THE EFFECTS OF PARATHYROID HORMONE, COLCHICINE, AND CALCITONIN ON THE ULTRASTRUCTURE AND THE ACTIVITY OF OSTEOCLASTS IN ORGAN CULTURE

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ABSTRACT

The ultrastructure of osteoclasts was examined in fetal rat bones after stimulation or inhibition of resorption in culture. A central ruffled border area completely encircled by a clear zone was considered to represent the resorbing system of the cell. The proportion of ruffled border and clear zone in osteoclast cross sections was compared with changes in bone resorption as measured by the release of previously incorporated radioactive calcium (^{45}Ca). In control cultures 55% of the osteoclast cross sections showed an area closely apposed to bone and this consisted mainly of clear zone; only 11% showed ruffled borders. Treatment with parathyroid hormone (PTH) increased ^{45}Ca release, increased the frequency of finding areas closely apposed to bone (79%), and markedly increased the frequency of the ruffled border area (64%).

Colchicine given concurrently with PTH decreased the number of osteoclasts. Colchicine or calcitonin treatment after PTH stimulation decreased the proportion of ruffled border area significantly by 1 h; this was followed by a decrease in ^{45}Ca release. These inhibited osteoclasts resembled osteoclasts from control, unstimulated cultures, suggesting that the cells had returned to their inactive state.

Colchicine-treated osteoclasts also showed a loss of microtubules and a massive accumulation of 100 Å filaments, suggesting that synthesis of microtubular subunits had increased.

INTRODUCTION

Ultrastructural studies of osteoclasts (Scott and Pease, 1956; Gonzales and Karnovsky, 1961; Dudley and Spiro, 1961) and time-lapse motion pictures (Gaillard, 1961; Goldhaber, 1961) have demonstrated that these cells actively resorb bone. The active site is considered to be the ruffled border area where deep invaginations of the cell membrane overlie a portion of bone which appears to be undergoing resorption (Scott and Pease, 1956; Gonzales and Karnovsky, 1961; Hancox and Boothroyd, 1963; Dudley and Spiro, 1961).

In descriptions of osteoclast ultrastructure little

attention has been paid to the clear zone. This zone is found adjacent to the ruffled border area and is so-called because it is free of cell organelles and filled with fine granular material (Schenk et al., 1967). It is closely apposed to the bone surface but this bone does not appear to be undergoing resorption.

Stimulation of bone resorption by parathyroid hormone (PTH) will increase the number of osteoclasts (Tatevossian, 1973). It is not known, however, what the effects of PTH on the fine structure of these cells are. Ultrastructural changes have been described when cultured calvaria were treated with calcitonin: the ruffled border was reported to disappear and this effect was considered specific (Kallio et al., 1972). However, no data were obtained on the clear zone. Recently we found that colchicine is another effective inhibitor of bone resorption in organ culture (Raisz et al., 1973). Colchicine prevents the development of PTH response, but can also, like calcitonin, inhibit resorption after PTH pretreatment, suggesting that it might have some direct effect on osteoclasts. In the present study, the effects of stimulation with PTH and inhibition with calcitonin and colchicine on osteoclast ultrastructure were compared. Special attention was paid to the clear zone and its relation to the ruffled border area.Changes in morphology were related to the activity of bone-resorbing cells by measuring the release of previously incorporated radioactive calcium from bone to medium in the same cultures.

MATERIALS AND METHODS

As described previously (Raisz et al., 1973), paired shafts of fetal radius and ulna were cultured in a chemically defined medium supplemented with bovine albumin (1 mg/ml) or 5% human serum, heat inactivated up to 60°C for 30 min. Bones were labeled with ^{45}Ca by injection of the mother 1 day previously. Three sets of morphologic experiments were performed: (*a*) Effects of continuous culture with colchicine. Bones were cultured for 48 h with or without PTH (1.6 μg/ml) and colchicine (10^{-6}–10^{-8} M). ^{45}Ca release was measured and the bones were prepared for light microscopy and the osteoclasts counted. (*b*) Acute effects of calcitonin and colchicine. Bones were precultured for 48 h with or without PTH and transferred to control medium or medium containing salmon calcitonin (100 mU/ml) or colchicine (10^{-6} M). Bones were fixed for electron microscopy and media analyzed for ^{45}Ca release at 1, 4, and 24 h after transfer. (*c*) Chronic effects of colchicine. Bones were precultured with PTH for 48 h and then transferred to control medium or medium with colchicine (10^{-6} M) for 24, 48, or 72 h, and analyzed as above.

