were found. In the fish exposed to low-calcium fresh water (F2) plasma calcium was slightly lower (P < 0.05) than in the freshwater controls (F1). Transfer of freshwater fish to seawater (F/S) was followed by a slight increase in plasma calcium (P < 0.01), to the same level as in seawater fish (S). In fish transferred from seawater to freshwater (S/F) plasma calcium underwent a small reduction (P < 0.01).

Removal of the Stannius bodies, but not sham-operation, resulted in moderate reductions of the plasma sodium levels. The difference between stanniectomized and sham-operated fish was significant in the F1, F3, and S/F fish (P < 0.01).

Plasma osmolarity of stanniectomized fish in all groups was not noticeably different from that in the sham-operated fish.

Prolactin Cells

The general structure of the prolactin cells of sticklebacks at light and electron microscope level has been described earlier (Leatherland, 1970; Benjamín, 1974; Wendelaar Bonga, 1978).

Freshwater (group F1). In sham-operated fish the prolactin cells showed the normal freshwater appearance. The cells contained large arrays of granular endoplasmic reticulum and prominent Golgi zones (Fig. 1). Many of the granules in the Golgi areas, classified as presecretory granules, were irregular in shape (Fig. 2). In these granules
Figs. 1 and 2. Prolactin cells of sham-operated freshwater fish.
Fig. 1. Ga, Golgi area; er, granular endoplasmic reticulum; st, agranular stellate cell processes (12,900 ×).
Fig. 2. Arrows, presecretory granules; mvb, multivesicular body (16,300 ×).
Figs. 3 and 4. Prolactin cells of stanniectomized freshwater fish.
Fig. 3. Lysosome-like bodies (26,000 ×).
Fig. 4. st, agranular stellate cell processes (12,900 ×).
Table I

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<th>Sodium (meq/liter)</th>
<th>Osmolarity (mosmol/liter)</th>
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<tr>
<td>Sham</td>
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<td>138 ± 2</td>
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The electron-translucent halo around the dense core was usually wider than that of the granules found elsewhere. Occasionally such a granule was found in contact with the Golgi lamellae. The mitochondria were large, and some contained cytoplasmic invaginations which appeared as inclusions in cross sections (see also Wendelaar Bonga, 1978). Membrane indentations indicative of exocytosis were scarce, but electron-dense deposits were commonly observed in the intracellular cleft between the prolactin cells and the surrounding agranular stellate cells. Such deposits have been interpreted as the extruded contents of secretory granules (Benjamin, 1974). Multivesicular bodies (Fig. 2) were present in small numbers, as were globules with moderate electron-dense contents, which were classified as lysosome-like bodies. Autophagic vacuoles were rarely found.

Removal of the Stannius bodies induced marked changes in cell morphology, as appears from the quantitative data presented in Fig. 5. Cell and nuclear volumes decreased by 30 and 50%, respectively (P < 0.01; Fig. 4). The extent of the granular endoplasmic reticulum, as well as the vol-

Fig. 5. Morphometrical data of prolactin cells in sham-operated and stanniectomized freshwater sticklebacks (F1), 16 days after the operation; a and b, cell and nuclear volumes as determined in paraffin sections (means ± SEM of seven fish per group); c–k, data of cytoplasmic organelles as determined in electron micrographs (means ± SEM of five fish per group); d, length of membranes of the granular endoplasmic reticulum per unit area of cytoplasm; e, g, h, and j, total surface of the organelles indicated per unit area of cytoplasm (%); f, number of presecretory granules per 100 μm² of cytoplasm; i and k, mean surface of a single profile of multivesicular bodies (i) and lysosomes (k).
volume fractions of Golgi areas, mitochondria, and multivesicular bodies were significantly reduced in comparison with the sham-operated controls \( (P < 0.01) \). If the reduction of the cytoplasmic volume is taken into account, these differences correspond to a reduction per cell of about 43% for the granular endoplasmic reticulum, 65% for the Golgi area, 58% for the mitochondria, and 85% for the multivesicular bodies. The number of presecretory granules per Golgi area was also considerably smaller. The fractional volume of the secretory granules had increased \( (P < 0.01) \), and phenomena indicative of granule release were no longer observed. The fractional volume \( (P < 0.01) \) and the mean size \( (P < 0.01) \) of the lysosome-like bodies were enhanced (Fig. 3). The increase of the fractional volume of the lysosome-like bodies amounts to 87% if calculated on a per cell basis. Autophagic vacuoles were commonly found.

