Effects of ovine prolactin on calcium uptake and distribution in Oreochromis mossambicus

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Flik, G., J. C. Fenwick, Z. Kolar, N. Mayer-Gostan, and S. E. Wendelaar Bonga. Effects of ovine prolactin on calcium uptake and distribution in Oreochromis mossambicus. Am. J. Physiol. 250 (Regulatory Integrative Comp. Physiol. 19): R161-R166, 1986.—Ovine prolactin stimulated the net uptake rate of Ca from the water by 96%, produced frank hypercalcemia, and increased total bone calcium content in fed rapidly growing freshwater male tilapia, Oreochromis mossambicus. It did not, however, alter the size of the readily exchangeable bone calcium pool. The increase in calcium accumulation resulted from an increase in whole-body Ca influx and a decrease in Ca efflux. It is concluded that prolactin exerts an important control over Ca exchange between the fish and its environment and that through its hypercalcemic action prolactin indirectly facilitates bone mineralization.

PROLACTIN functions as a hypercalcemic hormone in teleost fish. Injection of mammalian prolactin induces hypercalcemia in killifish (Fundulus heteroclitus), tilapia (Oreochromis mossambicus), sticklebacks (Gasterosteus aculeatus), rainbow trout (Salmo gairdneri), goldfish (Carassius auratus), American eel (Anguilla rostrata), and European eel (A. anguilla) (5, 22, 26, 28). Conversely, hypophysectomized killifish become hypocalcemic and exhibit tetanic seizures in Ca-deficient seawater; these disturbances are overcome either by supplying Ca to the water or by treating the fish with exogenous prolactin (3). The effectiveness of endogenous prolactin is evident from the observation that in tilapia ectopic transplants of homologous prolactin lobes induce hypercalcemia (26). Moreover, in this last species an inverse relationship exists between levels of ambient Ca and prolactin cell activity (31). Although these observations clearly suggest that prolactin has a hypercalcemic effect in teleost fish, the mechanisms involved in this hormone’s action are still poorly understood.

It is well established that fish have a remarkable capacity to extract Ca directly from the water (25). Indeed, it has been estimated that in tilapia and goldfish at least 80% of the Ca accumulated during growth is obtained from the surrounding aqueous environment (1, 7). The bulk of this uptake probably takes place via the chloride cells located in the branchial epithelium (24). It seems reasonable then to predict that branchial Ca uptake mechanisms are an important element of calcium homeostasis in fish, that they must be under some control, and that at least part of this control is mediated by prolactin. This prediction is supported by the observation that ovine prolactin enhances high-affinity transport Ca-ATPase activity in American eel branchial epithelial plasma membranes (10) and that prolactin synthesis is stimulated in tilapia exposed to low ambient Ca levels (28). Exposure to low ambient Ca levels stimulates Ca uptake from the water in killfish (18) and in tilapia (8). Concerning this last effect of low ambient Ca levels, there are, however, deviating observations: Hobe et al. (14) reported that Ca influx in rainbow trout and bullheads (Ictalurus nebulosus) was largely independent of ambient Ca levels.

The present study was undertaken to investigate the effects of prolactin on Ca exchange with the water in growing actively feeding tilapia. The hypothesis was that prolactin treatment would enhance net Ca uptake in these fish and that the treated fish would be able to deposit Ca in their bone more efficaciously. We analyzed the effects of ovine prolactin in both influx and efflux rates of Ca in freshwater male tilapia. The internal distribution of the Ca taken up was traced to determine the effect of prolactin on Ca compartmentalization in the fish and to obtain an estimate of the readily exchangeable Ca pools.

MATERIALS AND METHODS

Male tilapia, O. mossambicus, were obtained from laboratory stock and kept at 28°C in Nijmegen tapwater under conditions as described earlier (7). The Ca concentration of the water was 0.8 mM in all cases. The fish were fed and continued to grow throughout the experiments.

Only reagent grade chemicals (Sigma, St. Louis, MO) were used. Ovine prolactin (spec act 31.5 IU/mg protein) was a generous gift of the National Institutes of Health (Hormonal Division, Endocrinological Department, Bethesda, MD). The radiotracers 45Ca and 47Ca were pur-
chased (Amersham International, UK) as CaCl₂ in aqueous solution. Specific activities were 9.25-37.5 and >0.74 GBq/mol Ca for ⁴⁷Ca and ⁴⁵Ca, respectively.