For light microscopy, bones were fixed in Bouin-Hollande solution, and 4-μm paraffin sections were stained with hematoxylin and eosin. For electron microscopy, bones were fixed either: (*a*) at 0°C for 3 h in a mixture of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and 1% OsO_4 in cacodylate buffer in a ratio of 1:2 (modified from Hirsch and Fedorko [1968]), or (*b*) at room temperature for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 5 mM calcium chloride and for 2 h in 1% OsO_4 in the same buffer. Method *b* gave better preservation of microtubules. Both methods gave good visualization of the ruffled border and clear zone areas. After fixation the bones were dehydrated in graded alcohols, embedded in Epon, trimmed, and sectioned. In initial studies using 200-mesh grids most osteoclasts were not completely visualized because they disappeared under the grid bars. For this reason, the sections were collected on 75-mesh grids (hole opening 283 μm^2) coated with 0.4% Parlodion and reinforced with carbon. The sections were stained with 8% uranyl acetate in 50% ethanol and 0.2% lead citrate.

Sampling Technique

LIGHT MICROSCOPY: A measure of the number of osteoclasts per bone was obtained by selecting at random one section from the center of each shaft and counting all the osteoclasts in it. Osteoclasts were identified by their multiple nuclei and foamy acidophilic cytoplasm.

ELECTRON MICROSCOPY: One thin section of each cultured bone was selected at random and scanned. All cross sections of osteoclasts which were complete, that is, not partly obscured by grid bars, were registered, and the presence or absence of ruffled borders and clear zones (as defined in the Introduction) was recorded. Osteoclasts were identified by their abundant mitochondria and lack of rough endoplasmic reticulum. They did not have to contain multiple nuclei. With this sampling technique, the percentages of cross sections of osteoclasts that show ruffled borders and clear zones depends on the area occupied by these features in the cell and can be compared quantitatively. Statistical analyses were performed on morphology data using the chi-square test and on ^{45}Ca data using Student's *t*-test.

RESULTS

Ruffled borders were not a constant feature in osteoclast cross sections but in every one of the 241 osteoclast cross sections in which a ruffled border was present there were also always clear zones on either side. Some osteoclasts showed only

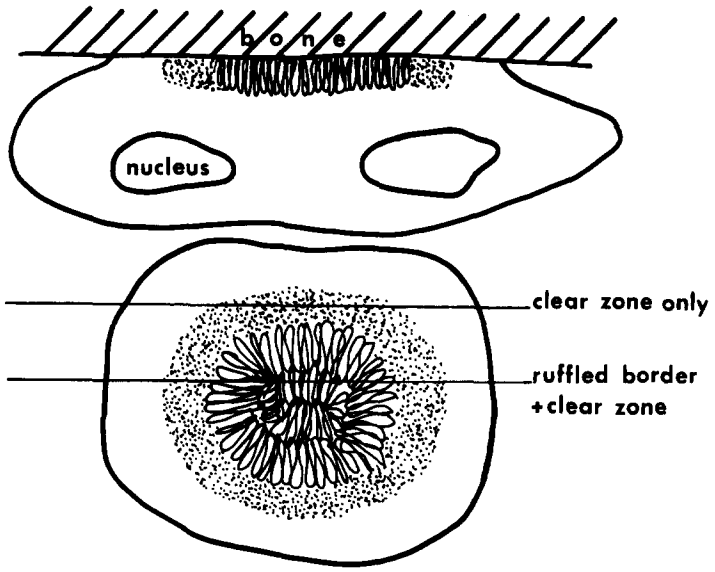

FIGURE 1 Diagrammatic representation of an osteoclast. A cross section through a ruffled border will always show adjacent clear zones if the ruffled border is completely surrounded by a clear zone.

clear zones apposed to bone surfaces. These findings are interpreted to mean that the clear zone completely encircles the ruffled border, as diagrammatically illustrated in Fig. 1. The bone surface under the ruffled border was irregular and often showed partial demineralization, loose crystals, and disrupted collagen fibers (Fig. 2). The clear zone was also in close contact with the bone surface and generally followed its contour for some distance away from the ruffled border area. However, in contrast to the ruffled border area, the bone under the clear zone normally showed no evidence of active resorption.

