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Calcitropic actions of prolactin in freshwater North American eel (Anguilla rostrata LeSueur)

GERT FLIK, JAMES C. FENWICK, AND SJOERD E. WENDELAAR BONGA
Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 25, 6525 ED Nijmegen, The Netherlands; and Department of Biology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada

Flvik, Gert, James C. Fenwick, and Sjoerd E. Wendelaar Bonga. Calcitropic actions of prolactin in freshwater North American eel (Anguilla rostrata LeSueur). Am. J. Physiol. 257 (Regulatory Integrative Comp. Physiol. 26): R74–R79, 1989.—In freshwater-acclimated American eels (Anguilla rostrata LeSueur), ovine prolactin and grafts of the part of the pituitary gland containing the prolactin cells induced hypercalcemia. The hypercalcemia was associated with increased uptake of calcium from the water (resulting from increased influx and decreased efflux) and with enhanced high-affinity Ca\(^{2+}\)-adenosinetriphosphatase (ATPase) activity in the gills, the putative biochemical correlate of the branchial Ca\(^{2+}\) pump. Kinetic analyses of ATPase-mediated Ca\(^{2+}\) transport in plasma membrane vesicles of branchial epithelium provided evidence that prolactin enhanced the maximum velocity of the Ca\(^{2+}\) pump. Prolactin treatments raised plasma cortisol levels slightly but significantly in eels. However, cortisol per se was not hypercalcemic in eels and did not stimulate the branchial Ca\(^{2+}\) pump. We conclude that the hypercalcemic potency of prolactin in fish relates to its stimulatory action on active Ca\(^{2+}\) transport in the gills.

cortisol; calcium fluxes; active calcium transport; plasma membranes

Fish lack parathyroid glands as a source of hypercalcemic hormone for calcium homeostasis. Instead fish depend on pituitary hormones for the maintenance of plasma calcium levels and more so when exposed to calcium-poor media (e.g., soft freshwater). As hypophysectomized killifish (Fundulus heteroclitus) become hypercalcemic and exhibit tetanic seizures in calcium-free water and as these symptoms are overcome by adding calcium to the water or treating the fish with prolactin (16), it seems reasonable to propose a hypercalcemic potency for prolactin in fish.

The hypercalcemic action of prolactin was subsequently confirmed for a variety of teleosts, including the North American eel (8). For tilapia (Oreochromis mossambicus), we have shown that prolactin-induced hypercalcemia is associated with enhanced branchial Ca\(^{2+}\) uptake from the water (8). Edery et al. (3) have given evidence for prolactin receptors in branchial epithelium of the same species. We advanced evidence that the hypercalcemic effect of prolactin resulted mainly from a dual action on the integument, namely, stimulation of branchial Ca\(^{2+}\) influx and reduction of the permeability of the gills to Ca\(^{2+}\), thus promoting net uptake of Ca\(^{2+}\) from the water. Physiological evidence for an involvement of prolactin in fish calcium metabolism was obtained from experiments in which an inverse relationship (activation of prolactin cells under hypocalcic conditions) was established between ambient calcium levels and prolactin cell activity (23). Recently, we presented substantial evidence that Ca\(^{2+}\) influx via fish gills is a transcellular process that requires the activity of an ATP-energized Ca\(^{2+}\)-stimulated adenosinetriphosphatase (ATPase) (19). In earlier studies on the North American eel, preliminary evidence was given that the activity of a plasma membrane bound high-affinity Ca\(^{2+}\) ATPase activity, the presumed biochemical correlate of the branchial Ca\(^{2+}\) pump, is enhanced during prolactin-induced hypercalcemia (5). Therefore, fish may provide a unique model for the study of calcitropic actions of prolactin and the underlying mechanisms, a model that may contribute significantly to the understanding of the regulatory role of prolactin in the calcium metabolism of higher vertebrates. It has been established that in mammals prolactin plays a major role in the control of Ca\(^{2+}\) transfer in gut (15, 20) and placenta (2). Fish offer a model in which calcitropic actions of prolactin can be studied in the absence of hypercalcemic actions of parathyroid hormone.