**Ca determinations.** Plasma total Ca was determined by atomic absorption spectrophotometry or by the use of a commercial Ca kit (Sigma) as described previously (7). Tissue total Ca was estimated after digestion of the tissue in concentrated HNO₃. Radiotracer activities were determined by γ-ray spectrophotometry (⁴⁷Ca) or by liquid scintillation counting (⁴⁵Ca).

**Ca²⁺ flux determinations.** Unidirectional Ca²⁺ fluxes between intact fish and water were determined and calculated as described in detail earlier (7). In brief, Ca²⁺ influx rates were calculated from ⁴⁷Ca²⁺ accumulated in the body over a 3-h period (by the use of a whole-body counter) and the water ⁴⁷Ca²⁺ specific activity. Efflux rates of Ca²⁺ from the fish were determined in two ways, yielding either total or branchial Ca²⁺ efflux. Total efflux rates were calculated on the basis of apparently constant whole-body ⁴⁷Ca²⁺ loss rates over a 20-h period and plasma ⁴⁷Ca specific activities in the middle of this period; in these fish ⁴⁷Ca²⁺ losses include urinary and intestinal losses. Branchial efflux rates of Ca²⁺ were determined on the basis of tracer appearance in the water and plasma ⁴⁵Ca specific activity 4 days after intraperitoneal injection of ⁴⁵Ca²⁺. In the latter type of efflux determination the fish were not fed for 1 day before the experiment, and the urinary bladder was emptied just before the experiment to exclude urinary and intestinal Ca²⁺ excretion during the flux measurements (7); a constant tracer appearance rate in the water was taken as evidence that no significant tracer excretion via urine or feces occurred (which would have led to fluctuating tracer appearance rates in the water). To assess whether the injection procedure for the hormone administration affected the flux rates, the observed flux rates in the fish that received control injections were compared with the flux rates calculated for these fish on the basis of the relationships for flux rates and body weight (W) reported for untreated fish (7); these were influx, Fin = 50 W₀·⁸⁰⁵ nmol Ca²⁺/h and efflux, Fout = 30 W₀·⁵₆₃ nmol Ca²⁺/h.

**Hormone administration.** Prolactin was dissolved in 50 mM HCl and was injected intraperitoneally with a 26-gauge needle fixed to a Hamilton precision syringe. The dosage was 0.1 IU/g fish per 48 h; the injected volume was 50 µl maximally. Control fish received equal volumes of solvent. Injections were given at fixed time intervals. Ca²⁺ flux rates were determined after a minimum of three hormone injections and always in the mornings of the day after the last injection. In the ⁴⁵Ca²⁺ efflux experiments the second injection consisted of a single combined injection of prolactin and tracer. The protocols for the different experiments are presented in Fig. 1.

**Calculations and statistics.** To compare flux rates of groups of fish with significantly different body weights (Wᵢ), individual flux rates were converted to flux rates related to the mean body weight of the pertinent groups (Wᵢ), taking into account the power relations for the respective fluxes and the body weights: F(Wᵢ) = aWᵢᵇ → F(Wᵢ) = aWᵢᵇ = F(Wᵢ) · (Wᵢ/Wᵢ)b.

To assess statistical significance of differences between mean values, the Mann-Whitney U test (one-tailed) was applied. A P value <0.05 was taken as significant. Linear regression analysis was performed according to the least-squares method.

**RESULTS**

**Tracer uptake and tracer retention.** As shown in Fig. 2A, ⁴⁷Ca²⁺ uptake from the water is significantly stimulated in prolactin-treated fish. ⁴⁷Ca²⁺ loss, however, after tracer loading from the water was not affected by prolactin treatment (Fig. 2B).