Control bones without PTH showed little active resorption and the osteoclasts showed ruffled borders in only 11% of the cross sections (Table I). These ruffled borders were only small areas with a few shallow infoldings of the cell membrane. The clear zone was extensive and was found in 55% of the cross sections, thus 44% showed clear zone without ruffled border.

The effect of PTH on osteoclast morphology persisted after the bones were transferred to control medium (see Table III below), consistent with our previous finding that brief exposure to PTH induces prolonged resorption (Raisz et al., 1972). Therefore, the data for 1–24 h post-PTH were pooled (Table I) to show that PTH treatment increased the percentage of cross sections with ruffled borders from 11 to 64%. Cross sections with clear zones increased to 79%, thus only 15% showed a clear zone without a ruffled border. Moreover, the ruffled borders were large with deep infoldings of the cell membrane.

Colchicine given with PTH was found to inhibit the development of the PTH response (Raisz et al., 1973). Table II demonstrates that this effect was accompanied by a progressive decrease in the number of osteoclasts with increased concentration of colchicine (10^{-8}–10^{-6} M) (Table II). Colchicine also decreased the number of osteoclasts in bones not treated with PTH. This decrease was not associated with a decrease in ^{45}Ca release. It is likely that the osteoclasts in these control bones were not actively resorbing since such bones show only a small proportion of ruffled border (Table I). Much of the ^{45}Ca release from these bones is ascribable to exchange.

Colchicine also inhibits bone resorption acutely after it has been stimulated by PTH (Raisz et al., 1973). When we examined the acute effects of colchicine and salmon calcitonin on osteoclast morphology of PTH-pretreated bones there were no obvious differences in distribution or appearance of mitochondria, vesicles, or ribosomal particles. However, there were marked changes in the ruffled borders and clear zones (Table III). After 1 h of either colchicine or calcitonin treatment, the percentage of cross sections showing ruffled border had decreased significantly. More-

