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Time-Related Changes in the Ultrastructure of Osteoclasts After Injection of Parathyroid Hormone in Young Rats

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Summary. The effects of parathyroid hormone (PTH) on the size of the osteoclasts, nuclei, ruffled borders, and clear zones in long bones of thyroparathyroidectomized (TPTX) rats were quantitated as a function of time. These data were compared with the number of osteoclasts in the bone and with plasma calcium levels. A significant increase in the average size of the ruffled borders was demonstrated 30 min after injection of 50 U of purified bovine PTH, and of the clear zones 30–90 min after PTH. This was followed at 90 min by an increase in the average size of the cells. The sizes of ruffled borders and clear zones dropped sharply to control levels after 6 h, whereas the size of the cells remained elevated up to 12 h and returned to control values at 24 h. Plasma calcium levels were increased, but not significantly, between 30 min and 6 h. An increase in the number of osteoclasts was significant after 12 h. Removal of the parathyroid glands did not diminish the normal activity of osteoclasts. In animals with intact glands injection of 50 U of PTH did not cause a significant change in cell size or resorbing apparatus. It is concluded that PTH acts to rapidly stimulate the bone resorptive activity of osteoclasts and to cause a delayed increase in their number.

Key words: Parathyroid hormone — Osteoclasts — Electron microscopy — Morphometry — Metaphysis.

Introduction

It is known that parathyroid hormone (PTH) increases osteoclastic bone resorption and that the ruffled border in the osteoclast is the site of bone resorption. When descriptive electron microscopic techniques have been used, changes in the size of the ruffled border have not been noticed after exposure to PTH [1–5], although changes have been seen in response to calcitonin [6–12]. In a quantitative electron microscopic study, however, we found that the addition of PTH to cultured fetal rat bones activated the osteoclasts [13, 14], resulting in an increase in the size of the cells in 3 h and an increase of the ruffled borders and clear zones in 1–6 h [14]. These effects were statistically significant long before an increase in the number of osteoclasts could be detected. We suggested that this effect on osteoclast activity might contribute to rapid osteoclast-mediated changes in calcium homeostasis. The recent finding that PTH causes a rapid activation of ruffled borders in intact egg-laying Japanese quails [15] supports this hypothesis.

The purpose of this study was to quantitate the effects of PTH on osteoclasts in thyroparathyroidectomized (TPTX) rats. We found that injection of PTH in these animals caused early effects on the morphology of the osteoclasts. We also investigated the effects of endogenous PTH on osteoclasts, but found no difference between osteoclasts in TPTX and sham-operated rats.

Methods

In experiment 1, three-week-old rats of the Wistar-Lewis strain were thyroparathyroidectomized. After 3 days, the animals were fasted for 10 h, blood was drawn by closed-chest cardiac puncture, and plasma calcium levels were determined by means of atomic absorption spectrophotometry [16]. All animals with plasma calcium levels below 8 mg/100 ml were considered to have had a successful TPTX and were included in the experiment. After 1 week the rats were again fasted for 10 h and then injected intravenously with 50 U of PTH (Sephadex-purified bovine PTH, 2500 U/mg, provided by the Medical Research Council, England) or with buffer [17]. At 0.5, 1.5, 3, 6, 12, and 24 h, 6

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rats (3 treated and 3 controls) were sacrificed, blood was withdrawn from the heart, and the proximal part of the radii of both front legs was removed. The metaphyses of both radii were dissected and cut into 3 to 4 pieces in a solution of 2.5% glutaraldehyde solution in 0.1 M cacodylic buffer on ice, fixed in a mixture of the glutaraldehyde solution and 1% OsO_4 [13], decalcified in 7.5% EDTA in the glutaraldehyde solution for 1 week, and prepared for electron microscopy as described previously [13].

In experiment 2, three-week-old rats were thyroparathyroidectomized or sham operated. The same procedures were followed as in the first experiment except that these animals were not fasted prior to PTH injection. At 5, 10, 30 or 90 min after injection of PTH, 3 treated and 3 control animals of the TPTX and sham-operated group were sacrificed.

