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

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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 Ca2+-adenosinetriphosphatase (ATPase) activity in the gills, the putative biochemical correlate of the branchial Ca2+ pump. Kinetic analyses of ATPase-mediated Ca2+ transport in plasma membrane vesicles of branchial epithelium provided evidence that prolactin enhanced the maximum velocity of the Ca2+ 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 Ca2+ pump. We conclude that the hypercalcemic potency of prolactin in fish relates to its stimulatory action on active Ca2+ 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 Ca2+ 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 Ca2+ influx and reduction of the permeability of the gills to Ca2+, thus promoting net uptake of Ca2+ 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 Ca2+ influx via fish gills is a transcellular process that requires the activity of an ATP-energized Ca2+-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 Ca2+-ATPase activity, the presumed biochemical correlate of the branchial Ca2+ 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 Ca2+ 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 Ca2+ uptake from the water in intact animals and active Ca2+ 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,
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 rack-beta 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 (rdp), also called the “prolactin lobe,” consists almost exclusively of prolactin cells. Adrenocorticotropic (ACTH) cells that border the rdp degenerate when explanted (1), and therefore rdp grafts may be used as a rather pure source of homologous prolactin. Two rdp s 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(hydroxymethyl)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, rdp s of recipient fish and rdp 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 $^{45}\text{Ca}$ (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 $^{45}\text{Ca}$ 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 Ca$^{2+}$ by the addition of CaCl$_2$, 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 $-80^\circ$C. Blood was centrifuged (15 s, 9,000 g) and plasma stored at $-20^\circ$C until further analysis.

To determine extraintestinal Ca$^{2+}$ influx, the (still frozen) intestinal tract was removed from partly defrosted animals and kept separate for $^{45}\text{Ca}$ 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 HNO$_3$ and neutralized with KOH. Quin duplicate samples were analyzed for $^{45}\text{Ca}$. Extraintestinal Ca$^{2+}$ influx ($F_{in}$) was calculated on the basis of total body (minus intestine) $^{45}\text{Ca}$ content after 3 h ($q_0$) and the water $^{45}\text{Ca}$ specific activity ($SA_w$); the water radioactive concentration did not change significantly during the 3-h flux period), according to $F_{in} = q_0/SA_w, 3$ (nmol/h). Ca$^{2+}$ influx was normalized to body weight and expressed as nanomoles per hour per 100 g fish.

For the determination of Ca$^{2+}$ efflux, eels were injected intraperitoneally with $^{45}\text{Ca}$ (6 GBq/100 g fish) in 500 $\mu$l saline (0.9% NaCl) 72 h before the start of the experiment. In a separate series of experiments it had been assessed that plasma $^{45}\text{Ca}$ 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 $^{45}\text{Ca}$ in the water (dq$_w$/dt) 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 $^{45}\text{Ca}$ specific activity ($SA_p$). Ca$^{2+}$ efflux ($F_{out}$) was calculated according to $F_{out} = (dq_w/dt)/SA_p$ (nmol/h), normalized to body weight and expressed as nanomoles per hour per 100 g fish. These techniques for the determination of unidirectional Ca$^{2+}$ 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 Ca$^{2+}$ 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 oriented; the vesicular space (determined by [1$^4$C]mannitol equilibration) is typically $\approx 2 \mu$l/mg protein.

By use of a rapid filtration technique and assay media in which Ca$^{2+}$ and Mg$^{2+}$ concentrations are carefully buffered (21), a homogenous, calmodulin-dependent ATP-driven Ca$^{2+}$ transporter ($K_{0.5} = 63$ nM Ca$^{2+}$, $V_{max} = 2.25$ nmol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$) (6) was demonstrated. Following the same procedures, we obtained very similar results for control fish ($K_{0.5} = 80$ nM, $V_{max} = 3.17$ nmol $\cdot$ min$^{-1} \cdot$ 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 $K_{0.5}$ and $V_{max}$ values were derived from Eadie-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 ± 0.28, 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$^+$-$\text{K}^+$-ATPase, Ca$^{2+}$-ATPase, or kinetics of plasma membrane Ca$^{2+}$ 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 Ca$^{2+}$ influx and efflux. In sham-operated eels Ca$^{2+}$ efflux is slightly but not significantly higher than in untreated eels; Ca$^{2+}$ influx is not affected. Eels with rpdi implants had significantly higher Ca$^{2+}$ influx and significantly lower Ca$^{2+}$ efflux than sham-operated eels.