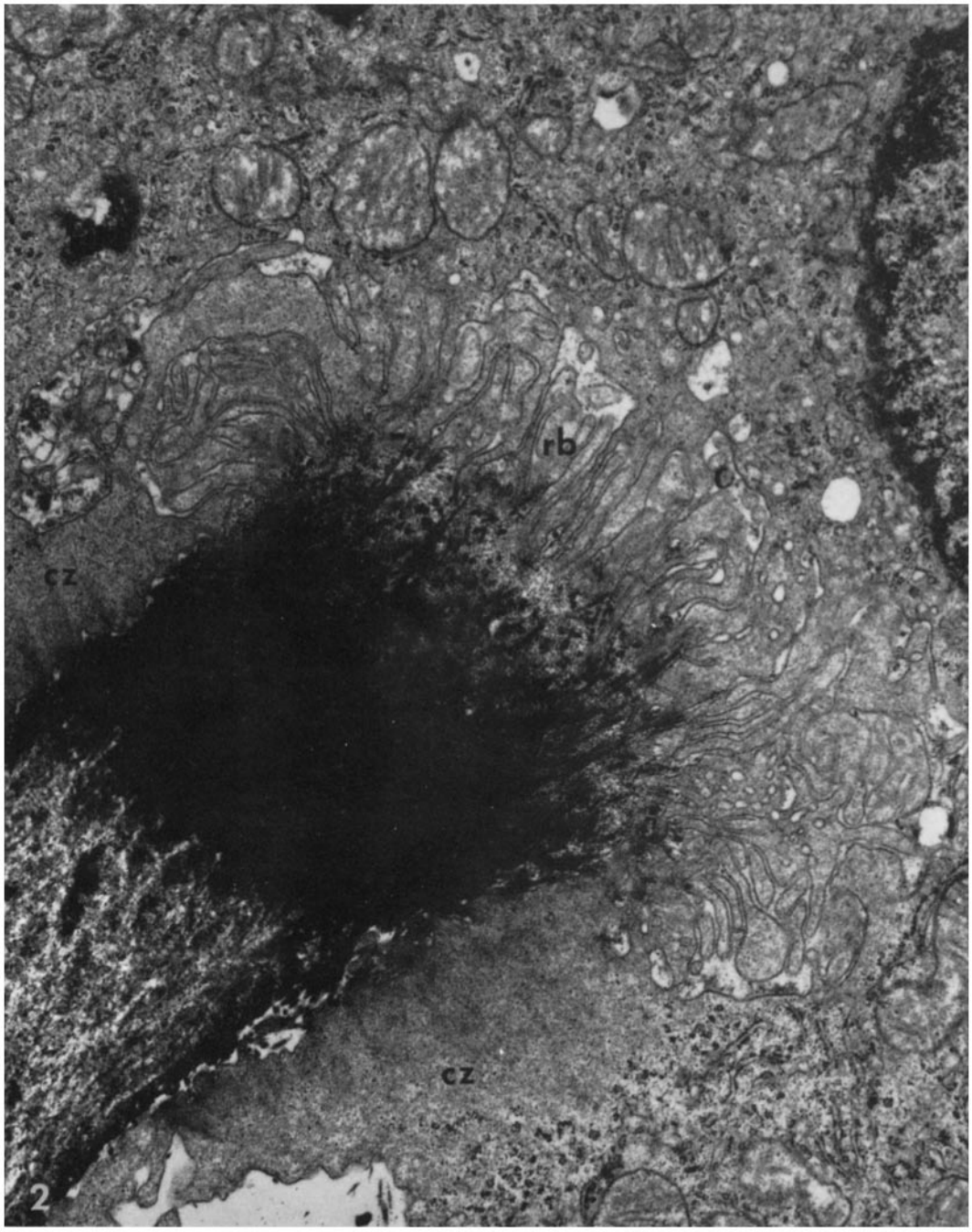

FIGURE 2 Ruffled border (*rb*) and adjacent clear zone (*cz*) cover a bony spicule. The bone under the ruffled border looks disrupted, while that under the clear zone does not. × 15,900.

over, in the calcitonin-treated cultures, although there were still 29% of osteoclast cross sections with ruffled borders, eight out of ten such osteoclasts showed a ruffled border with only a few shallow infoldings of the cytoplasmic membrane. As the frequency of ruffled borders decreased, the frequency of finding only a clear zone increased, suggesting that ruffled border was converted into

TABLE I

Frequency of Ruffled Borders (RB) and Clear Zones (CZ) in Osteoclasts after Culture in Control (Co) Medium or Medium with PTH (1.6 μg/ml) for 48 h and Subsequent Culture in Control Medium for 1, 4, and 24 h

	Number of		Number of osteoclast cross sections with		
	Bones	Osteoclasts	RB + CZ	CZ only	Total
Co → Co	18	90	10	40	50
			11%	44%	55%
PTH → Co	30	217	139*	33*	172*
			64%	15%	79%

The values are the numbers, absolute above and percent below, of cross sections through osteoclasts (one cross section per osteoclast) that show these areas. All cross sections showing ruffled borders also had clear zones.

* Significantly different from control → control, $P < 0.01$.

TABLE II

Effect of Colchicine on the Number of Osteoclasts and on ^{45}Ca Release from Fetal Rat Bones Cultured for 48 h with and without PTH (1.6 μg/ml)

Dose of colchicine	Osteoclasts/section		^{45}Ca release—cpm/bone	
	PTH	Control	PTH	Control
M				
0	18.2 ± 4.9*	10.6 ± 2.5	1,580*	930
10^{-8}	12.7 ± 2.1	10.0 ± 1.6	1,630*	840
10^{-7}	8.7 ± 1.2*	1.7 ± 0.2	1,010*	730
10^{-6}	2.2 ± 0.7	2.0 ± 0.6	860	850

Values are means (±SE) for osteoclast counts for five to six pairs of cultures.

* PTH-treated cultures significantly different from paired controls, $P < 0.05$.

clear zone. This is supported by the observation that some osteoclasts showed clear zone overlying bone which looked partially resorbed (Fig. 3).

