Effects of Prolactin on Na^+K^+ -ATPase Activity in the Nephron during Maturation in the Rat

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ABSTRACT. The effect of prolactin (PRL) on renal Na^+K^+ -ATPase was investigated in 7-d-old neonatal rats. Animals were treated by bromocriptine (Br; a blocker of endogenous PRL secretion), and the enzyme activity was compared with that of untreated controls. Na^+K^+ -ATPase was determined in renal sections in the medullary thick ascending limb of Henle's loop and in the distal tubule by cytochemistry. In the distal tubule, Na^+K^+ -ATPase activity was significantly lower in Br-treated animals than in controls (330 ± 169 versus 558 ± 146 pmol inorganic phosphate/mm/h, respectively); values did not differ in the medullary thick ascending limb of Henle's loop between Br-treated and control animals (132 ± 74 versus 165 ± 113 pmol inorganic phosphate/mm/h, respectively). *In vitro* effects of PRL were investigated by determining the enzyme activity after incubation of renal sections from Br-treated and untreated animals with different concentrations of PRL. Results suggest that PRL may affect renal Na^+K^+ -ATPase activity in the distal tubule in the neonatal period but do not support a major role of PRL in the enzyme maturation. (*Pediatr Res* 31: 207–210, 1992)

Abbreviations

PRL, prolactin
mTAL, medullary ascending limb of Henle's loop
DT, distal tubule
Br, bromocriptine

Mechanisms by which fluid and salt balances are preserved in the fetal and neonatal periods are not completely understood. The development of fetal and neonatal sodium excretion ability (1, 2) and its relationship with hormones involved in volume regulation such as the renin-angiotensin-aldosterone system (3, 4) vasopressin (5, 6), and, more recently, atrial natriuretic factor (6) have been investigated and are still controversial. PRL is a hormone with apparently little effect in the control of fluid volume in mature animals (7, 8); however, its role has been recently questioned in fluid and electrolyte homeostasis throughout vertebrate phylogeny (9) and its elevated levels (10) during gestation and the neonatal period.

Renal Na^+K^+ -ATPase, the driving force of tubular reabsorption, plays a key role in fluid and electrolyte homeostasis; in a previous study (11), we reported in mature animals a parallelism in enzyme activity and PRL levels *in vivo* and PRL stimulation of the sodium pump *in vitro* in a range of concentrations similar to those observed during maturation, suggesting that the hormone may control the enzyme activity at this period. In the

present study, we first investigated the effect of *in vivo* changes in PRL secretion on renal Na^+K^+ -ATPase in 7-d-old rats. Groups of neonatal rats were treated with Br, a drug that blocks PRL secretion, for 5 d and renal Na^+K^+ -ATPase activity in these treated animals was compared with that of untreated animals. The hormone effects were investigated *in vitro* by determining the enzyme activity in kidney sections from Br-treated and control animals after incubation with increasing concentrations of ovine PRL.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were obtained and allowed to deliver naturally at our research facilities. They were observed at least twice daily to determine the exact parturition time; day birth counted as d 1. Pups were kept with their dam. All animals had free access to the standard laboratory diet (Soufirat, UAR, Epinay sur Seine, France) and tap water. Litters ($n = 3$) were reduced to five pups, and treatment for a given litter was determined randomly. No animals died. Ten male adult rats (300 g) were used as a reference group (adult control).

Br Treatment. On d 7, three pups from each litter (Br) were treated with Br (2-Br-ergocriptine mesylate; Sandoz Pharmaceuticals, Hanover, NJ). Because in preliminary experiments intraperitoneal and s.c. injections induced necrosis, the drug was given by suckling. In a previous study (11), administration of 1 mg/kg body wt/d (intraperitoneally) was sufficient to inhibit PRL secretion. However, inasmuch as the drug absorption in the neonatal period is unknown, the dose was increased to 5 mg/kg body wt/d; the drug and an equal weight of tartaric acid were dissolved in absolute ethanol and then mixed with 0.9% saline to the desired concentration. Each pup received by suckling 0.05 mL of the preparation each day for five d. Control pups received vehicle only. After 5 d of treatment (d 12), animals were killed.

Determination of Na^+K^+ -ATPase Activity. Renal enzyme activities were determined by quantitative cytochemistry (12) adapted to image analysis (13).

