DIRECT IN VIVO DEMONSTRATION BY RADIOAUTOGRAPHY OF SPECIFIC BINDING SITES FOR CALCITONIN IN SKELETAL AND RENAL TISSUES OF THE RAT

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ABSTRACT

An in vivo binding assay using radioautography was employed to visualize calcitonin receptors in rat tissues. At 2 min after intravenous injection of biologically active ^{125}I-salmon calcitonin, free hormone was separated from bound hormone by intracardiac perfusion with lactated Ringer's followed by fixation with 2.5% glutaraldehyde. Various tissues were removed and processed for light and electron microscope radioautography. These were compared to tissues removed from animals that received identical amounts of labeled hormone with a large excess of unlabeled calcitonin. Among the tissues investigated, kidney and bone demonstrated labeling. In kidney, most silver grains were located over vesicles below the brush border of cells of the proximal convoluted tubules. These grains were still present after simultaneous injection of excess unlabeled hormone and most likely represented binding to sites involved with ingestion and degradation of hormone from the urinary filtrate. In contrast, grains localized to the basal surfaces of distal convoluted tubule cells were significantly reduced in number in control animals and represented sites of saturable, specific hormone binding. In bone, specific binding sites were found only at the periphery of osteoclasts. These labeled cells were located at resorption sites examined in tibia, humerus, and alveolar bone. This demonstration of the localization of ^{125}I-calcitonin *in situ* provides a new approach for studying the interaction of calcium-regulating hormones with their target cells.

The action of calcitonin in lowering serum calcium (6) and modulating the levels of other ions in serum (1) has implicated the presence of target cells in the intestine, kidney, and bone. These target organs were examined using a technique that provides direct in vivo visualization by radioautography of peptide hormone binding sites in cells and tissues (3–5). Thus, it was possible to identify histologically the cells responsible for binding calcitonin. The technique involves injecting an experimental rat intravenously with biologically active ^{125}I-labeled salmon calcitonin. The labeled hormone is allowed to circulate for 2 min, after which the unbound hormone is washed out of the body by intracardiac perfusion with lactated Ringer's solution. The tissues are then fixed by perfusion with 2.5% glutaraldehyde. The control rat receives radioactive hormone mixed with a large excess of unlabeled hormone. The competition for specific and saturable binding sites favors

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the occupation of these sites by unlabeled hormone. Consequently, in radioautographs prepared from experimental and control rats, sites within corresponding cells and tissues that are labeled in experimental rats, but unlabeled in the controls, represent specific binding.

MATERIALS AND METHODS

Hormone Preparation and Assessment of Biological Activity

Synthetic salmon calcitonin (sCT; ~2,500 mU/μg; a generous gift of Sandoz Co. Ltd., Basel, Switzerland) was iodinated with $^{125}I^-$ by the chloramine T method (10) immediately before each experiment. This procedure results in a labeled preparation that retains full biological activity because of the absence of methionine and tryptophan residues within the salmon molecule. These residues, present in the calcitonin of other species, are susceptible to oxidation during iodination (15), with resultant biological inactivation of those molecules. Labeled hormone was separated from free iodide with beads of AG-1 × 10 (200–400 mesh, chloride form, Bio-Rad Laboratories, Richmond, Calif.) and was purified from reactants with microfine silica (QUSO G32, Philadelphia Quartz Co., Valley Forge, Pa.). For intravenous injection, the labeled hormone was eluted in a mixture of 20% (vol/vol) acetone and 1% (vol/vol) acetic acid, evaporated to dryness under a stream of nitrogen, and redissolved in 2.5% (wt/vol) bovine serum albumin in 25 mM Tris-HCl buffer at pH 7.4. The biological integrity of identically labeled hormone has previously been confirmed by demonstrating specific in vitro binding and adenylate cyclase stimulation in membranes derived from fetal rat calvaria and from adult rat kidneys (8, 14, 15). In further experiments to determine the in vivo biological activity of the ^{125}I-labeled sCT, duplicate male Sherman rats were injected intravenously with either ^{125}I-sCT (specific activity, 710 μCi/μg; 25×10^6 or 250×10^6 cpm) or unlabeled sCT (30 or 300 ng) or buffer alone (2.5% (wt/vol) bovine serum albumin in 25 mM Tris-HCl, pH 7.4). At 1 h after injection the animals were exsanguinated through the dorsal aorta, and plasma calcium was determined (Fig. 1) on a Sequential Multiple Analyzer with Computer (Technicon Instruments Corp., Tarrytown, N.Y.) by the method of Gitelman (7).