The morphologic effects at the end of the first hour of treatment were not accompanied by a measurable decrease in the rate of ^{45}Ca release from 0 to 1 h. However, by 4 h of calcitonin or colchicine treatment, when the frequency of ruffled borders had decreased still further, cumulative (0–4 h) ^{45}Ca release had decreased significantly At 24 h, the colchicine effect was still well sustained but the calcitonin-treated bones showed a significant increase in ruffled border area and in ^{45}Ca release. This presumably represents the phenomenon of escape which we have observed previously with continuous calcitonin treatment of PTH-stimulated bones in culture (Wener et al., 1972).

In the third set of experiments the effects of colchicine were examined after longer time periods (Table IV). Increased ^{45}Ca release was maintained in PTH-pretreated cultures, until the bones were almost completely resorbed, and inhibition was maintained with colchicine. This could not be ascribed to a progressive toxic effect since osteoclast viability seemed well maintained morphologically. The percentage of cross sections with ruffled borders decreased and, at 48 and 72 h, no ruffled borders were found in colchicine-treated bones, but clear zones were still present. In PTH-pretreated bones without colchicine the proportion of ruffled borders also decreased with time so that at 48 and 72 h the difference between bones transferred to control and colchicine medium was no longer significant (Table IV).

Colchicine disrupts microtubules and binds to their subunits; these can then be seen as filaments with a diameter of 100 Å (Wisniewski et al., 1968; Ishikawa et al., 1968). In control and PTH-treated osteoclasts only a few microtubules were found in any one cell and the filaments were present but sparse. After colchicine treatment

TABLE III

Effect of 1, 4, or 24 h of Colchicine (Colch, 10^{-6} M) or Salmon Calcitonin (SCT, 1 mU/ml) on Ruffled Border (RB) and Clear Zone (CZ) of Osteoclasts and on ^{45}Ca Release from Bones Precultured for 48 h with PTH (1.6 μg/ml), Compared with Bones Precultured in Control Medium (Co)

Time	Treatment	Number of		Percent sections of osteoclasts with			^{45}Ca release
		Bones	Osteoclasts	RB + CZ	CZ only	Total	cpm/bone
h							
1	PTH → Co	6	54	68	9	77	120
	PTH → Colch	6	32	19*	44	63	140
	PTH → SCT	6	35	29*	40	69	110
	Co → Co	6	34	11*	47	58	60*
4	PTH → Co	11	85	58	20	78	430
	PTH → Colch	16	88	15*	49	64	350*
	PTH → SCT	6	30	10*	39	49	320*
	Co → Co	6	30	10*	47	57	260*
24	PTH → Co	13	78	68	15	83	1,960
	PTH → Colch	11	74	18*	53	71	880*
	PTH → SCT	6	33	39*,‡	45	84	1,440*
	Co → Co	6	26	12*	38	50	620*

The number of osteoclasts represent the number of cross sections seen and does not indicate the frequency of osteoclasts per bone.

Values for ^{45}Ca release are weighted means for two to ten cultures representing 12–40 bones.

* Significantly different from PTH → control group, $P < 0.01$.

‡ Value at 24 h significantly higher than at 4 h, $P < 0.01$.

microtubules had largely disappeared and were replaced by numerous 100 Å filaments. After 24 h these filaments had become so abundant that they formed bands running between the cell organelles. After 72 h there was a still further increase so that in many osteoclasts these filaments occupied large areas of the cytoplasm (Fig. 4). This phenomenon was also found in other cell types but not in those cells undergoing mitosis and showing metaphase arrest.

DISCUSSION

In this study we have found that the ruffled border with a surrounding clear zone together constitute the resorbing system of the osteoclast. Clear zones are also present when the ruffled borders are sparse and resorption is minimal. They could function to hold the osteoclast to the bone surface. When resorption becomes active under a ruffled border the clear zone may help to seal off the resorbing site (Schenk et al., 1967).

The percentage of cross sections of osteoclasts that show a certain feature indicates the average of all cells examined, but does not tell us exactly how that feature is distributed. The low proportion of cells showing a ruffled border in control cultures could mean either that this area is small in all cells, or that it has disappeared completely from some cells and that other cells are unaffected. We found that osteoclasts in control bones usually showed small, poorly developed ruffled borders with only a few infoldings in the membranes, while PTH-treated osteoclasts had large, highly developed ruffled borders. Thus, the first possibility is the most likely one.