Tissue sampling. Immediately after the animals were killed, kidneys were removed, decapsulated, and frozen to -70°C in an *n*-hexane bath. Enzyme activities were determined on unfixed kidney sections (8 μm) obtained in a Bright low temperature (-20°C) cryostat (Instrument Company, Ltd., Huntingdon, UK) with an automatic cryotome device to ensure constancy of slice thickness. The kidney fragment was oriented in the microtome so that sections were parallel to the cortico-papillary axis. Each slice was then surrounded by a plastic ring to retain the cytochemical media.

Cytochemical procedure. The procedure used was derived from that described by Chayen *et al.* (12) in which a lead acetate complex precipitates phosphates liberated by the enzyme from ATP. After a 10-min preincubation in a medium containing a 40% (wt/vol) solution of Polypep (Sigma 5115, Sigma Chemical Co., St. Louis, MO) in 0.2 M Tris buffer (pH 7.5) to remove free phosphates that otherwise could produce a background color-

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tion, the preincubation medium was removed and replaced by a prewarmed incubation medium consisting of a 40% solution of Polypep in 0.2 M Tris buffer containing sodium chloride (150 mM), magnesium sulfide (20 mM), potassium chloride (5 mM), low calcium vanadate free ATP (16.5 M), levamisole (1 mM), and lead acetate complex (32 mg/mL), final pH adjusted to 7.5. Alternate sections were treated with the same medium with ouabain octohydrate (1 mM) added. After 15 min at 37°C, the incubation medium was suctioned off and the slices were rinsed in cold 0.2 M Tris buffer (pH 7.5) to stop the reaction. Finally, after immersion in sulfide-saturated water (1 min), sections were washed with distilled water, allowed to dry, and mounted in Z5 medium (14). Na⁺K⁺-ATPase activity was calculated as the difference between total ATPase activity (determined without ouabain) and ouabain-insensitive ATPase activity (determined with ouabain).

In vitro experiments. In these experiments, ovine prolactin (oPRL; NIADDK, Baltimore, MD; 30.5 UI/mg, dissolved in 0.03 M NaHCO₃ in 0.15 M NaCl, pH adjusted to 7.5) at various concentrations (12.5, 125, and 1250 ng/mL; 530, 5320, and 53 180 pmol/L) or vehicle (oPRL) was added to the Polypep solution on kidney sections from control and Br-treated animals during the preincubation step; after 20 min at 37°C, the preincubation medium was suctioned off and replaced by the incubation medium, as described in the standard procedure.

All reagents were purchased from Sigma.

Quantification. Absorption determinations of the cytochemical reaction product were performed *in situ* by video densitometry (13), using an image analysis system consisting of a computer image processor (NS 1500; Nachet Vision, Evry, France), an Apple IIe microcomputer, and a camera (Lhesa LH 4015; Lhesa Electronique, Cergy Pontoise, France) fixed on an optical microscope (Leitz Orthoplan; Leitz, Rueil-Malmaison, France) with a ×40 objective.

Appropriate algorithms were developed allowing, successively: 1) automatic selection of identified tubule sections, 2) measurement of the chromophore absorption on each point of the frame (1 pixel = 1 μm²) of the precipitate present in the tubule section, 3) integration of the individual absorption values thus obtained over the whole tubule section area, and 4) determination of simple morphologic parameters such as tubule diameter estimated from the tubule section area. Before each absorption measurement, filters of known absorbance (Melles Griot, Saint Quentin en Yvelines, France) were assayed to check that transformation from gray levels to absorbances was correct; absorbances were yielded relative to background that was subtracted as a blank. Absorbances were measured at a 420-nm wavelength.

Identification of structures. Na⁺K⁺-ATPase and ouabain-insensitive ATPase activities were determined in the mTAL and in DT present in the juxtamedullary cortex. The various segments of the nephron studied were characterized by their morphologic appearance (15) and localization within the cortex or medulla, inasmuch as the sections were performed along the cortico-papillary axis. Among all tubular structures present on a given video frame (280 × 192 μm), all structures identified as pertaining to a definite nephron segment were automatically analyzed, provided that the tubule section was transversal; five to 10 tubule sections were thus quantified by video frame (for each segment), and five video frames were analyzed by slice. Na⁺K⁺-ATPase activity was determined from the difference between the mean of total ATPase activities (three slices) and the mean of ouabain-insensitive activities (three slices).

Expression of results. The algorithm used yields the value of the absorbance integrated on the area of the tubule section (ΣA_T). Enzyme activities, proportional to the amount of chromophore and, therefore, to the absorbance of the precipitate present in the tubule section within the slice were standardized for time

of incubation and tubule length as follows:

Enzymatic activity (absorbance units/mm tubule/h)

$$= \Sigma A_T \cdot e \cdot 10^3 \cdot 60 / t$$

where *e* is the slice thickness in μm, and *t* is the time of incubation in min. A standardization curve (16) allowed the transformation of absorbance units into more conventional biochemical units.