In experiment 3, eleven 3-week-old rats were TPTX and 5 rats were sham operated. All TPTX animals had serum calcium levels below 7.4 mg/100 ml indicating successful parathyroidectomy. The animals were fasted overnight, and 24 h after the operation blood was drawn from the abdominal aorta and the proximal metaphyses of the tibiae were dissected and prepared for electron microscopy.

Methods of Sampling and Quantitation

For estimation of the number of osteoclasts in the metaphyses of the dissected radii, we selected 6 pieces of bone at random from each animal. One random section from each piece of bone was examined with the light microscope at a magnification of 400×. Using an ocular grid to determine field size (0.04 mm^2), we counted the number of osteoclasts in 2–4 nonoverlapping fields directly below the hypertrophic chondrocytes. The average number of osteoclasts per field was calculated for each animal and used to make comparisons of the population of osteoclasts in the proximal radial metaphysis in the different groups. The numbers of osteoclasts were not counted in experiment 2 since an increase has never been reported after a time as short as 90 min.

In two populations of cells with different volumes, the average profile (cross section) of the larger cells will also be larger if selected randomly [18]. This correlation also holds for the cell components. Hence significant differences in the average area of cell profiles and their components represent significant differences in the average volumes of the cells and their components. Such a method is therefore valid for comparing size differences between groups of cells.

Cell profiles were collected in the electron microscope as follows: Four pieces of bone from the same site as that used for counting numbers of osteoclasts were selected at random from the 3 animals of each experiment or control group. One thin section from each piece of bone containing a clear unfolded area was selected at a magnification at which cell types could not be recognized. At a higher magnification, this section was scanned from left to right and from top to bottom. All osteoclast profiles encountered were recorded on a 35 mm film at a magnification that allowed the profile to fill up the frame. If a ruffled border or a clear zone were present, these were photographed separately at a higher magnification, again to fill up the space allotted by the film. In each group at least 25 cell profiles were collected and pooled from the 3 different animals per group, each profile being taken from a different osteoclast. The negatives of the recorded cell profiles were projected at a fixed magnification onto a digitizing table interfaced with a computer. The areas of interest, i.e., cell area, nuclear area, ruffled border area, and clear zone area, were traced with a sensor. The computer was programmed to compensate for the different magnifications at which the pictures were taken and calculated the traced areas in square microns. Cell and nuclear areas are bounded by a membrane and are therefore easy to recognize. Ruffled border area was defined as the area of osteoclast adjacent to the bone characterized by highly convoluted membranes. Clear zone area was defined as the area adjacent to bone and ruffled border filled with a filamentous or amorphous material excluding the usual cellular organelles. An example of the tracing of ruffled border and clear zone area is given in Fig. 1. The means and standard errors of the different cell areas within each group were calculated and differences were tested for statistical significance using the Student's t test for unpaired data. Details and a discussion of this method of morphometry have been reported elsewhere [19].

Method for Converting Area Data to Volume Data

The mean volume of cells in a population can be compared to the mean cell volume in another population by using the relationship $V = \beta \cdot A^{3/2}$, in which V represents the mean volume of cells or their components, A the mean area in cell profiles, and B a correction factor that depends on the shape of the cells [18]. Assuming that osteoclasts have roughly the same shape independent of their size, the following relationship can be extrapolated between the volumes of cells in two populations:

$$\frac{V_1}{V_2} = \frac{A_1^{3/2}}{A_2^{3/2}}$$

These values are average figures for cell volume that are accurate to the extent that the shapes of the cells are similar. Since it is more likely that the shapes are not quite similar, these values represent approximations of the real average volumes. Nevertheless, approximations were made of the rate of change of the volumes of cells and their components under these experimental conditions.