To address the question of which mechanisms underlie the hypercalcemic actions of prolactin in the freshwater eel, we studied branchial Ca\(^{2+}\) uptake from the water in intact animals and active Ca\(^{2+}\) transport in branchial plasma membranes of fish made hypercalcemic by prolactin treatments. In the course of these experiments we observed that plasma cortisol levels were elevated in the prolactin-treated fish. To investigate the possibility that the effects of prolactin were indirect and caused by cortisol (the latter hormone has hypercalcemic effects in trout) (19), we also examined administration of cortisol for calcemic effects.

MATERIALS AND METHODS

Sexually immature female eels, Anguilla rostrata LeSueur, with a body weight ranging from 100 to 300 g were collected at the eel ladder of the Cornwall Power Dam in the St. Lawrence River, Cornwall, Ontario, Canada. In the laboratory, the fish were kept in running dechlorinated city of Ottawa tapwater (0.45 mM Ca, R74 0363-6119/89 $1.50 Copyright © 1989 the American Physiological Society
12°C) under a photoperiod of 16 h of light alternating with 8 h of darkness. Experiments were carried out in November and December. The animals were not fed.

**Analytical Methods**

Plasma total calcium content was determined with a commercial colorimetric calcium-kit (Sigma cat. no. 586). Combined calcium phosphate standards (Sigma cat. no. 360-11) were used as reference. Plasma cortisol was determined with a commercial radioimmunoassay kit (Amersham, Amerlex IM). Radiotracer activities were determined with a LKB Rackbeta LSA equipped with a disintegrations per minute program.

**Hormone Treatments**

Ovine prolactin was purchased from Sigma (St. Louis, MO; cat. no. L7135; 31.5 IU/mg) and dissolved in 50 mM HCl. The hormone was administered continuously for 10 days by means of Alzet osmotic minipumps implanted intraperitoneally. The dosage was 0.066 IU·g⁻¹·day⁻¹; controls received vehicle (50 mM HCl) infusions. At the end of the experiments the infusion rate of the pumps was checked by measuring the pump content (maximum infusion duration at 12°C for these pumps was calculated to 42 days), and a 10% deviation was accepted. The stability of the hormone preparation was checked electrophoretically by comparison of freshly dissolved prolactin with prolactin recovered from the pumps on completion of the experiments; silver-stained electrophoreographs did not reveal qualitative differences. Control and experimental animals were kept in the same tanks during the experiment and were marked by the incisions made for the implantation of the pumps (left for controls, right for prolactin).

*Prolactin lobe grafts.* In fish, the rostral pars distalis (rpd), also called the “prolactin lobe,” consists almost exclusively of prolactin cells. Adrenocorticotropic (ACTH) cells that border the rpd degenerate when explanted (1), and therefore rpd grafts may be used as a rather pure source of homologous prolactin. Two rpds from freshly dissected pituitary glands of weight-matched freshwater donor eels were implanted under the renal capsule in the mesonephric region of the recipient fish. Recipients were anesthetized with tris(hydroxy-methyl)aminomethane (Tris)-buffered (pH 7.4) tricaine methanosulfate (MS-222, 2 g/l). Shams were treated similarly but received no grafts. The incisions in the renal capsule, muscle, and skin were sutured carefully. After 10 days the grafts were analyzed for biosynthetic activity in vitro, using radiolabeling techniques (23). In short, rpds of recipient fish and rpd grafts were preincubated for 30 min in Hanks’ balanced salt solution (HBSS, Sigma) at room temperature and subsequently incubated for 4 h in 50 μl HBSS to which 3.3 GBq [³H]-leucine had been added. Tissue was washed in HBSS and homogenized in 500 μl 0.05 N HCl and centrifuged at 9,000 g for 10 min; the supernatant radioactivity was determined on triplicate 25-μl samples, and the remaining 425 μl were lyophilized. After reconstitution with water, samples with equal radioactive concentration were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (13). Production of prolactin in vitro (Fig. 1) was taken as criterion for a successful graft.