**Ca²⁺ fluxes (Table 1).** Prolactin treatment increased Ca²⁺ influx significantly by 39%. Measured influx rates in the control group (411 ± 128 nmol Ca²⁺/h) did not differ significantly from Ca²⁺ influx rates calculated according to Fᵢ = 50 W₀·⁸⁰⁵ nmol Ca²⁺/h (397 ± 32 nmol Ca²⁺/h; U = 29, P > 0.05), which indicates that the handling and the injection procedure did not affect influx measurements. Branchial Ca²⁺ efflux rates were 38% lower in the prolactin-treated fish than in the control fish. Branchial efflux rates in the control fish (149 ± 49 nmol Ca²⁺/h) did not differ significantly from efflux rates calculated according to Fₑᵢt = 30 W₀·⁵₆₃ (128 ± 11 nmol Ca²⁺/h; U = 31, P > 0.05), and this was taken as evidence that the procedure itself did not noticeably influence the flux measurement. Total body efflux rates of Ca²⁺ were reduced by 35% in prolactin-treated fish.

No significant differences existed between the mean body weights of the groups of fish used for influx and total efflux determinations. This allows the net Ca²⁺ uptake rate from the water (Fₑᵢt) to be calculated directly from the observed flux values as Fₑᵢt = Fᵢt - Fᵢ, being 411 - 178 = 233 nmol Ca²⁺/h for the controls and 571 - 115 = 456 nmol Ca²⁺/h for the prolactin-treated fish; in the prolactin-treated group Fₑᵢt is increased by 96%.

Mean body weights of the groups of fish used for branchial efflux experiments were significantly higher (P < 0.01 for controls and prolactin-treated fish) than those of the groups of fish used in the influx experiments.

To allow the calculation of net branchial Ca²⁺ influx rates (Fₑᵢt) as Fₑᵢt = Fᵢt - Fᵢ, measured individual influx rates were first normalized to the mean body weight (13.2 g) of the group of fish used for branchial efflux experiments, according to Fᵢt (13.2) = Fᵢt(Wᵢ) · x(13.2/Wᵢ)₀·⁸⁰⁵. Normalized influx rates, Fᵢt(13.2), were 480 ± 120 nmol Ca²⁺/h for controls and 683 ± 195 nmol Ca²⁺/h for prolactin-treated fish. Net branchial Ca²⁺ uptake rates then come to 480 - 149 = 331 nmol Ca²⁺/h for controls and to 683 - 92 = 591 nmol Ca²⁺/h for prolactin-treated fish, Fₑᵢt being increased by 79% in the latter group.

In all cases the prolactin-treated tilapia showed significantly increased plasma Ca levels (Tables 1 and 3).

**Tissue Ca analyses (Tables 2 and 3).** Prolactin treatment significantly increased specific activities (SA) of ⁴⁷Ca of vertebral bone and scales, but plasma ⁴⁷Ca SA of prolactin-treated fish, although somewhat higher, was not significantly different from those of control fish (values were 354 ± 49 and 385 ± 52 counts · min⁻¹ · µmol⁻¹ Ca for controls and prolactin-treated fish, respectively).

The relative specific activities (SAᵢ = 100 × SA_tissue/
SAplasma) for vertebrae and for scales in prolactin-treated 45Ca2+-exposed (72 h) fish were increased by 17 and 19%, respectively (Table 2). Thus prolactin stimulates the deposition in the bone of Ca2+ taken up from the water.

Table 3 lists the effects of prolactin on the Ca content and the 45Ca SA, values of plasma and three types of bone, 80 ± 3 h after tracer injection. The SA, values, reflecting the readily exchangeable Ca pool of the tissue, were not affected by prolactin treatment. However, skeletal, dermal, and scalar bone showed significant increases in Ca content in prolactin-treated fish.

**DISCUSSION**

**Prolactin-induced hypercalcemia.** Ovine prolactin produced a state of frank hypercalcemia in freshwater-adapted tilapia. This confirms earlier reports concerning tilapia (26) and other teleosts, including sticklebacks (G. aculeatus) (26), American eels (A. rostrata) (11), rainbow trout (S. gairdneri) (15), and the killifish (F. heteroclitus) (22). We believe that the effect observed was a physiological rather than a pharmacological response to prolactin, because the fish used in the present study were kept in water with a Ca2+ concentration of 0.8 mM, at which concentration the endogenous prolactin production is submaximal (28). Also, in tilapia kept under identical conditions, homologous prolactin can cause hypercalcemia, as judged by the effect of ectopically implanted prolactin lobes (27). In our opinion, prolactin must therefore be considered a hypercalcemic hormone in teleosts. Since the degree of bone mineralization had also increased, the prolactin-stimulated Ca2+ uptake from the water must have caused this hypercalcemia.