Results

Injection of PTH in TPTX, fasted animals (experiment 1) caused a rise in plasma calcium levels that reached a peak by 3 h, and leveled off to control values by 6 h (Table 1). This rise was not statistically significant, but it should be noted that these values were the average of only 3 animals. The number of osteoclasts per field increased sharply between 6 and 12 h to values twice the controls (Table 1) and remained elevated for 24 h. PTH increased the average area of osteoclast profiles significantly by 1.5 h ($P < 0.01$, Fig. 2). The nuclear area increased at approximately the same rate, causing the average nuclear/cell ratio to remain approximately constant (Table 2). Between 12 and 24 h, the areas of cell and nucleus returned to control values. The average area of ruffled borders and clear zones in osteoclast profiles enlarged significantly compared to controls as early as 30 min after injection of PTH ($P < 0.05$ and $P < 0.005$, respectively). This increase dropped sharply to control values at 6 h (Figs. 3 and 4). The

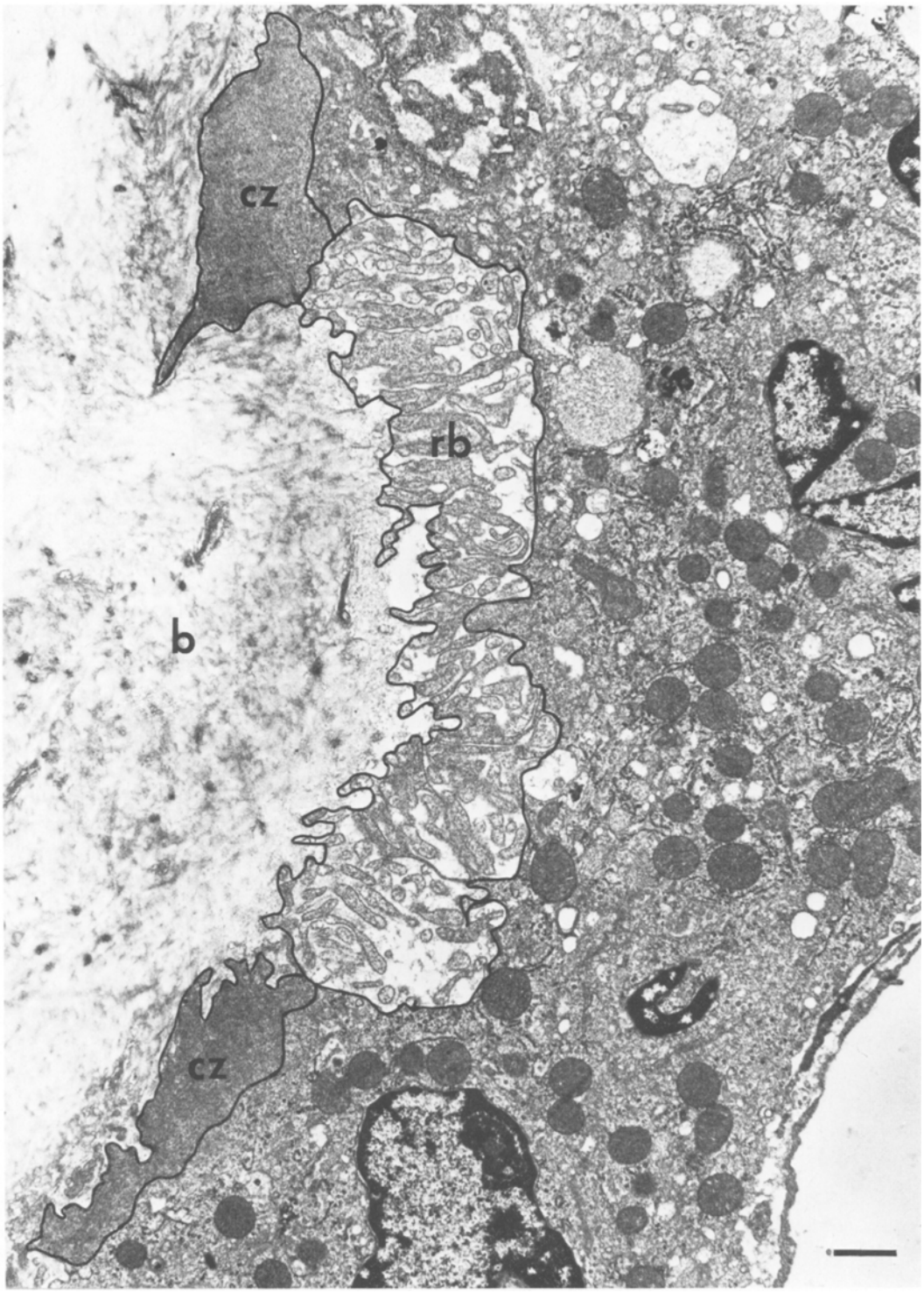

Fig. 1. Ruffled border (rb) and clear zone (cz) of an osteoclast outlined as they would be for measurement. b, decalcified bone; ×9360. Scale is 1 μm

proportion of the cell area occupied by ruffled borders in osteoclasts exposed to PTH was much higher than the control values at 0.5 h and 3 h, indicating that ruffled borders had enlarged at a much faster rate than the cell in these early time periods (Table 2). In contrast, clear zones enlarged at the same rate as the cell (Table 2).

