

Calcium Regulating Hormones during the Estrous Cycle of the Rat (41539)

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Abstract. Plasma levels of calcium (Ca), phosphate (P), calcitonin (CT), parathyroid hormone (PTH), and prolactin (PRL) were measured at 8, 13, and 17 hr during the 4-day estrous cycle of the rat. Ca levels fell throughout the day during proestrus (PE) and estrus (E). In contrast Ca rose transiently during diestrus (D1, D2). P levels fluctuated inconsistently at all stages of the cycle with the exception of E where P levels were significantly higher at 13 hr. CT levels showed an increase during D1 and D2 and fell to their lowest values during E, at 13 hr. Daily fluctuations in each stage were also recorded. Variations of PTH levels during the estrous cycle were minor. PRL levels increased sharply during PE. No direct relationship between PRL secretion and CT secretion could be established. These results indicate that CT but not PTH varies specifically in relation to the estrous cycle. They suggest that there is a link between sexual hormones and CT, apparently independent of plasma calcium levels.

Among the several factors affecting calcitonin (CT) levels, sex has recently been shown to exert an important influence. For instance, Roos *et al.* (1) and Peng and Garner (2) have observed higher levels of CT in female than in male rats during aging. CT levels also vary according to the reproductive state. Circulating hormone increases during gestation (3) and lactation (4) and decreases after ovariectomy (5, 6), the lowest values are reached during estrous (7). This last finding has prompted us to investigate both calcium (Ca) and phosphate (P) levels during the estrous cycle in the rat in relation to CT, parathyroid hormone (PTH), and prolactin (PRL) fluctuations. As diurnal variations of CT levels have been reported in both male and female rats (1) blood samples were obtained at three different times in the day.

Materials and Methods. *Animals.* One hundred 3-month-old female rats (Wistar Cf) weighing 250 g were housed under controlled conditions of temperature, humidity, and light (light on 0600 to 1800 hr). Estrous cycles were monitored according to vaginal smears taken daily between 8 and 9 hr. Only animals showing three or more regular 4-day cycles were used. During the experimental period, free access to food and water was allowed. Blood samples were obtained by retroorbital sinus puncture using heparin as an anticoagulant.

Plasma was immediately separated at 4° and Ca and P estimated directly. Aliquots were immediately frozen until assayed for CT, PTH, and PRL.

Calcium and phosphate estimations. Ca was estimated by atomic absorption spectrophotometry. P was measured by a colorimetric microtechnique (8).

Radioimmunoassays. CT was estimated by a sensitive assay for human CT which shows a total cross-reaction with murine CT (9, 10). In brief 50 μ l of rat plasma was incubated in the presence of antiserum against human CT (HCT) at a final dilution of 1/250,000 in phosphate-buffered albumin in a total volume of 400 μ l for 6 days, then 100 μ l of ¹²⁵I-HCT (specific activity 200 μ Ci/ μ g) was added and incubation continued for another 5 days. Free and bound hormone were separated by absorption on dextran-coated charcoal. Standard curves were performed using synthetic HCT and protein effects controlled by the addition of affinity stripped plasma (50 μ l) (11).

A sensitive RIA for PTH was developed using antibodies obtained in the goat (12) against 1-34 N terminal fragment of human PTH (HPTH) prepared by a solid-phase method (13) according to the sequence proposed by Niall *et al.* (14). Synthetic 1-34 HPTH was used both as standard and tracer after iodination by chloramine-T (15). Antiserum was used at a final dilution of 1/250,000 in barbital buffer, 0.05 M, pH 8.6, containing 17% of PTH free (affinity stripped) human

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plasma. Incubation was carried out for 4 days at 4° and then tracer (specific activity 100 $\mu\text{Ci}/\mu\text{g}$) was added and incubation continued for 3 more days; 100 μl of plasma sample or various quantities of standard 1-34 HPTH solutions in a final volume of 500 μl were used for estimating unknowns or establishing standard curves. Free and bound hormone were separated by a modified dextran charcoal method (16). Nonspecific adsorption was controlled by the incubation of each unknown in the absence of antibody.

