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DIURNAL VARIATION OF PLASMA CALCIUM AND CALCITONIN FUNCTION IN THE RAT

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SUMMARY

1. These experiments were designed to investigate the normal physiological role of calcitonin in the control of blood calcium.

2. The rat can be adapted to an artificial cycle of alternating 12-hr periods of light and darkness. Since rats eat only in the dark, calcium absorption is confined to the dark period.

3. In rats thus adapted, the plasma calcium and phosphate levels show cyclic variation related, among other factors, to the calcium intake.

4. The variation observed is modified by the removal of the thyroid gland. This cannot be attributed to deprivation of thyroxine and triiodothyronine because these experiments were carried out within 2 days of thyroidectomy.

5. Thyroidectomy raises the plasma calcium and phosphate levels, but only if performed during the dark-fed period. This suggests the existence of a diurnal rhythm in endogenous calcitonin function.

6. The magnitude of the falls in plasma calcium and phosphate, produced by exogenous calcitonin, also varies with the time of day. This shows that the rat's responsiveness to calcitonin also varies diurnally.

INTRODUCTION

Previous studies of the role of calcitonin in the control of plasma calcium have relied on two experimental approaches. Either the hormonal response to calcium challenge has been measured, or the impaired ability of thyroidectomized animals to restore normal calcium levels after such a challenge has been assessed (Milhaud & Perault-Staub, 1968; Care, Cooper, Duncan & Orimo, 1968; Lee, Deftos & Potts, 1969; Gray & Munson, 1969; Cooper, Hirsch & Munson, 1970; Munson & Gray, 1970).

However, none of these studies has established whether calcitonin

secretion plays an important role in regulating plasma calcium under normal conditions of life.

The rat, eating *ad libitum* and adapted to 12 hr of alternating light and darkness, exhibits diurnal cycles in food intake (Siegel & Stackey, 1947) and, consequently, in calcium absorption.

We have used rats adapted in this way to study the physiological significance of calcitonin, with two experimental approaches.

1. The assessment of spontaneous diurnal variation in plasma calcium level, and the way in which this variation is affected by thyroidectomy.

2. Demonstration of a diurnal rhythm in the acute effect of thyroidectomy on plasma calcium level and in the magnitude of responses to exogenous calcitonin.

Since calcitonin is known to affect plasma levels of phosphate as well as calcium (Hirsch, Voelkel & Munson, 1964), both these variables have been measured.

METHODS

Animals. Experiments were performed on male Wistar CF rats 50 days old (120–140 g). They were adapted to a 12 hr cycle of alternating artificial light and darkness, fed only during the dark part of the cycle with an equilibrated diet (0·6 % Ca, 0·6 % PO_4 as P) and drank tap water *ad libitum*.

Rats, received 20 days old, were randomly divided in two groups and housed, during 1 month, in two parts of a special room arranged so that one group was in the dark when the other one was in the light.

Surgical procedures. All operations were conducted under ether anaesthesia. One month before thyroidectomy, the parathyroid glands were auto-transplanted to the sternohyoid muscle. One week later, plasma calcium was measured: levels of 10·0 mg/100 ml. or more indicated that the transplanted parathyroid glands were functional. Rats were thyroidectomized by blunt dissection (TX) and control animals sham operated.

Hormone. Partially purified porcine calcitonin extract was prepared in the laboratory by the method of Milhaud, Moukhtar, Bourichon & Perault (1965) and injected intravenously (100 M.R.C. mU per rat). Control animals received the vehicle alone (acetate buffer 0·1 M, pH 4·6). The hypocalcemic and hypophosphataemic response to calcitonin extract was measured 1 hr after injection.

Blood collection and analysis. Blood samples were obtained by retro-orbital puncture with heparinized pipettes. Plasma was immediately separated by centrifugation: calcium was determined by flame photometry and phosphate by the colorimetric method of Chen, Toribara & Warner (1956).

Statistical analysis. Allocation of treatments and distribution of rats into experimental groups were conducted randomly. The mean values were compared using Student's t test. Data were considered significant at the $P < 0{\cdot}05$ level.

RESULTS

Diurnal variations of plasma calcium and phosphate levels

The rats which were bled in groups of five or six, fall into three series, described according to the treatment employed as 'Normal' (intact), 'Control' (parathyroid-transplanted and sham-thyroidectomized) and 'TX' (parathyroid-transplanted and thyroidectomized). One group from each treatment series was sacrificed every 3 hr throughout the 24. Sufficient rats were used in the study to avoid bleeding any animal more than once.