Estimations of the changes in the average volume of osteoclasts, their ruffled borders and clear zones, were derived from the changes in average areas of

Table 1. Effect of PTH on plasma calcium and on the number of osteoclasts in thyroparathyroidectomized rats

		0.5 h	1.5 h	3 h	6 h	12 h	24 h
Plasma calcium	Co	7.1 ± 0.6	7.1 ± 0.7	6.1 ± 0.5	6.7 ± 0.4	6.0 ± 0.3	5.9 ± 0.6
	PTH	7.7 ± 0.3	8.6 ± 0.8	7.6 ± 0.5	7.2 + 0.6	6.3 ± 0.4	5.1 ± 0.6
Number of osteoclasts	Co	2.5 ± 0.4	2.8 ± 0.2	3.1 ± 0.3	3.1 ± 0.1	2.4 ± 0.2	2.4 ± 0.2
	PTH	2.3 ± 0.2	2.8 ± 0.2	3.3 ± 0.1	3.8 ± 0.2	4.9 ± 0.3[a]	4.7 ± 0.3[a]

Values for plasma calcium are mean ± SE for 3 rats. Values for numbers of osteoclasts are mean ± SE per ocular field for 6 pieces of bone from the radial metaphysis of each of 3 rats

[a] Significantly different from corresponding control ($P < 0.01$)

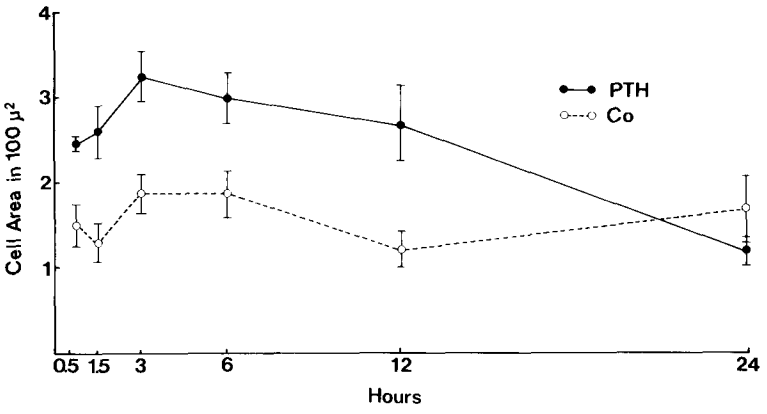

Fig. 2. Changes in the average size of osteoclast profiles as a function of time in bones from thyroparathyroidectomized rats injected with PTH or buffer (Co). Each point represents the mean of 23–26 cell profiles pooled from 3 rats/group, and the vertical bars represent SEM

osteoclast profiles, and are shown in Table 3. Within 3 h after injection of PTH the cell volume as well as their nuclei increased approximately twofold compared to control osteoclasts, the volume of the clear zones increased more than threefold, and the volume of ruffled borders was 14 to 21 times larger. Ruffled borders and clear zones resumed control values between 3 to 6 h whereas cell and nucleus returned to control values between 12 and 24 h (Table 3).