The marked increase in frequency and development of ruffled border after treatment with PTH suggests that osteoclasts can modulate from a resting phase to an active phase. It also means that osteoclast counts in light microscope sections may not be a satisfactory index of the rate of bone resorption. This could explain the discrepancy we observed between osteoclast count and calcium release in control bones compared with those treated with colchicine alone (Table II).

In the present study there was usually a good correlation between changes in the proportion of ruffled borders in osteoclast cross sections and

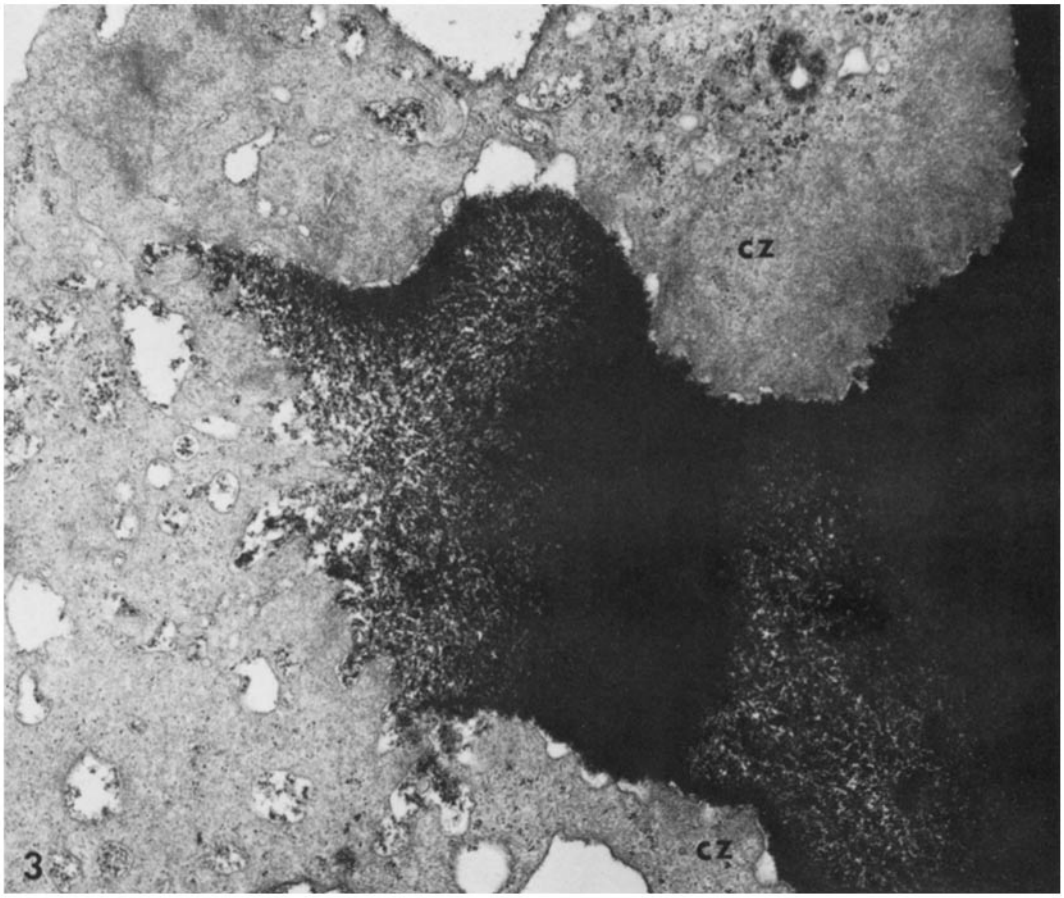

FIGURE 3 Osteoclast after treatment with colchicine for 24 h. The upper and lower surfaces of the spicule show typical clear zones (*cz*). The bone surface on the left appears disrupted, but there is no typical ruffled border. This may represent an area of previous ruffled border activity. × 18,600.