*Cortisol treatment.* Cortisol (sodium hydrocortisone 21-hemisuccinate, 400 μg/100 g fish; vehicle volume 40 μl/100 g fish per injection) was injected intramuscularly, once a day for 7 days. Controls received vehicle (0.9% wt/vol NaCl). Injections were given between 1500 and 1600 h. Experiments were carried out on the morning after the last injection.

**Ca²⁺ Flux Determinations**

For the determination of Ca²⁺ fluxes between fish and water, the eels were individually housed in flux boxes (V = 2 liters) and provided with a flow of well-aerated water.
Transfer to the boxes was at least 24 h before the start of the experiment to minimize influences of handling stress. At the start of an influx experiment, the water flow through the box was discontinued and 45Ca (1.6 GBq/l) was added to the water via a hole in the cover of the box. Rapid mixing of the isotope with the water was guaranteed by aeration of the water. The water 45Ca specific activity was monitored every 15 min for a 3-h period. Then the tracer containing water was rapidly removed by flushing the flux box with tap water, and the eel was anesthetized in a slightly acidic solution of MS-222 (2.5 g/l; pH 6.0) and rinsed with tap water made up to 10 mM Ca2+ by the addition of CaCl2, to facilitate the removal of tracer adsorbed to the external integument. A blood sample was taken by cardiac puncture and the eel quick-frozen at −80°C. Blood was centrifuged (15 s, 9,000 g) and plasma stored at −20°C until further analysis.

To determine extraintestinal Ca2+ influx, the (still frozen) intestinal tract was removed from partly defrosted animals and kept separate for 45Ca analysis. By doing so, tracer intake as a result of drinking was excluded from integumental uptake (18). The rest of the fish was microwave cooked (1.5 min) and blended with water (1 ml/g body wt) in a commercial blender. Triplicate samples (~10 g, weighed to the nearest 3 decimals) were ashed overnight at 600°C; ashes were dissolved in concentrated HNO3 and neutralized with KOH. Quin­tuple samples were analyzed for 45Ca. Extraintestinal Ca2+ influx (Fin) was calculated on the basis of total body (minus intestine) 45Ca content after 3 h (q0) and the water 45Ca specific activity (SAw); the water radioactive concentration did not change significantly during the 3-h flux period), according to Fin = q0/SAw.3 (nmol/h). Ca2+ influx was normalized to body weight and expressed as nanomoles per hour per 100 g fish.

For the determination of Ca2+ efflux, eels were injected intraperitoneally with 45Ca (6 GBq/100 g fish) in 500 µl saline (0.9% NaCl) 72 h before the start of the experiment. In a separate series of experiments, it had been assessed that plasma 45Ca levels do not change significantly over a 5-h period (the duration of the efflux measurement) between 72 and 96 h after injection of the tracer. At the start of the experiment, the water flow through the flux box was discontinued. The rate of appearance of 45Ca in the water was derived from the radioactivity in 5-ml water samples (in triplicate) taken with 1-h intervals over a 5-h period. Next, the fish was anesthetized, and a blood sample was taken by cardiac puncture and plasma prepared as described above. Plasma was analyzed for 45Ca specific activity (SAp). Ca2+ efflux (Fout) was calculated according to Fout = (dq/dt)/SAp (nmol/h), normalized to body weight and expressed as nanomoles per hour per 100 g fish. These techniques for the determination of unidirectional Ca2+ fluxes have been validated previously (7). Whole body fluxes determined in this way reflect branchial fluxes (the gills form up to 90% of the fish’s integument that is in direct contact with the water).