In experiment 2, fed TPTX or sham-operated rats were injected with PTH or buffer and sacrificed after 5, 10, 30, or 90 min. In the TPTX animals the effects were similar to those of experiment 1. After injection with PTH average cell areas were above control values at each time point and became significantly larger than control values by 90 min (Table 4). The average nuclear area was significantly larger after 5 min, and larger but not significantly, at later time points (Table 4), resulting in a constant nuclear/cell ratio. The ruffled border area was significantly larger than in control osteoclasts after 30 min and the clear zone area after 90 min (Table 4).

Table 2. Percentage of average cell area occupied by average nuclear area, ruffled border area, and clear zone area as a function of time in osteoclast profiles from TPTX rats injected with PTH or vehicle (Co)

			0.5 h	1.5 h	3 h	6 h	12h	24 h
Nucleus / Cell	(%)	Co	12.4	12.5	10.6	11.0	16.2	7.7
		PTH	11.8	9.8	8.2	12.5	13.8	14.6
Ruffled border / Cell	(%)	Co	3.4	3.2	1.8	2.1	2.0	1.2
		PTH	12.4	8.2	7.7	0.8	0.9	1.0
Clear zone / Cell	(%)	Co	3.3	3.9	6.2	4.2	3.2	4.0
		PTH	4.8	4.9	4.1	2.1	3.1	4.6

Information on the effects of endogenous PTH on osteoclasts was derived from comparison of osteoclasts in TPTX and sham-operated controls (Table 4), and was supported by the results of experiment 3 (Table 5). No differences were seen in cell area or area of nucleus, ruffled border, or clear zone in osteoclasts between TPTX and sham-operated controls, indicating that the activity of osteoclasts during remodeling is not governed by PTH. When PTH was injected into sham-operated animals, no significant increases could be found between cell areas and ruffled border areas in osteoclast profiles and the clear zone was significantly larger compared with control only at 30 min. Nevertheless, the ruffled borders were usually somewhat larger in the PTH-treated animals.

Discussion

This study demonstrated that osteoclasts can be activated as early as 30 min after injection of PTH in

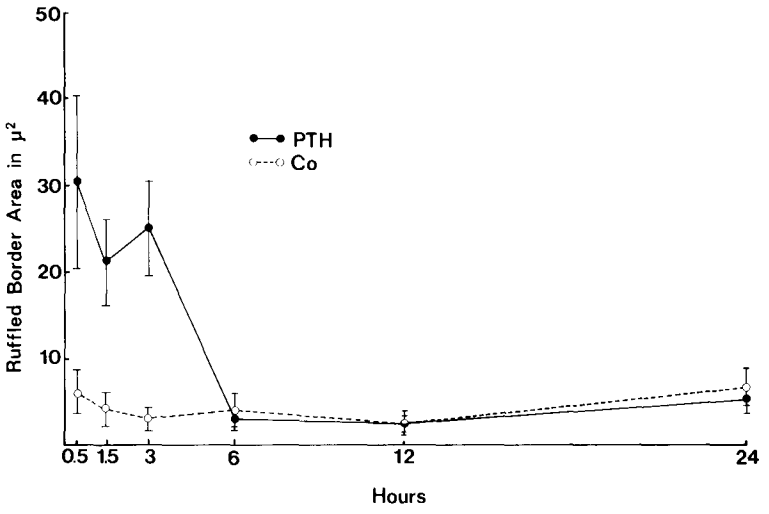

Fig. 3. Changes in the average size of ruffled border area as a function of time in the same osteoclast profiles as represented in Fig. 2