Statistical Analysis. Results are expressed as mean ± SD; comparison between groups was performed by using analysis of variance; *p* values less than 0.05 were considered significant.

RESULTS

Effect of Br treatment on weight gain. Treatment by Br did not impair the animals' growth, inasmuch as mean weight before (Br 12.2 ± 0.6 versus control 12.4 ± 0.8 g) and after treatment (Br 20.1 ± 1.8 versus control 20.8 ± 1 g) did not differ in Br-treated and controls animals.

Effect of Br treatment on renal ATPase activities. Morphology of renal structures did not differ strikingly at optical microscope examination of kidney slices from Br-treated or untreated animals; development of the cortical zone occurred normally and tubule diameter in mTAL (Br 19.3 ± 2.4 versus control 19.1 ± 2.0 μm) and in DT (Br 25.6 ± 2.9 versus control 25.2 ± 2.6 μm) were similar. Enzymes activities are reported in Table 1; Na⁺K⁺-ATPase activity in DT was significantly (*p* < 0.05) lower in Br-treated animals (59% of control values) than in controls; Na⁺K⁺-ATPase activity in mTAL and ouabain-insensitive ATPase activities in both segments did not differ significantly in Br-treated and control animals.

Effect of PRL addition in vitro. The effect of PRL on ATPase activities *in vitro* was studied by adding different concentrations (0, 12.5, 125, and 1250 ng/mL; 0, 530, 5320, and 53 180 pmol/L) of the hormone in the preincubation medium (Fig. 1). PRL addition had no effect on enzyme activities in kidney slices from control rats. In kidney slices from Br-treated animals, PRL (12.5 ng/mL; 530 pmol/L) addition increased significantly (*p* < 0.05) Na⁺K⁺-ATPase activity in DT by 98%, reaching the activity level observed in controls (Br 615 ± 67; control 557 ± 54 pmol inorganic phosphate/mm/h; NS). Maximal stimulation was observed when 1250 ng/mL (53 180 pmol/L) PRL was added, the activity increasing 2-fold. In contrast, PRL addition did not increase enzyme activities significantly in mTAL; no significant correlation could be found between PRL concentration and Na⁺K⁺-ATPase activity in mTAL or DT. Ouabain-insensitive activity remained unchanged in all cases.

DISCUSSION

In the present study, we found that a 5-d *in vivo* inhibition of PRL secretion by Br in 7-d-old rats induced a decrease in renal Na⁺K⁺-ATPase activity in the DT, whereas enzyme activity

Table 1. ATPase activities in different segments of nephron in adult control rats (C Ad; *n* = 10) and in control (C; *n* = 6) and Br-treated (Br; *n* = 9) young rats (d 12) killed after 5 d of treatment (mean ± SD)*

	mTAL	DT
Na-K-ATPase (pmol Pi/mm/h)		
C Ad	1270 ± 640	1374 ± 235
C (d 12)	165 ± 113	558 ± 146
Br (d 12)	132 ± 74	330 ± 169†
Ouabain-insensitive ATPase (pmol Pi/mm/h)		
C Ad	1558 ± 688	2719 ± 921
C (d 12)	337 ± 157	1088 ± 306
Br (d 12)	397 ± 73	1175 ± 351

* Pi, inorganic phosphate.

† *p* < 0.01 Br (d 12) vs C (d 12).

Fig. 1. Effect of oPRL addition *in vitro* on Na^+K^+ -ATPase activity. PRL was added during the preincubation step at various concentrations (12.5, 125, or 1250 ng/mL) on kidney slices from control (empty columns) or Br-treated (hatched columns) 12-d-old rats. Na^+K^+ -ATPase activity was determined in the mTAL and in the DT. Control slices (oPRL) were preincubated with PRL solvent only. *, $p < 0.05$; ***, $p < 0.001$ vs oPRL.

remained unchanged in the mTAL. Although these results suggest that PRL may affect the pump activity in the neonatal period, it cannot be assessed from these *in vivo* experiments that the changes observed in ATPase activity are related to a decrease in plasma PRL concentration. On one hand, inasmuch as plasma PRL concentration could not be determined, insensitivity to Br or incomplete inhibition of PRL secretion cannot be excluded. Insensitivity to dopamine has been reported in the early neonatal period in rats, but the response increased by d 5 (17). The drug absorption may have been insufficient, inasmuch as Br was administered by suckling; nursing pups may imbibe some PRL from their mother's milk, which will not be affected by the drug. On the other hand, even if PRL secretion was correctly inhibited by the dopamine agonist, the specificity of the effect cannot yet be assessed because it might have been produced by a direct or indirect dopamine agonist effect. Aldosterone may be under dopamine control (18), and it has been proposed (4) that aldosterone contributes to some extent to the relatively high distal tubular Na reabsorption in the immature nephron; dopamine itself binds to proximal tubule and influences transepithelial Na transport (19).