**Prolactin and Ca2+ influxes.** The Ca2+ influx rates presented in this study represent whole-body influx rates. However, we advanced arguments that in freshwater tilapia whole-body influx in fact can be equated with branchial influx (7). The prolactin-induced hypercalcemia in freshwater tilapia is thus accompanied by a stimulation of branchial Ca2+ influx. This observation corroborates our report on stimulation of branchial transport Ca2+-ATPase activity during prolactin-induced hypercalcemia in American eels (11). A similar enzyme activity was demonstrated in the gills of tilapia, and moreover, this enzyme activity may drive transbranchial transport of Ca2+ from the water to the blood (9). It seems reasonable, therefore, to state that prolactin treatment stimulates active transport mechanisms in the gills and thereby promotes Ca2+ influx. Whether the stimulatory effect of prolactin on branchial Ca2+ uptake mechanisms is direct or indirect, e.g., through the action of steroids, requires further investigation. Fleming and co-workers (6) have given evidence that in F. kansae prolactin stimulates the production of cortisol, the major mineralocorticoid in fish. It has been suggested that branchial chloride cell densities in tilapia are positively correlated with circulating cortisol levels (12). Our unpublished measurements of chloride cell numbers in the branchial area show that, after...
injection of ovine prolactin or after implantation of tilapia prolactin cell implants, chloride cell densities are increased in these fish. Recently, Edery et al. (4) reported that also the extrabranchial efflux is reduced by prolactin treatment. The extrabranchial routes mainly concern the intestine and urinary tracts. Our approach in determining of extrabranchial routes.

In fish residing in hypocalcic fresh waters, \( \text{Ca}^{2+} \) efflux will result from passive diffusion of \( \text{Ca}^{2+} \) through the integument and from urinary and intestinal outflow of \( \text{Ca}^{2+} \). As discussed earlier (7), integumental \( \text{Ca}^{2+} \) efflux essentially equals branchial efflux of \( \text{Ca}^{2+} \). Data on total body \( \text{Ca}^{2+} \) efflux and integumental \( \text{Ca}^{2+} \) efflux, then, allow discrimination between branchial and extrabranchial efflux rates. Branchial and total body efflux rates determined in the present study for control fish agree well with those reported elsewhere (7, 8). The effect of prolactin on net total body \( \text{Ca}^{2+} \) uptake, which is greater than the effect on net branchial \( \text{Ca}^{2+} \) uptake, suggests that also the extrabranchial efflux is reduced by prolactin treatment. The extrabranchial routes mainly concern the intestine and urinary tracts. Our approach in determining \( \text{Ca}^{2+} \) efflux rates does not allow distinction between the contribution to \( \text{Ca}^{2+} \) efflux of both routes. Both may be implicated, however, for it has been shown that prolactin exerts osmoregulatory effects not only on the gills but also on the intestine (19), kidney (13), and urinary bladder (3) of freshwater fish; such observations are in agreement with the presence of prolactin receptors in

![FIG. 2. A: whole-body \( ^{45}\text{Ca}^{2+} \) uptake from water in prolactin- and solvent-treated tilapia. Whole-body tracer content (\( q_t \)) is expressed as fraction of water total tracer content at \( t = 0 \) (\( q_t/q_{\text{tot}} \)). Values are means \( \pm SD \) (n = 9). \( *P < 0.01 \). B: whole-body tracer retention in prolactin- and solvent-treated tilapia after tracer loading from water. Whole-body tracer content at time \( t' \) is expressed as fraction of total amount of tracer in body at \( t_0 \) (\( q_t/q_{\text{tot}}; t_0 = t_2 \) of Fig. 2A). Whole-body \( \text{Ca}^{2+} \) efflux rates were calculated from interpolated mean plasma \( ^{45}\text{Ca} \)-specific activities at \( t' = 62 \text{ h} \) and whole-body tracer loss over time period \( t' = 52-72 \text{ h} \). For each experimental group 4 fish were killed at \( t' = 52 \text{h} \) and 9 fish at \( t' = 72 \text{h} \).
branchial, intestinal, and renal tissue of freshwater tilapia (4). From a quantitative point of view, however, our data indicate that, although the effects of prolactin on Ca^{2+} fluxes may extend to extrabranchial sites, the gills are, in this respect, the most important target for this hormone.