PRL was measured by a radioimmunoassay for rat prolactin using reagents obtained from NIAMDD. Preparation of tracer (specific activity 150 $\mu\text{Ci}/\mu\text{g}$) and incubation conditions were carried out according to the experimental protocol recommended by NIAMDD.

All radioimmunoassay data were computed using a spline function (17).

Statistical treatment of data. Variance analysis was used for all data showing homogeneity of variances (Bartlett's test) (18). In case of variance heterogeneity (PRL) a *t* test was used after due reduction of the number of degrees of freedom (18).

Results. *Plasma calcium levels.* Highly significant fluctuations (*F* test, $P < 0.01$) were recorded during estrous cycle and during the day. Ca levels were higher at 8 hr PE as compared to 8 hr D1 and D2, and higher at 8 hr E as compared to 8 hr D2 ($P < 0.05$) (Table I). At all stages of the cycle, Ca levels declined between morning and evening. This fall was significant for PE between 8 and 17 hr (P

< 0.05) and for D1 and D2 between 13 and 17 hr ($P < 0.05$) but not significant for E.

Plasma phosphate levels. P changes were similar during the day for all the stages (Table I). A significant rise was observed during E between 8 and 13 hr ($P < 0.02$).

Plasma CT levels. No differences were observed at 8 hr for all the stages (Fig. 1). High values were observed at 13 hr D1 and D2 (D1/E, $P < 0.01$; D2/E, $P < 0.02$; D1/PE, $P < 0.05$). At 17 hr D1, the increase in CT values persisted (D1/E, $P < 0.01$, D1/PE, $P < 0.02$, D1/D2, $P < 0.01$) (Fig. 1).

Plasma PTH levels. The pattern was devoid of significant fluctuations during the cycle or during the day (Fig. 1).

Plasma PRL levels. PRL levels rose during PE, with maximum values at 17 hr, 8 hr/17 hr, $P < 0.002$ (Fig. 1). No significant change was observed during the rest of the cycle; the values were very low (17 ng/ml).

Discussion. Our previous results showed that CT levels fluctuated during the estrous cycle of the rat (7). It appears from the data reported here that the fluctuations in levels of Ca are not correlated with those of CT; the physiological secretion of CT seems to be independent of variations of blood calcium levels. The data reported here also confirm our previous findings that PTH levels were not significantly modified during the cycle. Thus the restricted fluctuations in Ca levels cannot be ascribed to compensatory changes in PTH secretion. It is important to point out that we have used an N-terminal assay directed toward the biologically active part of the mol-

TABLE I. PLASMA CALCIUM AND PHOSPHATE LEVELS (mg/100 ml)

	8 hours		16 hours		17 hours	
	Ca	P	Ca	P	Ca	P
PE	11.42 \pm 0.12	5.18 \pm 0.19	11.18 \pm 0.15	5.80 \pm 0.12	10.88 \pm 0.13 ^a	5.63 \pm 0.27
E	11.21 \pm 0.25	5.20 \pm 0.16	11.02 \pm 0.15	6.14 \pm 0.18 ^d	10.83 \pm 0.17	5.48 \pm 0.27
D1	10.91 \pm 0.31 ^a	5.51 \pm 0.26	11.19 \pm 0.11	5.62 \pm 0.09	10.64 \pm 0.04 ^b	5.45 \pm 0.14
D2	10.76 \pm 0.11 ^a	5.22 \pm 0.30	11.21 \pm 0.16	5.60 \pm 0.22	10.62 \pm 0.23 ^c	5.61 \pm 0.21

Note. Values are the mean \pm SEM for groups of five animals. PE, proestrus; E, estrus; D1, diestrus Day 1; D2, diestrus Day 2. Data were subjected to variance analysis.

^a Significantly different from PE, 8 hr ($P < 0.05$).

^b Significantly different from D1, 13 hr ($P < 0.05$).

^c Significantly different from D2, 13 hr ($P < 0.05$).

^d Significantly different from E, 8 hr ($P < 0.02$).