TABLE IV

Effects of 24, 48, and 72 h of Colchicine (Colch, 10^{-6} M) on Ruffled Border (RB) and Clear Zone (CZ) of Osteoclasts and on ^{45}Ca Release from Bones Precultured for 48 h with PTH (1.6 μg/ml)

Time	Treatment	Number of		Percent sections of osteoclasts with			^{45}Ca release
		Bones	Osteoclasts	RB + CZ	CZ only	Total	cpm/bone
h							
24	PTH → Co	7	41	37	34	71	2,003
	PTH → Colch	8	24	17*	71	88	769*
48	PTH → Co	7	43	26	51	78	2,839
	PTH → Colch	7	9	0	33	33	976*
72	PTH → Co	7	18	22	61	83	2,474
	PTH → Colch	8	25	0	64	64	884*

The number of osteoclasts represent the number of cross sections seen and does not indicate the frequency of osteoclasts per bone.

* Significantly different from PTH → Co, $P < 0.05$.

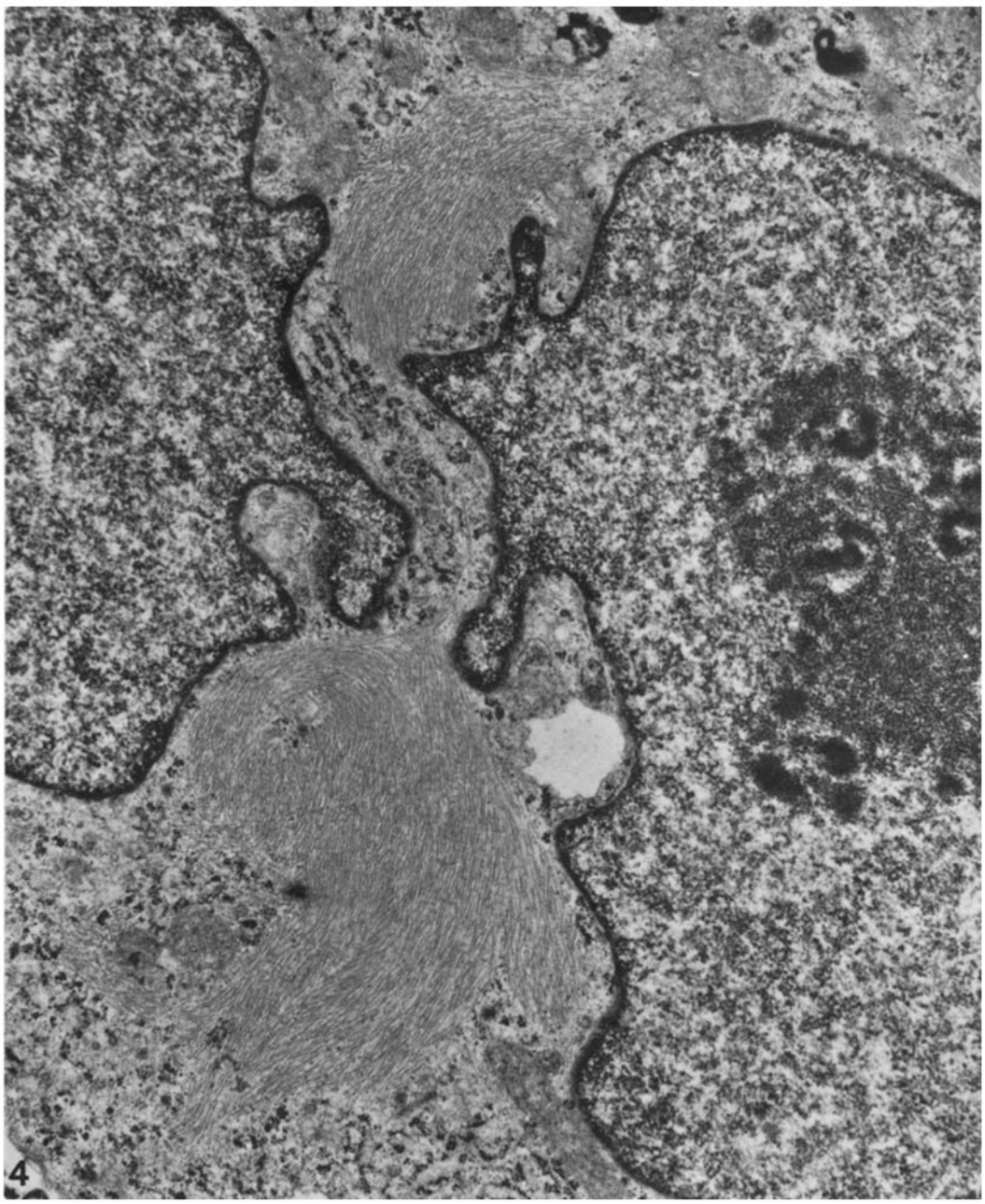

FIGURE 4 Representative micrograph showing vast bands of 100 Å filaments in the cytoplasm of an osteoclast after treatment with colchicine for 72 h. × 18,200.

changes in ^{45}Ca release. The only exception was 1 h after colchicine or calcitonin when ^{45}Ca release during that hour could not yet be shown to decrease, but ruffled borders at the end of that hour were markedly affected. This could indicate that the change in ruffled borders preceded the change in calcium release.

The values for frequency of ruffled borders and clear zones after treatment with colchicine or calcitonin were similar to those obtained from

osteoclasts cultured continually in control medium: the cells seemed to have returned to their resting phase. Since the appearance of the osteoclasts was so similar with two different drugs and in control cultures, we must conclude that the morphologic effects were not specific for any one agent. In an earlier study of osteoclast morphology in calcitonin-treated mouse calvaria, Kallio et al. (1972) reported that all control osteoclasts had ruffled borders and that none showed ruffled borders after treatment with calcitonin. This system differs from ours in that control bones (not treated with PTH) show increasing osteoclastic activity in culture. The effects of calcitonin were similar to those we observed in our study, except that the differences were more extreme; this could have resulted from limited nonrandom sampling of the osteoclasts in that study.