Changes in ambient calcium (groups F2 and F3). Comparison of the sham-operated fish exposed to low-calcium fresh water (F2) with their sham-operated controls (F1) shows that reduction of external calcium is followed by a significant enlargement of prolactin cells and nuclei \( (P < 0.05; \text{Fig. 6}) \). The same holds true for the corresponding stanniectomized specimens \( (P < 0.01) \). But, as in the F1 fish, the values of the stanniectomized F2 fish were well below those of the sham-operated F2 fish \( (P < 0.01) \).

Calcium enrichment of the medium (group F3) led to a reduction of prolactin cell and nuclear size in the sham-operated fish \( (P < 0.001) \), but did not noticeably affect the prolactin cells in the stanniectomized fish.

Transfer to seawater or freshwater (groups F/S, S, and S/F). Transfer to seawater (F/S) induced a reduction in size of prolactin cells and nuclei in sham-operated fish \( (P < 0.001) \), but did not affect the prolactin cells in the stanniectomized fish if compared with the freshwater group (F1). The values for the transferred fish were similar to those of the fully adapted sham-operated and stanniectomized seawater fish (S). Transfer of seawater fish to fresh water after operation (S/F) was followed by an increase in cell and nuclear volume in the sham-operated fish only \( (P < 0.01) \).

DISCUSSION

Stanniectomy and Plasma Calcium, Sodium, and Osmolarity

The data presented show that removal of the Stannius bodies offers the opportunity to study prolactin cells in vivo under conditions of enhanced plasma calcium and reduced plasma sodium levels. A distinct hypercalcemia such as that occurring in operated sticklebacks has been reported for each of the few species studied after stanniectomy: goldfish (Ogawa, 1968), killifish (Pang, 1971; Pang et al., 1973a), and several species of eel (Fontaine, 1964; Butler, 1969; Chan, 1972; Bailey and Fenwick, 1975). In eel the increase of plasma calcium is partly accounted for by a rise of ionic calcium and partly by enhancement of the protein-bound calcium fraction (Bailey and Fenwick, 1975).

After exposure to low-calcium fresh water the plasma calcium level in stanniectomized sticklebacks was still considerably higher than that of the sham-operated controls. This seems an indication that the hypercalcemia following stanniectomy is primarily due to mobilization of internal calcium stores, such as bone tissue. However, in eel and killifish the supplementary calcium is likely to be of external origin. Following stanniectomy there occurs hypertrophy of the chloride cells in the gills (Chartier et al., 1977) as well as stimulation of branchial calcium-activated ATPase (Fenwick, 1976) and branchial calcium influx (Fenwick and So, 1974).

A slight decline in plasma sodium after removal of the Stannius bodies, as we
Fig. 6. Cell and nuclear volumes of prolactin cells in sham-operated (open bars, S) and sham-operated (open bars, F) mice transferred from fresh water to seawater (F/S) and from seawater to fresh water (S/F). SEM = ± SEM.
found in sticklebacks, has been observed in eel (Fontaine, 1964; Chan et al., 1967) and killifish (Pang, 1971), but not in goldfish (Ogawa, 1968). In the eel evidence has been obtained that the reduction of plasma sodium was due to a loss of capacity to reabsorb sodium in the kidneys (Rankin et al., 1967).

Stannicotomy did not lead to appreciable changes in plasma osmolarity in sticklebacks. A slight reduction has been reported for eels (Butler, 1969).

**Prolactin Cells in Stanniectomized Fish**

The results of the quantitative analysis of the prolactin cells in freshwater sticklebacks show that removal of the Stannius bodies leads to reduction of prolactin cell activity. In the operated fish there was not only a marked decrease of cell and nuclear volumes, but the intracellular synthetic apparatus, represented by endoplasmic reticulum and Golgi areas, was also strongly reduced. The decrease of the number of presecretory granules in the Golgi areas similarly points to a decreased synthetic activity. The absence of indications of exocytosis shows that hormone release too had decreased. A similar conclusion can be drawn from the observed reduction of the number and size of the multivesicular bodies. The latter structures are likely derived from membrane remnants of secretory granules which are pinched off from the outer cell membrane after exocytosis (Geuze and Kramer, 1974). Their number and size may therefore be considered as indicators of hormone release. The increase in number and size of the lysosome-like bodies and the frequent occurrence of autophagic vacuoles in the stanniectomized fish indicate that the atrophy of the prolactin cells is due to enhanced autophagic digestion. The prolactin cells of the stanniectomized fish showed the same structural signs of reduced secretory activity that characterize prolactin cells of freshwater fish transferred to seawater; this is the case for sticklebacks (Wendelaar Bonga, 1978) as well as many other species (Schreibman et al., 1973).