As shown in Table 2, both prolactin treatments stimulated high-affinity Ca$^{2+}$-ATPase activity in leaky plasma membrane preparations. No effect of either pro-
FIG. 2. Kinetics of ATP-driven Ca\(^{2+}\) uptake in eel gill plasma membrane vesicles. Initial rates of ATP-dependent Ca\(^{2+}\) uptake (V, in nmol-min\(^{-1}\)-mg P\(^{-1}\); 1-min determinations) were corrected for ATP-independent uptake. Free Ca\(^{2+}\) (S) was varied between 7.5 \times 10^{-8} and 10^{-6} M. Inset, Eadie-Hofstee transformation of data that were used to calculate kinetic parameters V\(_{\text{max}}\) and K\(_{\text{m}}\) (Ca\(^{2+}\)). Mean values ± SD are given for 6 experiments.

<table>
<thead>
<tr>
<th>TABLE 1. Effects of prolactin treatments on blood plasma mineral composition and cortisol content</th>
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<tbody>
<tr>
<td>Untreated</td>
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<tr>
<td>n</td>
</tr>
<tr>
<td>Na</td>
</tr>
<tr>
<td>K</td>
</tr>
<tr>
<td>Mg</td>
</tr>
<tr>
<td>Ca</td>
</tr>
<tr>
<td>Cl</td>
</tr>
<tr>
<td>P(_{i})</td>
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<tr>
<td>Cortisol</td>
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</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.

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 Ca\(^{2+}\) from the water in vivo and with enhanced Ca\(^{2+}\) 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 \(\mu\)g/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 \(\mu\)g/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
TABLE 2. Effects of prolactin treatments on enzymic characteristics of eel gill plasma membranes

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Controls</th>
<th>oPRL</th>
<th>ePRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺-K⁺-ATPase V₂₅</td>
<td>51.5±13.3</td>
<td>49.0±24.3</td>
<td>53.9±9.8</td>
<td>55.2±14.7</td>
</tr>
<tr>
<td>% Recovery</td>
<td>22.4±7</td>
<td>24±8</td>
<td>19.4±4</td>
<td>21±6</td>
</tr>
<tr>
<td>Enrichment</td>
<td>15±1.3</td>
<td>15.8±2.9</td>
<td>14.4±7.1</td>
<td>16.2±4.3</td>
</tr>
<tr>
<td>Ca²⁺-Mg²⁺-ATPase V₂₅</td>
<td>2.23±0.43</td>
<td>2.47±0.99</td>
<td>3.23±0.55</td>
<td>4.46±0.55</td>
</tr>
<tr>
<td>Vesicular space</td>
<td>2.21±0.43</td>
<td>2.57±0.33</td>
<td>2.39±0.48</td>
<td>2.58±0.42</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 8 in all cases. ATPase activities were determined in the presence of optimum saponin concentrations (0.3 mg/ml vesicle suspension). Enzyme specific activities are expressed in nmol P-h⁻¹·mg⁻¹ protein⁻¹; relative recovery is percent activity recovered in plasma membrane fraction of initial homogenate of tissue; enrichment is ratio of specific activity in plasma membrane fraction to specific activity in initial homogenate; vesicular space is expressed in µm²/mg protein and was calculated from vesicle mannostol content after 2-h incubation. * P < 0.01.

TABLE 3. Prolactin and plasma membrane Ca²⁺-transport

<table>
<thead>
<tr>
<th></th>
<th>Vₘₐₓ</th>
<th>Kₘₐₓ</th>
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<tr>
<td></td>
<td>nmol·min⁻¹·mg⁻¹</td>
<td>nM Ca⁺⁺</td>
</tr>
<tr>
<td>Controls</td>
<td>3.17±0.10</td>
<td>80±25</td>
</tr>
<tr>
<td>oPRL*</td>
<td>4.98±0.15</td>
<td>103±31</td>
</tr>
<tr>
<td>ePRL†</td>
<td>4.94±0.32</td>
<td>152±76</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 for oPRL-treated eels, n = 8 for controls and ePRL-treated eels. * Ovine prolactin, 0.066 IU/g/day, Alzet minipumps, 10 days; † Eel prolactin lobe grafts, 2 per fish, 10 days. ‡ Significantly different from controls, P < 0.001.

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 altering 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 hypercortisolinaemia 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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REFERENCES