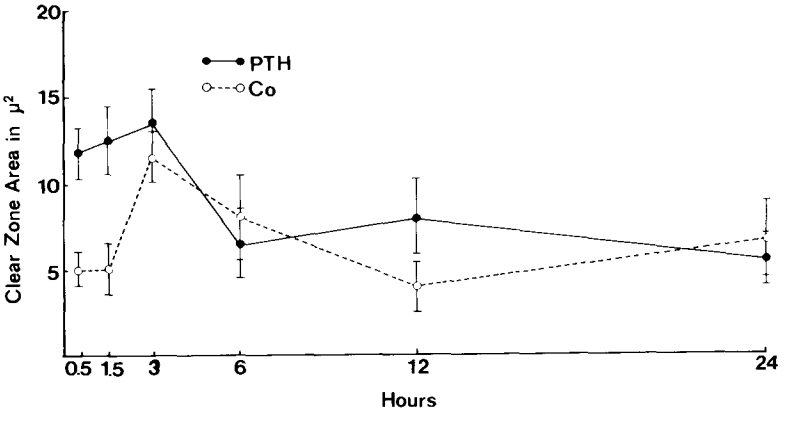

Fig. 4. Changes in the average size of clear zone area as a function of time in the same osteoclast profiles as represented in Fig. 2

TPTX rats. This activation coincided with small changes in plasma calcium and was manifested predominantly in the resorbing area of the osteoclast, the ruffled border, which increased at a greater rate than the cell or the clear zone.

It has been suggested that a major role of PTH in calcium homeostasis might be indirect by stimulating the production of $1,25(OH)_2D_3$ in the kidney [20, 21]. Injection of PTH in TPTX rats caused a significant rise in $1,25(OH)_2D_3$ in the serum after 12 h and normal serum levels were restored after 36 h [21]. In intact chicks repeated injections with PTH for 48 h resulted in a significant rise in the enzyme 25HCC-1-hydroxylase, whereas a single injection of PTH showed only a marginal increase in activity after 3 h [20]. Although $1,25(OH)_2D_3$ levels were not measured in our experiments, the level in the serum of the TPTX rats was probably low [21]. Nevertheless, activation of osteoclasts was accomplished 30 min after injection of PTH, which is before $1,25(OH)_2D_3$ could have been produced. Therefore, our data suggest that PTH can activate osteoclasts directly without synthesis of $1,25(OH)_2D_3$. At the same time the production of $1,25(OH)_2D_3$ in the kidney is stimulated which may, at a later time, further stimulate bone resorption. This supports the idea suggested earlier [14] that this early activation of osteoclasts by PTH is an important mechanism in calcium homeostasis.

The activation of osteoclasts by PTH occurred in cells that were actively involved in the remodeling process of the metaphysis during normal growth. Removal of the parathyroid glands did not diminish this activity suggesting that endogenous PTH is not required for the functioning of osteoclasts.

Table 3. Estimations of the changes in the volumes of osteoclasts, nuclei, ruffled borders, and clear zones in TPTX rats after injection with PTH (treated/controls)

		0.5 h	1.5 h	3 h	6 h	12 h	24 h
Cell	T/C	2.1	2.8	2.3	2.0	3.2	0.6
Nucleus	T/C	1.9	1.9	1.6	2.4	2.5	1.6
Ruffled border	T/C	14.6	11.7	21.0	0.5	1.0	0.5
Clear zone	T/C	3.7	3.9	1.3	0.7	3.0	0.8

Table 4. Plasma calcium and areas (μm^2) of cell, nucleus, ruffled border, and clear zone in profiles of osteoclasts of TPTX or sham-operated rats as a function of time after injection with PTH or buffer (Co)