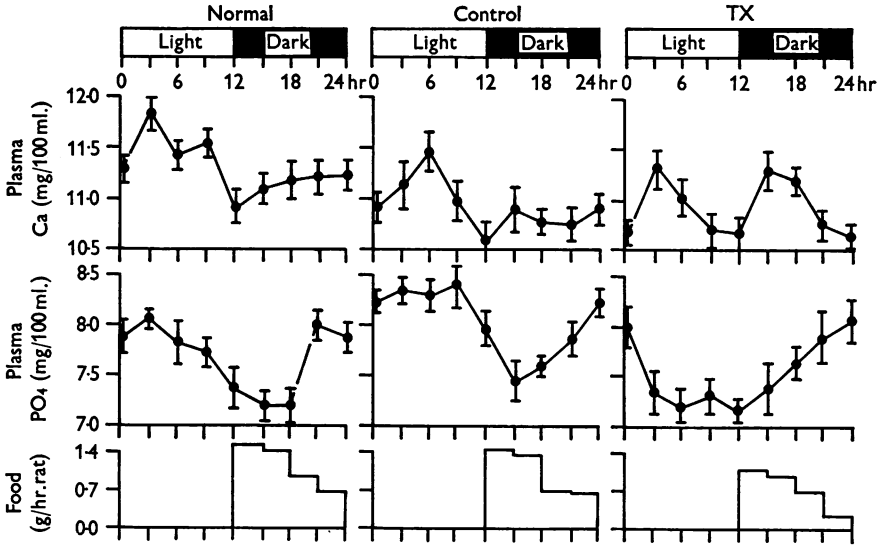

Fig. 1. 24-hr pattern of plasma calcium and phosphate level in normal (N), 2-days thyroidectomized (TX) and their control rats. Each point represents the mean value of plasma calcium or phosphate concentration of five or six rats and the vertical line shows the S.E. Each group of rats was used only once. The light-unfed period takes place from 0–12 hr and the dark-fed period from 12 to 24 hr. The bottom part of the Figure shows the mean food intake in g/hr.rat.

It can be seen from Fig. 1 that in each series the mean plasma calcium and phosphate levels varied during the 24 hr. As summarized in Table 1, the range of variation was about 0·9 mg/100 ml. for both calcium and phosphate, and the differences between maxima and minima were significant ($P < 0{\cdot}01$). The range of variation was not significantly altered by thyroidectomy.

In both 'Normal' and 'Control' rats, the plasma calcium level showed one maximum and one minimum per 24 hr. Times are indicated on a

24-hr clock with 0 hour representing the end of the 'dark-fed' period (DFP) and the start of the 'light-unfed' period' (LUP). The maximum food intake occurred early in the DFP, that is between 12 and 18 hr, and the maximum calcium level occurred more than 12 hr later, during the succeeding LUP. Average calcium levels during the DFP and LUP, which are also shown in Table 1, differed significantly. The minimum calcium level occurred at 12 hr, immediately preceding the start of feeding. In 'TX' rats, however, the calcium level rose and fell twice per 24 hr cycle, the maxima occurring at 3 and 16 hr.

TABLE 1. Some characteristics of daily plasma and phosphate levels in normal, 2-days thyroidectomized (TX) and their control rats (taken out of the Fig. 1). Average value during the LUP (calculated from 3, 6, and 9 hr) and during the DFP (calculated from 15, 18 and 21 hr); daily maximum and minimum value. All values are the mean ± S.E. (number of rats)

	Plasma calcium (mg/100 ml.)			
	Average value during			
Surgery	LUP	DFP	Max. value	Min. value
Normal	11·62 ± 0·076 (15)	11·23 ± 0·073 (15)	11·8 ± 0·11 (5)	10·9 ± 0·16 (5)
Control	11·19 ± 0·102 (18)	10·84 ± 0·080 (18)	11·5 ± 0·19 (6)	10·6 ± 0·11 (6)
TX	11·03 ± 0·096 (19)	11·10 ± 0·091 (16)	11·3 ± 0·14 (6)	10·7 ± 0·11 (6)
	Plasma phosphate (mg/100 ml.)			
	Average value during			
Surgery	LUP	DFP	Max. value	Min. value
Normal	7·86 ± 0·083 (15)	7·45 ± 0·120 (15)	8·1 ± 0·10 (5)	7·2 ± 0·15 (5)
Control	8·34 ± 0·069 (18)	7·64 ± 0·094 (18)	8·4 ± 0·19 (6)	7·5 ± 0·22 (6)
TX	7·30 ± 0·084 (19)	7·65 ± 0·133 (16)	8·1 ± 0·19 (5)	7·2 ± 0·07 (6)

Considering plasma phosphate, the levels in both 'Normal' and 'Control' rats were high for at least 9 hr out of the 24. They fell towards the end of the LUP and during the first few hours of feeding, reaching a minimum at 15–18 hr and returning to a high level by the end of the DFP. In 'TX' rats, on the other hand, plasma phosphate levels were low for 12 hr out of the 24. They rose towards the end of the DFP, as in the other series, but declined again between 0 and 3 hr and remained low for the rest of the LUP. Table 1 shows that the average values during LUP and DFP were significantly different in 'Normal' and 'Control' series, but not in 'TX' series.