**Prolactin Cell Activity and Calcium**

The low activity of prolactin cells in seawater-adapted fish has been attributed to the high sodium content and the high osmolarity of the environment (Wigham and Ball, 1977). Previous experiments in this laboratory, however, led to the conclusion that in sticklebacks the high ionic calcium concentration of seawater is the main factor responsible (Wendelaar Bonga, 1978). The results of the transfer experiments reported here confirm this conclusion, at least for the sham-operated controls. In these fish prolactin cell activity was indeed inversely related to the calcium content of the medium. But in stanniectomized fish such a relationship was not observed. This is best illustrated by the results of the experiments whereby sticklebacks were transferred from seawater to fresh water (S/F). Whereas in the controls such transfer apparently activated the prolactin cells, no change could be observed in the cells of the stanniectomized fish. This lack of response in stanniectomized fish to the external calcium concentration may be attributed to their extremely high internal calcium levels. In the stanniectomized fish the plasma calcium concentration was almost the same in the F1, F3, F/S, S, and S/F groups. The values for prolactin cell and nuclear volumes were also similar in these groups. And since the expected activating effect of lowered external calcium on the prolactin cells of the S/F group was apparently blocked by the higher internal calcium level, it could be argued that in sticklebacks the effect of external calcium on prolactin cells is mediated by the internal calcium concentration. However, this possibility is only partly supported by the results of transfer to low-
calcium fresh water (group F2). In the sham-operated fish of this group the absence of adequate calcium supplies led to a reduction of plasma calcium but a marked increase of prolactin cell and nuclear volumes, as in normal sticklebacks (Wendelaar Bonga, 1978). In the stanniectomized fish exposed to low-calcium fresh water the plasma calcium levels were significantly higher than in freshwater control fish. Consequently, a firm reduction of prolactin cell activity was expected. But in fact the cells and their nuclei were as large as in freshwater controls. Thus, despite the high internal calcium concentration the prolactin cells still appear to react to the extremely low external calcium levels. It is suggested therefore that prolactin cell activity is influenced separately by internal as well as external calcium levels. This suggestion is consistent with the dual control mechanism of prolactin cell activity in teleosts, involving inhibiting nervous input and direct effects of plasma factors on these cells, as proposed by Nagahama et al. (1975).

An inverse relationship between prolactin cell activity and plasma sodium or osmolality, as suggested by the results of in vitro studies of teleost prolactin cells (Nagahama et al., 1974, 1975; Baker and Ingleton, 1975) or of pituitary transplants (Wigham and Ball, 1977), was not observed during the present experiments.

**Endocrine Aspects of Calcium and Sodium Metabolism**

A hypercalcemic endocrine factor like the parathyroid hormone of higher vertebrates has not been demonstrated in teleosts. But prolactin has been found to possess a hypercalcemic activity in fish (Pang et al., 1973b) and possibly also in other vertebrate groups (Boschwitz and Bern, 1971; Mahajan et al., 1975). Injection of mammalian prolactin raises plasma calcium in eel (Olivereau and Lemoine, 1973), killifish (Pang et al., 1973b), and sticklebacks (Wendelaar Bonga et al., 1978). The present results indicate that in sticklebacks plasma calcium levels exert a negative feedback control on the release of prolactin.

It is unlikely, however, that prolactin is responsible for the hypercalcemia following stanniectomy, since we observed a pronounced reduction of prolactin cell activity in stanniectomized fish. The endocrine basis of the postoperative hypercalcemia remains therefore unclear. It is hard to understand that the removal of the hypocalcemic hormone of the Stannius bodies is the only factor accounting for the extremely high calcium levels.

It may well be that the observed decline of plasma sodium in the stanniectomized fish is due to reduced prolactin secretion. Prolactin is known to promote sodium reabsorption in the kidneys, a process that is impaired in stanniectomized eel (Rankin et al., 1967). The possibility can not be excluded, however, that the reduction of plasma sodium is a direct effect of the removal of the Stannius bodies. In sticklebacks the Stannius bodies contain a cell type that is different from the presumptive hypocalcin producing cells. Its activity is inversely correlated with external and internal sodium and potassium content (Wendelaar Bonga et al., 1976, 1977) and it may be involved in the endocrine control of sodium metabolism.

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