Isolation of Plasma Membranes and Ca2+ Transport Assays

The procedures for the isolation of plasma membranes from eel gills and for the marker enzyme assays used in this study have been described in detail elsewhere (6). Characteristics of the membrane preparation used are 1) a specific enrichment in Na+-K+-ATPase activity compared with the crude homogenate of the tissue and 2) a minor contamination with mitochondrial fragments, endoplasmic reticulum, or Golgi membranes. An estimated 59% of the membranes is resealed, and 33% of the membranes is inside-out orientated; the vesicular space (determined by [14C]mannitol equilibration) is typically ~2 µl/mg protein.

By use of a rapid filtration technique and assay media in which Ca2+ and Mg2+ concentrations are carefully buffered (21), a homogenous, calmodulin-dependent ATP-driven Ca2+ transporter (Kd0.5 = 63 mM Ca2+, Vmax = 2.25 nmol ⋅ min−1 ⋅ mg−1) (6) was demonstrated. Following the same procedures, we obtained very similar results for control fish (Kd0.5 = 80 nM, Vmax = 3.17 nmol ⋅ min−1 ⋅ mg−1; Fig. 2, Table 3).

Calculations and Statistics

Values are expressed as means ± SD, unless otherwise stated. Statistical analysis of the data was carried out using Student’s t test or the Mann-Whitney U test, where appropriate. P < 0.025 was taken to indicate significance. Apparent Kd0.5 and Vmax values were derived from Eade-Hofstee transformations of the data; to this end a nonlinear regression data analysis program was used (13). Linear regression analysis was based on the least-squares method.

RESULTS

As shown in Table 1, prolactin treatment of North American eels produced significant hypercalcemia and a concomitant hypomagnesemia. Prolactin did not affect other plasma ions investigated. Prolactin lobe implants induced a hypercalcemia that was comparable to that induced by infusion of ovine prolactin. Plasma cortisol levels had increased significantly after prolactin treatment.

After cortisol treatment plasma cortisol levels were 5.7 ± 2.5 and 465 ± 109 ng/ml for control and experimental eels, respectively. Concomitantly a slight but significant hypocalcemia was observed (3.01 ± 0.21 and 2.73 ± 0.23 mM Ca for controls and cortisol-treated fish, respectively; n = 8, 0.01 < P < 0.025). Cortisol had no significant effects on plasma Na, K, Cl, or Mg levels. No significant effects were observed with respect to Na+-K+-ATPase, Ca2+-ATPase, or kinetics of plasma membrane Ca2+ transport after cortisol treatment; control values did not differ from values presented here for untreated or control fish.

As shown in Fig. 3, untreated eels have similar whole body Ca2+ influx and efflux. In sham-operated eels Ca2+ influx is slightly but not significantly higher than in untreated eels; Ca2+ influx is not affected. Eels with rpd implants had significantly higher Ca2+ influx and significantly lower Ca2+ efflux than sham-operated eels.

As shown in Table 2, both prolactin treatments stimulated high-affinity Ca2+-ATPase activity in leaky plasma membrane preparations. No effect of either pro-
TABLE 1. Effects of prolactin treatments on blood plasma mineral composition and cortisol content

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Controls</th>
<th>oPRL</th>
<th>ePRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
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<td>Na</td>
<td>154±6</td>
<td>157±6</td>
<td>155±7</td>
<td>161±6</td>
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<td>K</td>
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<td>2.2±0.3</td>
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<td>Mg</td>
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<td>1.13±0.14</td>
<td>0.89±0.08*</td>
<td>0.85±0.07*</td>
</tr>
<tr>
<td>Ca</td>
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<td>3.23±0.28*</td>
<td>3.29±0.24*</td>
</tr>
<tr>
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<td>165±39</td>
</tr>
<tr>
<td>Pi</td>
<td>1.24±0.21</td>
<td>1.21±0.19</td>
<td>1.10±0.09</td>
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</tr>
<tr>
<td>Cortisol</td>
<td>1.8±1.2</td>
<td>3.5±2.7</td>
<td>7.1±3.8*</td>
<td>7.0±5.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of animals. Plasma mineral content is expressed in mM, cortisol levels in ng/ml plasma. oPRL, ovine prolactin infusions by means of Alzett minipumps, 0.066 IU · g⁻¹ · day⁻¹ for 10 days; ePRL, 2 eel prolactin lobe grafts implanted in the renal capsule, 10 days. * P < 0.01.