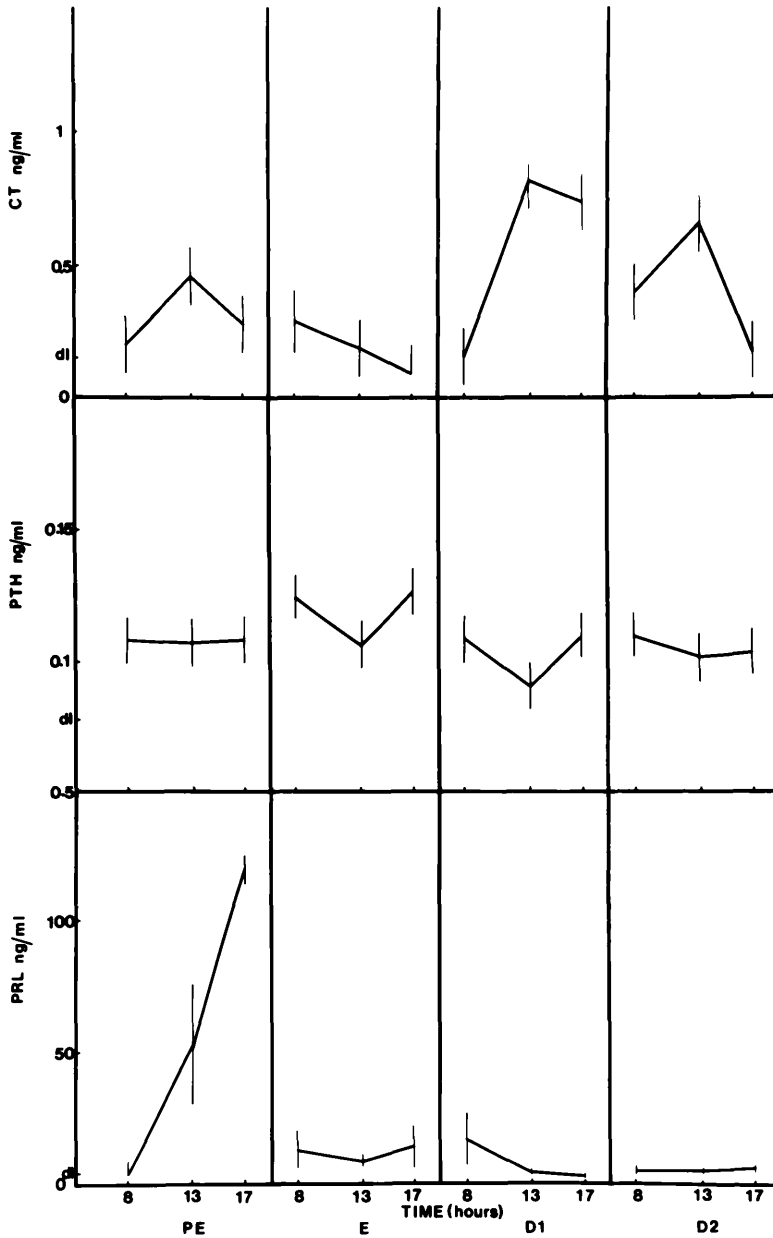


FIG. 1. Plasma PRL, CT, and PTH levels during 4-day estrous cycle. PRL levels rose during PE, reaching the highest value at 17 hr; 13 hr/17 hr, $P < 0.05$; 8 hr/17 hr, $P < 0.002$. PTH values showed no significant variations during the estrous cycle. CT values pattern differed with the hour of the day and with the day of the cycle. Higher values were observed on D1 and D2 at 13 hr: D1/E, $P < 0.001$; D2/E, $P < 0.02$; D1/PE, $P < 0.05$; and at 17 hr on D1: D1/E, $P < 0.01$; D1/PE, $P < 0.02$; D1/D2, $P < 0.01$. Values shown are the mean \pm SE (vertical lines) for a group of five rats. PE, proestrus; E, estrus; D1, diestrus Day 1; D2, diestrus Day 2.