To investigate further the role of PRL on the enzyme, experiments were performed in which PRL was added *in vitro* directly on kidney sections: the specific restoration of Na^+K^+ -ATPase activity in DT by PRL addition strongly suggests that the decrease induced by Br in this segment is due to the decrease in PRL secretion and confirms that mTAL is not sensitive to the hormone. The highest response was observed for 125 ng/mL (5320 pmol/L), a level of concentration observed in plasma in the neonatal period.

In a previous study performed in mature animals (11), similar treatment by Br and PRL addition *in vitro* induced similar effects in the renal pump activity, but in both segments. Differences in the target of tubular response between young and mature rats may be related to the heterogeneous immaturity of neonatal kidney in the rat. The formation of new nephrons is completed by the 7th or 8th postnatal day in this species (20), but functional development and structural differentiation of nephrons continues until the 35th to 40th postnatal day (21). Differences in

maturity between segments could be related to differences in the nephron from which they originate. Because nephrogenesis proceeds centrifugally, juxtamedullary nephrons are more mature than the more superficial cortical nephrons that begin to develop during the first postnatal week. Although elongation of Henle's loop is slow, it cannot be excluded that on d 12 some mTAL in which determinations were performed originated from cortical nephrons, because the medulla may contain both cortical and juxtamedullary loops; these structures are likely to be less mature than the DT studied at the same period, whose juxtamedullary origin is easy to ascertain because of their localization within the cortex.

Another possibility is that the differences observed may be linked to intranephronic heterogeneity of tubular maturation. In the present study, the pump activity represented 15% of mature activity on d 12; these results are in accordance with previously reported data in this segment (22); in DT, although activity was also lower than in the mature animal, it represented a higher fraction of mature activity: 40% on d 12. Although no data are available, to our knowledge, on the developmental pattern of enzyme activity in this part of the nephron, results are in accordance with previous work using micropuncture techniques (23) showing that the sodium reabsorption capacity of the developing nephron is more efficient in DT than in more proximal segments; together these results suggest a functional intranephronic heterogeneity in nephron maturation. Therefore, within the nephron unit, relative immaturity of the mTAL *versus* DT could explain its lack of responsiveness to PRL.

Altogether, the results presented suggest that PRL can increase the pump activity in DT in young rats. Presence of PRL receptors in the kidney have been reported, but their precise localization has not been established clearly. Most labeling studies demonstrate that receptors are situated in proximal tubules, but specific binding has been found in distal structures. No data are available on the presence and developmental pattern of this putative PRL receptor; however, receptors for other hormones (*e.g.* aldosterone, oxytocin, arginine-vasopressin) are present in the neonatal period, although their localization may differ. The mechanisms by which the hormone controls the enzyme activity cannot be

drawn from these preliminary experiments. The second messenger for PRL has not been established. PRL could affect the enzyme either directly or through another mediator (increase in intracellular sodium, for example). This study does not allow discrimination of an increase in catalytic site activity or the number of pump units. Although morphologic parameters were unchanged, PRL could act by favoring the growth or differentiation of structures necessary to the enzyme expression (for example, the basal membrane). However, an effect through maturation of structures is unlikely, inasmuch as morphologic parameters were unaffected; moreover, the time course of PRL effect *in vitro* (increasing activity within 20 min) argues against a maturation process.

Finally, the question of the physiologic significance of the possible control by PRL of the pump activity during the neonatal period must be addressed. PRL concentration is high in the fetal and early neonatal period. Different authors have suggested a role for fetal PRL in water and salt conservation (24). Other reports have suggested that it could also control the neonatal reservoir (25). Our data suggest that by increasing pump activity PRL may contribute to the relative overcapacity of DT to reabsorb sodium, and, therefore, may contribute to the difficulties of inducing natriuresis in volume-expanded young rats. The decrease in plasma PRL concentration during neonatal life could contribute to the water loss that occurs during this period, independent of an adequate fluid intake.

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