Thus the presence of the thyroid gland does not affect the magnitude of the daily fluctuations of plasma calcium and phosphate levels, but modifies the pattern of the oscillations.

Acute effect of thyroidectomy on plasma levels of calcium and phosphate

In a second experiment, blood was drawn 0 and 2 hr after thyroidectomy or after a sham operation, from groups of seven rats whose parathyroids had previously been transplanted (similar to the 'control' rats of the previous experiment). The thyroidectomy was performed at one of four selected times in the 24 hr, that is at 3, 6, 15 or 18 hr. The resulting mean changes in plasma levels of calcium and phosphate are shown in Fig. 2.

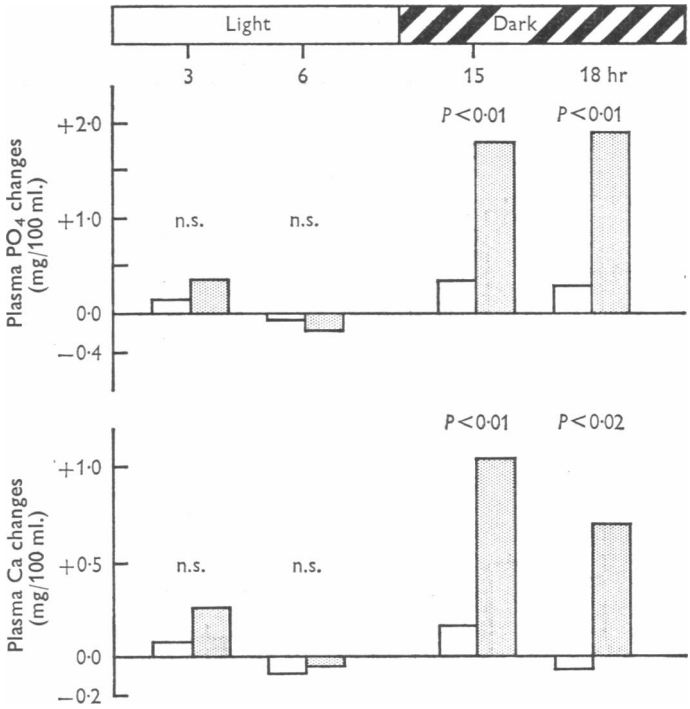

Fig. 2. Acute effect of thyroidectomy or sham operation on plasma calcium and phosphate levels as a function of periods of the day. Operations were done 3 and 6 hr after the beginning of the LUP or DFP. Each bar represents the mean value of plasma calcium and phosphate changes in seven rats, 2 hr after operation. Open histogram: sham operated; stippled histogram: thyroidectomized.

The sham operation was always without effect, and thyroidectomy was without effect during the LUP, that is at 3 and 6 hr. However, thyroidectomy caused significant elevation of both plasma calcium and phosphate when performed during the DFP, at 15 or 18 hr.

Thus, thyroidectomy performed during the 'dark-fed' period causes the changes which would be expected if calcitonin were normally secreted to prevent hypercalcaemia at that time.

Activity of exogenous calcitonin on plasma calcium and phosphate levels

In a third experiment, groups of 'Normal', 'Control' and 'TX' rats were given a large dose of porcine calcitonin intravenously, at about the middle of the DFP and LUP (at 5 and 17 hr). Blood was drawn 1 hr after the calcitonin injection, and the mean changes in plasma levels of calcium and phosphate are shown in Fig. 3.