ecule. In humans, a distinct increase of PTH values has been reported during the midcycle when compared to values during the follicular

and luteal phase, at which time the rise of CT levels was less distinct and consistent (19). However, Baran *et al.* (20) found no changes

in PTH and CT levels in the human female during the menstrual cycle. These differences in hormonal secretion during the sexual cycle in man and rat could be species specific inasmuch as CT levels are higher in female rats as compared with those in age-matched males (1); the reverse has been found in humans (21, 23). In the female rat, CT levels increase during aging, whereas they diminish with age in women (24). The fluctuations in CT levels during E might be due to changes in the secretion of hypophyseal hormone as a selective increase of PRL levels in man has been reported after exogenous PTH administration (25), while CT infusion at pharmacological doses reduced PRL levels (26). From our results no direct relationship can be found between PTH and CT with PRL levels under physiological conditions. Furthermore the fluctuations of Ca levels were not correlated with the important surge in PRL levels during PE.

Though circulating FSH and LH levels have not been measured in our experiences, fluctuations in CT levels are presumably not directly related to LH and FSH secretion as these hormones are increased during proestrus (27) while the principal fluctuations in CT occur during estrus and diestrus.

During the estrous cycle important fluctuations of food intake and body weight have been reported (28), food intake is decreased during estrus and increased during diestrus. Therefore the fall in CT level during estrus and the rise during diestrus could be in relation to changes in food intake during the estrous cycle. However, it has been shown (29) that modifications of the feeding schedule of male rats does not alter CT circadian rhythm. More probably the variations in plasma CT levels during the estrous cycle are the consequence of a regulation exercised by the central nervous system and/or hypothalamus. The identification of specific receptors for calcitonin in the central nervous system and in the hypothalamus in particular (30) tends to support this hypothesis.

Previous investigations on the secretion of CT in female rats have advocated a protective role of the hormone during periods of Ca loss such as pregnancy and lactation. Our results suggest that during a physiological state in which presumably the skeleton is not sub-

jected to major Ca losses, the important fluctuations in CT secretion could reflect a role of the hormone during the estrous cycle.

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1. Roos BA, Cooper CW, Frelinger AL, Deftos LJ. Acute and chronic fluctuations of immunoreactive and biologically active plasma calcitonin in the rat. *Endocrinology* **103**:2180-2186, 1978.
2. Peng TC, Garner SC. Sex difference in serum calcitonin level in rats as related to feeding, fasting and age. *Endocrinology* **107**:289-293, 1980.
3. Garel JM, Jullienne A. Plasma calcitonin levels in pregnant and newborn rats. *J Endocrinol* **75**:373-382, 1977.
4. Toverud SU, Cooper CW, Munson PL. Calcium metabolism during lactation: Elevated blood levels of calcitonin. *Endocrinology* **103**:472-479, 1978.
5. Cressent M, Bouizar Z, Pidoux E, Moukhtar MS, Milhaud G. Effet de l'ovariectomie sur le taux de calcitonine plasmatique chez le rat. *CR Acad Sci D* **289**:501-504, 1979.
6. Cressent M, Bouizar Z, Moukhtar MS, Milhaud G. Effect of ovariectomy on circulating calcitonin levels in the rat. *Proc Soc Exp Biol Med* **166**:92-95, 1981.
7. Cressent M, Bouizar Z, Taboulet J, Moukhtar MS, Milhaud G. Calcitonin secretion during the estrous cycle of the rat. *Biomedicine* **33**:129-130, 1980.
8. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. *Anal Chem* **28**:1756-1758, 1956.
9. Milhaud G, Tharaud D, Jullienne A, Moukhtar MS. Radioimmunoassay of rat calcitonin. In Taylor S, ed. *Endocrinology, Proceedings, 3rd International Symposium London, 1971*. London Heinemann, pp380-385, 1972.
10. Moukhtar MS, Tharaud D, Jullienne A, Raulais D, Calmettes C, Milhaud G. Immunological similarity of human and rat calcitonin confirmed by immunofluorescent methods. *Experientia* **29**:552-555, 1973.
11. Moukhtar MS, Jullienne A, Tharaud D, Taboulet J, Milhaud G. Extraction et concentration de la calcitonine plasmatique humaine par des anticorps couplés sur support solide. *CR Acad Sci D* **276**:3445-3448, 1973.
12. Desplan C, Jullienne A, Moukhtar MS, Raulais D, Rivaille P, Milhaud G. Isolation of circulating immunoreactive human parathyroid hormone. Comparison with a 1-34 N terminal synthetic fragment. *Proc Soc Exp Biol Med* **157**:241-244, 1978.
13. Rivaille P, Staub JF, Jullienne A, Raulais D, Milhaud G. Strategy in the solid phase synthesis and purification of peptide hormones. Control methods applied to the active fragment of human parathyroid hormone. In Wolman Y, ed. *Peptides*. New York, Wiley, pp.221-225, 1975.