In our studies colchicine has been used as an inhibitor of bone resorption in two different ways and appeared to have two different effects. When given concurrently with PTH, it decreased osteoclast number. When given after PTH-stimulated resorption was established, it inhibited resorption acutely and decreased ruffled border frequency. Colchicine is considered to be specific in that it binds with high affinity to microtubular proteins (Borisy and Taylor, 1967) and disrupts microtubular function. Microtubules are usually considered to be involved in transport processes from one region of the cell to another (Freed and Lebowitz, 1970; Malawista, 1965; MacGregor and Stebbings, 1970), or from the interior to the exterior (Lacy, et al., 1968; Williams and Wolff, 1972). It is difficult to explain the acute effect of colchicine on the ruffled border as a microtubular effect. There are few microtubules in osteoclasts, and these do not appear to be localized near the ruffled border region. It is possible that colchicine affects not only microtubular function but acts directly on cell membranes. In liver cells, colchicine has been shown to bind to nuclear membrane (Stadler and Franke, 1972). There are other cell systems in which the effects of colchicine seem to be related to membrane function (Vasiliev et al., 1970; Harris and Krane, 1971; Berlin and Ukena, 1972; Ukena and Berlin, 1972). While these responses could be explained by altered microtubular control of membrane function, a direct involvement of the drug in membrane transport has not been ruled out. While the effect of colchicine on the differentiation of osteoclasts could involve microtubular function, it could also be readily explained as a membrane effect preventing the aggregation of precursor cells.

Colchicine binds to a microtubular subunit (Weisenberg et al., 1968), and this prevents the aggregation of subunits into microtubules. Morphologically these subunits are thought to be represented by filaments with a width of 100 Å (Wisniewski et al., 1968; Ishikawa et al., 1968). A massive accumulation of these filaments was observed in colchicine-treated osteoclasts which was far too great to represent merely the breakdown of microtubules which were previously present. Moreover, there was with time a progressive increase in 100 Å filaments. One explanation could be that the lack of complete microtubules stimulated the cells continuously to synthesize microtubular protein and form subunits, which could not form microtubules because of binding of colchicine to the subunits. In flagella it has been demonstrated that the synthesis of microtubular protein continues after treatment with colchicine (Rosenbaum et al., 1969). In our cultures, cells in metaphase arrest, in which protein synthesis is decreased, did not show an increase of 100 Å filaments. This supports the concept that the accumulation of filaments in interphase cells represents recently synthesized microtubular proteins.

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