			5 min	10 min	30 min	90 min
Plasma calcium	TPTX	Co				9.2 ± 0.53
		PTH				9.8 ± 0.17
	Sham	Co				10.6 ± 0.19
		PTH				10.8 ± 0.29
Cell	TPTX	Co	358.5 ± 57.5	341.7 ± 43.3	349.3 ± 37.9	313.3 ± 45.8
		PTH	510.7 ± 53.2	425.4 ± 52.6	415.4 ± 53.9	508.1 ± 65.3[a]
	Sham	Co	349.4 ± 40.2	375.6 ± 46.3	477.7 ± 53.6	307.0 ± 40.0
		PTH	418.2 ± 59.9	343.7 ± 43.6	399.4 ± 43.9	423.1 ± 75.5
Nucleus	TPTX	Co	35.2 ± 8.0	36.5 ± 7.7	49.3 ± 9.0	42.2 ± 11.1
		PTH	63.8 ± 9.6[a]	54.4 ± 8.9	50.8 ± 9.8	72.0 ± 13.9
	Sham	Co	31.8 ± 8.3	37.8 ± 7.3	58.9 ± 8.9	34.7 ± 7.5
		PTH	40.4 ± 8.4	30.8 ± 6.8	42.1 ± 8.2	52.8 ± 9.5
Ruffled border	TPTX	Co	11.8 ± 4.3	6.2 ± 2.0	7.8 ± 2.3	10.0 ± 3.2
		PTH	17.1 ± 1.5	8.3 ± 3.3	22.0 ± 4.1[b]	23.5 ± 4.7[a]
	Sham	Co	11.4 ± 3.2	5.4 ± 1.9	11.0 ± 3.4	10.3 ± 3.2
		PTH	14.6 ± 4.6	11.8 ± 3.3	14.0 ± 2.9	19.5 ± 7.0
Clear zone	TPTX	Co	11.6 ± 2.2	11.3 ± 3.6	9.6 ± 1.8	10.1 ± 2.5
		PTH	16.7 ± 2.0	8.1 ± 2.4	14.4 ± 2.4	20.3 ± 3.8[a]
	Sham	Co	6.5 ± 1.6	7.8 ± 2.5	7.4 ± 1.6	11.4 ± 3.4
		PTH	7.5 ± 1.6	11.7 ± 2.7	17.5 ± 3.6[a]	11.7 ± 2.8

Values for areas are the mean ± SE of 25-30 osteoclast profiles pooled from 3 rats
[a] Significantly different from corresponding control; $P < 0.05$
[b] $P < 0.01$

The osteoclasts in animals with intact glands were not activated by PTH as much as in the TPTX animals. Calcitonin, which inhibits osteoclasts, is secreted when plasma calcium levels rise [22], but this occurs *after* a PTH effect on intestine, kidney, or bone. On the other hand, calcitonin levels also rise with increases in circulating gastrin [23], and these animals were not fasted. An alternate explanation of the difference of responsiveness to PTH of osteoclasts in sham-operated and TPTX rats could be a decrease in receptor sites with increased concentration of endogenous PTH. A progressive loss of receptors on target cells has been reported with increasing concentrations of hormones such as insulin and growth hormone [24].

Table 5. Serum calcium (mg/100 ml) and areas (μm^2) of cell, nucleus, ruffled border, and clear zone in profiles of osteoclasts of TPTX or sham-operated rats

	Sham	TPTX
Serum calcium	10.0 ± 0.3	6.3 ± 0.2[a]
Cell	211.9 ± 21.8	271.8 ± 28.8
Nucleus	20.3 ± 4.1	19.5 ± 5.1
Ruffled border	9.4 ± 2.6	7.4 ± 2.4
Clear zone	3.9 ± 1.1	6.0 ± 1.5

Values for areas are the mean ± SE of 35 or 48 osteoclast profiles
[a] Significantly different from corresponding control ($P < 0.001$)

We conclude that PTH has an immediate effect on the activation of osteoclasts in bone which is not mediated by the production of $1,25(OH)_2D_3$. This mechanism may play an important part in calcium homeostasis when the calcium regulatory mechanisms in kidney and intestine do not suffice. Endogenous PTH is not required for the osteoclastic function of bone remodeling during growth.

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