Our Ca^{2+} efflux data implicate that integumental permeability to Ca^{2+} is reduced by prolactin. Earlier (7) we concluded that in tilapia this permeability to Ca^{2+} is determined by the constitution and numbers of the paracellular pathways of the epithelium. In the same species the significance of the paracellular routes for the permeability to ions was first shown for Na^{+} efflux by Dharmamba and Maetz (2). Prolactin decreases branchial permeability to water and ions in tilapia (2), freshwater Japanese eel (A. japonica) (21), European eel (A. anguilla) (16), killifish (F. heteroclitus) (17) and green molly (Poecilia latipinna) (5). We now suggest that the control of integumental permeability to Ca^{2+} is a pivotal event in the action of prolactin on hydromineral regulation; the central role of the Ca^{2+} ion in this action may be further illustrated by the interrelationship between endogenous prolactin synthesis rates and ambient Ca^{2+} levels (28, 32), which latter, in turn, determine integumental permeability to Ca^{2+} (8).

Prolactin and internal Ca^{2+} reservoirs. The hypercalcemia and increased Ca content of the bony tissues, after prolactin-stimulated Ca^{2+} uptake in tilapia, show that in this species plasma Ca^{2+} freely exchanges with the bone and can be stored there. From the present results it may be concluded that prolactin-induced hypercalcemia enhances bone mineralization. Clearly, the acellular bone of tilapia is intimately associated with calcium metabolism and acts as an internal compartment for Ca^{2+} storage. No separate determination of plasma-free and protein-bound Ca^{2+} was carried out in this study; it has been shown, however, that in tilapia prolactin-induced hypercalcemia is accompanied by elevated levels of free Ca^{2+} (2). Apparently, it was the plasma-free Ca^{2+}, increased by the prolactin treatment, that was deposited into the bony tissues. These phenomena in tilapia resemble the Ca^{2+} exchange process in otoliths of rainbow trout, reported by Mugiya (20). Mugiya showed that the degree of calcium deposition in the otoliths was positively correlated with total plasma Ca levels. From his in vitro studies he concluded that the Ca^{2+} exchange between the otoliths and the endolymp parallels the fluctuations in free Ca^{2+} levels in the endolymp.

In our study the analysis of the prolactin effect on bone was restricted to analysis of the bone Ca contents. It has been demonstrated earlier that prolactin treatment of tilapia does not affect the lining cells (osteoblasts) of its bone, at least when judged on the basis of the ultrastructural features of this tissue (26). The observed effect of prolactin on fish bone, therefore, seems noncellular and is different from that exerted by, e.g., growth hormone on bony tissue in mammals. Growth hormone stimulates the activity of the lining osteoblasts and thereby promotes growth of bone. However, in tilapia both calcitonin and 24,25-dihydroxyvitamin D_{3} exert their effect on bone through activation of the lining cells (29, 30), which indicates that, in fish also, lining cells are involved in bone metabolism. The SA, values for bones in the controls and in the prolactin-treated tilapia are very similar, which indicates that the increase in bone Ca content is not noticeably accompanied by a relative increase in the bone readily exchangeable Ca pool. This then further supports our conclusion that true storage of Ca^{2+} in bone occurs when tilapia are treated with prolactin.

In conclusion, the results presented in this paper confirm that prolactin is a hypercalcemic hormone in freshwater teleosts and show that in tilapia the hypercalcemic effect of prolactin results from mainly a dual action on the integument, viz. stimulation of branchial Ca^{2+} uptake and reduction of integumental permeability to Ca^{2+}. In terrestrial vertebrates calcium homeostasis depends on parathormone that is hypercalcemic and stimulates cell-mediated Ca resorption from the bone. In fish, calcium metabolism differs essentially from that in land-living vertebrates, and it seems that in freshwater fish calcium homeostasis depends on the control of Ca^{2+} exchange with the water by the hypercalcemic hormone prolactin.

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