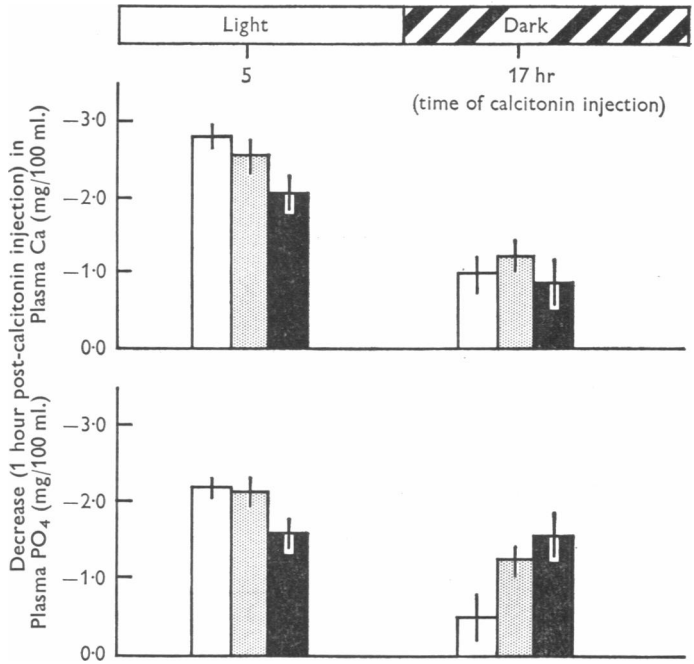

Fig. 3. Hypocalcaemic and hypophosphataemic responses to porcine calcitonin in normal (N), 2-days thyroidectomized (TX) and their control rats, 5 hr after the beginning of the LUP or DFP. Open histogram: normal; stippled histogram: thyroidectomized; filled histogram: control. Each bar represents the mean difference between the treated and untreated rats, 1 hr after injection. Vertical lines show the S.E. of the difference.

Calcitonin caused significant hypocalcaemia in all groups, but the fall was smaller during the DFP than during the LUP. The hypophosphataemic response to calcitonin was similarly smaller during the DFP than the LUP

in animals which possessed their thyroid gland, but in thyroidectomized rats the fall in plasma phosphate concentration was the same during the two periods.

DISCUSSION

The experimental data show three points in particular.

1. *Evidence on calcium homoeostasis from the diurnal changes in plasma calcium and phosphate concentration*

The experiments show that the plasma calcium level of rats is not constant during the 24 hr. The pattern (Fig. 1), but not the magnitude (Table 1) of the daily fluctuations depends on the presence of the thyroid gland.

In thyroidectomized rats, a peak in the plasma calcium levels appears 3 hr after the start of feeding, that is, just after the time of maximum calcium absorption. This second peak is absent in rats possessing their thyroid glands, which show a single calcium peak, occurring during the fasting period. As this change occurred within 48 hr of thyroidectomy and it is weeks before rats show evidence of deprivation of thyroxine and triiodothyronine (Tata, Ernster, Lindberg, Arrhenius, Pedersen & Hedman, 1963), the effect is attributed to lack of control by calcitonin secretion.

2. *Evidence for a physiological role of calcitonin in controlling plasma levels of calcium and phosphate*

The involvement of calcitonin in control of the plasma calcium level has never been proved under normal conditions of life. Indeed, it was reported that removal of the thyroid or ultimobranchials did not produce obvious changes in calcium metabolism and had no effect on the plasma calcium level (Talmage, Neuenschwander & Kraintz, 1965; Brown, Perey & Jowsey, 1970). We show here, however, that thyroidectomy does have such an effect if it is performed during the period of normal food intake (Fig. 2). The effect of thyroidectomy on plasma calcium and phosphate levels is the opposite of the effect of calcitonin injection, and the results strongly suggest that thyroid gland, through its calcitonin, normally plays an effective role in controlling the plasma level of calcium, and perhaps of phosphate. They provide evidence for daily cyclic variation in calcitonin function: nevertheless, as our experimental conditions would not have detected very transient variations of calcitonin secretion, we cannot reject the possibility of short periods of increased calcitonin secretion to protect against spontaneous small calcium challenges (Cooper *et al.* 1970), to occur even during the LUP.

3. *Evidence for a diurnal rhythm in responsiveness to exogenous calcitonin*

A diurnal variation in the hypocalcaemic response to injected calcitonin was observed in 'TX' as well as 'Normal' and 'Control' rats. It probably results from diurnal variation in the rate of bone resorption, calcitonin having been shown to cause hypocalcaemia principally by inhibiting this calcium flux (Milhaud, Perault & Moukhtar, 1965). Variation in the sensitivity of calcitonin receptors seems much less probable, and it may be assumed that calcitonin has no acute effect on calcium absorption (Robinson, Matthews & MacIntyre, 1968).

In the case of 'Normal' and 'Control' rats, sensitivity to exogenous calcitonin may well depend on the circulating endogenous level of the hormone. However, in 'TX' animals one must think in terms of some other variable: calcitonin deficiency may be balanced by any unidentified factors acting on calcitonin bone receptor. However, such a diurnal variation in the rate of bone resorption is difficult to reconcile with the constant hypophosphataemic response to calcitonin in the 'TX' rat: kidney (Kenny & Heiskell, 1965) and bone are both involved in the hypophosphataemic response to calcitonin.

In conclusion, the results presented here agree with the postulated role of calcitonin in the control of plasma calcium level and, moreover, specify some aspects of this hormonal function. They disclose a calcitonin function, which varies rhythmically in relation to the periods of the day. Further studies are required to establish whether this rhythm is a direct consequence of the times of illumination or feeding or is mediated indirectly by some 'endocrine' clock.

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