Prolactin treatment on Na⁺-K⁺-ATPase activity in plasma membranes or on the [¹⁴C]mannitol vesicular space was observed. Recoveries and enrichment factors for Na⁺-K⁺-ATPase activities in the plasma membrane fractions obtained from controls and experimental animals were not affected by prolactin treatments.

As shown in Table 3, prolactin treatments stimulate the maximum velocity of the plasma membrane ATP-driven Ca²⁺ transport but do not effect the affinity of the transporter for Ca²⁺.

DISCUSSION

The data presented here document the hypercalcemic potency of prolactin in eel and clarify the molecular mechanism that underlies the hypercalcemic action of this hormone. We show that ovine and eel prolactin induce hypercalcemia in association with enhanced uptake of Ca²⁺ from the water in vivo and with enhanced Ca²⁺ transport capacity in the gills in vitro.

Prolactin-Induced Hypercalcemia

The prolactin-induced hypercalcemia in freshwater eel corroborates previous reports on the effects of prolactin on the same species (5) as well as on a variety of other teleost species (8, 10, 17). One could argue that the dose of ovine prolactin used (0.066 U/g or 2.1 μg/g) evokes a pharmacological rather than a physiological response. But it is our experience, and that of others, that ovine prolactin is effective in fish only at μg/g-doses” and that it takes at least 3 to 5 days before prolactin effects become noticeable (8, 10, 11, 14, 22). In a recent study (11) it was reported that chum salmon prolactin was a...
hundred times more potent than ovine prolactin in a Na+-retention test, using Fundulus heteroclitus. In another species though, the Japanese eel Anguilla japonica, similar doses of chum prolactin or ovine prolactin are required to provoke hypertensive and hypercalcemic effects. These data were interpreted to indicate either a relative low prolactin dependence of freshwater eel or an insensitivity of the eel to heterologous prolactin. But since we observed that two ectopic prolactin lobes (one lobe had no significant hypercalcemic effect; data not shown) produce the same effect as a treatment with ovine prolactin it does not seem too presumptuous to conclude that the lobes are producing prolactin and that the hypercalcemic effect is due to excess prolactin produced by the rpd grafts. It follows then that the eel is rather sensitive to homologous prolactin. As it is unlikely that two rpd grafts produce as much as 2 μg prolactin g−1 day for a 10-day period (the estimated prolactin content of the freshly dissected prolactin lobes is 20 μg/mg prolactin lobe protein; the total protein content of the two prolactin lobe grafts was 53.6 ± 18.3 μg; n = 6), we conclude that eel prolactin is much more potent in eels than ovine or salmon prolactin. Our experiments showing hypercalcemic effects of ectopic prolactin lobes in freshwater eel corroborate similar experiments with freshwater tilapia (24). Unfortunately, no specific assays are available at the moment for eel prolactin. No direct proof can be given therefore to indicate that the ectopic prolactin lobes induced hyperprolactinemia.

It is interesting to note that both ovine and homologous prolactin induced hypercortisolinemia. This observation corroborates a report by Fleming et al. (4), who gave evidence that prolactin stimulates cortisol production in Fundulus kansae. We conclude therefore that, in the case of the rpd grafts, it was not the adrenocorticotropic hormone (ACTH) cells that evoked this response. The fact that ectopic ACTH cells, which are under predominant stimulatory control in situ, degenerate rapidly (1) further adds to this contention. The mild hypercortisolinemia observed in prolactin-treated eels may reflect an antagonistic response of the interrenal to the hyperprolactinemia, an interrelationship that has been suggested for other teleost species as well (25).