14. Niall HD, Saner RT, Jacobs JW, Kentmann HT, Segre GV, O'Riordan JLH, Aurbach GD, Potts JT Jr. The amino acid sequence of the amino terminal 37 residues of human parathyroid hormone. *Proc Nat Acad Sci USA* **71**:384-388, 1974.
15. Hunter WM, Greenwood FC. Preparation of ¹³¹I labelled human growth hormone of high specific activity. *Nature (London)* **194**:495-496, 1962.
16. Herbert V, Lan KS, Gottlieb CW, Bleicher SJ. Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab* **25**:1375-1384, 1965.
17. Marschner I, Erhardt F, Scriba PC. Calculation of the radioimmunoassay standard curve by spline function in RIA and related procedures in medicine. International Atomic Energy Authority, Istanbul Meeting, September 1973, Vol I:pp111-120, 1974.
18. Norman TJ, Bailey MAM. *Statistical Methods in Biology*. London, English Universities Press, 1959.
19. Pitkin RM, Reynolds WA, Williams GA, Hargis GK. Calcium regulating hormones during the menstrual cycle. *J Clin Endocrinol Metab* **47**: 626-632, 1978.
20. Baran DT, Whyte MP, Haussler MR, Deftos LJ, Slatopolsky E, Avioli L. Effect of the menstrual cycle on calcium regulating hormones in the normal young woman. *J Clin Endocrinol Metab* **50**:377-379, 1980.
21. Heath H, Sizemore GW. Plasma calcitonin in normal man. Differences between men and women. *J Clin Invest* **60**:1135-1140, 1977.
22. Parthemore JG, Deftos LJ. Calcitonin secretion in normal human subjects. *J Clin Endocrinol Metabol* **47**:184-188, 1978.
23. Hillyard CJ, Stevenson JC, MacIntyre I. Relative deficiency of plasma calcitonin in normal women. *Lancet* **1**:961-962, 1978.
24. Shamonki IM, Frumar AM, Tataryn IV, Meldrum DR, Davidson BH, Parthemore JG, Judd HL, Deftos LJ. Age-related changes of calcitonin secretion in females. *J Clin Endocrinol Metab* **50**:437-439, 1980.
25. Isaac R, Merceron RE, Caillens G, Raymond JP, Ardaillou R. Effect of parathyroid hormone on plasma prolactin in man. *J Clin Endocrinol Metabol* **47**:18-23, 1978.
26. Isaac R, Merceron R, Caillens G, Raymond JP, Ardaillou R. Effects of calcitonin on basal and thyrotropin-releasing hormone-stimulated prolactin secretion in man. *J Clin Endocrinol Metab* **50**:1011-1015, 1980.
27. Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17 β throughout the 4-day estrous cycle of the rat. *Endocrinology* **94**:1704-1708, 1974.
28. Tarttelin MF, Gorski RA. Variations in food and water intake in the normal and acyclic female rat. *Physiol Behav* **7**:847-852, 1971.
29. Hirsch PF, Hagaman JR. Feeding regimen, dietary calcium and the diurnal rhythms of serum calcium and calcitonin in the rat. *Endocrinology* **110**:961-967, 1982.
30. Rizzo AJ, Goltzman D. Calcitonin receptors in the central nervous system of the rat. *Endocrinology* **108**:1672-1677, 1981.

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