### Prolactin and Ca²⁺ Fluxes

The effects of rpd grafts on Ca²⁺ uptake in the freshwater eel agree strongly with the effects of ovine prolactin on Ca²⁺ uptake in the freshwater tilapia (8), namely, stimulation of whole body Ca²⁺ influx and inhibition of efflux. The whole body flux measurements in fish as performed in this study reflect branchial fluxes (7). Apparently, prolactin exerts its effects on the gills by stimulating Ca²⁺ influx from water to blood and inhibiting Ca²⁺ efflux from blood to water. As the chloride cells of the epithelium mediate branchial Ca²⁺ influx (19), it follows that prolactin enhances Ca²⁺ transport mechanism(s) in chloride cells to promote Ca²⁺ influx and reduces the epithelial permeability to Ca²⁺ to reduce passive Ca²⁺ efflux.

### Prolactin and Plasma Membrane Ca²⁺ Transport

The plasma membrane preparations obtained from control and prolactin-treated eels were very similar as judged from recovery and enrichment factors for Na⁺-K⁺-ATPase as well as from their vesicular space. The ratio Na⁺-K⁺-ATPase over Ca²⁺-ATPase, however, was significantly lower in prolactin-treated eel (untreated: 23.1, controls: 19.8, ovine prolactin: 16.7, and rpd grafts: 12.4), indicating increased density of the Ca²⁺-transporting enzyme in the plasma membranes. This conclusion seems further justified by our observation that both prolactin treatments stimulated Ca²⁺ transport in plasma membrane vesicles by enhancing the maximum velocity without affecting the affinity for Ca²⁺. The fact that only the 33% “inside-out” vesicles of the membrane preparation contribute to ATP-driven Ca²⁺-transport does not alter this conclusion: correction for percentage inside-out does not alter the apparent affinity for Ca²⁺ and increases transport velocities by a constant factor 3. Apparently an increased density of transporting enzymes underlies the enhanced Ca²⁺ transport capacity in the plasma membranes and this could explain the slow onset of prolactin effects.

It appears difficult to us to evaluate whether the slight hypercortisolinemia after prolactin treatment contributed to the effects observed in the plasma membranes. In freshwater trout a similar dose of cortisol as given here to the eel exerts a hypercalcemic effect and stimulates branchial Na⁺-K⁺-ATPase and transport Ca²⁺-ATPase activity (9). Such an effect of cortisol appears species dependent as we failed to obtain similar results.
in the eel. One could argue then that the cortisol levels induced by intramuscular injection are very high and the effects therefore pharmacological. However, the injection procedure per se raised plasma cortisol (1.8 ± 1.2 vs. 5.7 ± 2.5 ng/ml for untreated and saline-injected eels, respectively; P < 0.025) to levels observed in prolactin-treated eels, without significant effects on any of the blood or biochemical parameters tested. Unfortunately we cannot conclude whether the calcitropic effects of prolactin are direct, but an involvement of cortisol seems unlikely to us.

To our knowledge, this is the first report showing prolactin dependence of a plasma membrane-bound Ca\(^{2+}\) ATPase in a Ca\(^{2+}\) transporting epithelium. Foskett et al. (10) have shown that prolactin decreases chloride secretion and conductance of seawater tilapia opercular membranes and that these effects of prolactin on the epithelium are specifically exerted by influencing the chloride cells therein. We suggest that part of the calcitropic actions of prolactin in freshwater fish, namely, the stimulation of Ca\(^{2+}\) influx, may be mediated specifically via the chloride cells of the gills.

Prolactin stimulates intestinal Ca\(^{2+}\) absorption and placental Ca\(^{2+}\) transfer in pregnant ewes (2) and intestinal Ca\(^{2+}\) transport in vitamin D-deficient rats (15). The fascinating possibility that prolactin exerts its calcitropic effects in higher vertebrates in a way analogous to that in fish, namely, via stimulation of active Ca\(^{2+}\) transport mechanisms, needs further investigation.

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Address for reprint requests: G. Flik, Dept. of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 25, 6525ED Nijmegen, The Netherlands.

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