Other control experiments were done with porcine insulin (24.4 U/mg; Connaught Laboratories, Toronto, Ontario) that was also iodinated by the chloramine T procedure as previously described (3–5). Synthetic bovine parathyroid hormone [bPTH(1–34), 6,000 U/mg], also used as a control, was purchased from Beckman Instruments, Inc. (Palo Alto, Calif.) and $Na^{125}I$, 17 μCi/mg was obtained from New England Nuclear (Boston, Mass.).

Animal Procedures

For studies concerning the in vivo localization of calcitonin binding sites by radioautography, three experiments were performed on six male Sherman rats (Table I). Under Nembutal anesthesia, the experimental rats were injected through the external jugular vein with 0.1 ml of freshly prepared ^{125}I-sCT (Table I). After 2 min (range, 2 min to 2 min, 55 s) the animals were perfused (by gravity at ~15 ml per 10 s) through the left ventricle with lactated Ringer's solution (Abbott Diagnostics, Diagnostic Products, North Chicago, Ill.) for ~30 s (range, 24–60 s) to remove unbound hormone. This was immediately followed by perfusion for 10–15 min with 2.5% glutaraldehyde in 0.05 M Sorensen's phosphate buffer containing 0.1% (wt/vol) sucrose at pH 7.3. Control animals received the same quantity of ^{125}I-sCT but with an excess of unlabeled sCT (Table I).

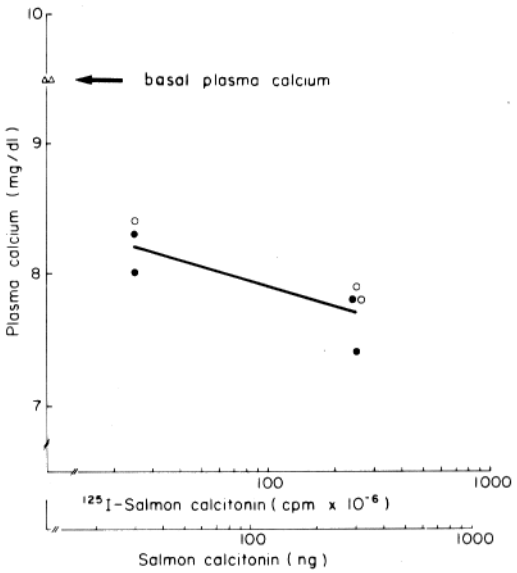


FIGURE 1 Changes in plasma calcium produced 1 h after intravenous injection of ^{125}I-salmon calcitonin (●) and unlabeled salmon calcitonin (○). Basal plasma calcium (△) was determined 1 h after injection of buffer only.

In other control experiments assessing the specificity of this method, ^{125}I-sCT was injected into male Sherman rats, with or without 50 μg of unlabeled bPTH(1–34). Also, ^{125}I-insulin (140×10^6 dpm, specific activity 160 μCi/μg) was injected, with or without excess (50 μg) unlabeled insulin. Finally, $Na^{125}I$ (200×10^6 dpm) was injected alone. At 2 min after the injection in each case, the animals were perfused, and fixed according to the procedures described above for calcitonin localization by radioautography.

Immediately after perfusion, kidney, liver, stomach, duodenum, tibia, humerus, and incisors (within the alveolar bone) were dissected and placed in the same fixative at 4°C. Nonmineralized tissues were subsequently washed in 0.15 M phosphate buffer at pH 7.3 for 50 min and postfixed for 2 h in 1% O_sO_4 with veronal-acetate buffer. After acetone dehydration, blocks were infiltrated and embedded in Epon. Mineralized tissues were decalcified in 4.13% disodium EDTA for 14–16 d at 4°C (18) and washed for 24 h in 0.15 M phosphate buffer before being processed as above.

For light microscope radioautography, 0.5-μm-thick sections were prestained with iron hematoxylin, coated with Kodak NTB2 emulsion (Kodak Canada Ltd., Toronto, Ont.) (13), and exposed for various intervals. For electron microscope radioautography, silver-to-gray interference color sections were cut and prepared according to Kopriwa (12).

Quantitative Analysis of Radioautographs

KIDNEY: Silver grains were counted with the light microscope

over proximal (Table II) and distal (Table III) convoluted tubules from similarly exposed radioautographs of experimental and control animals. The diameter of each tubule counted was measured in two axes at right angles to each other. Average diameters were calculated and compared between several rats in experimental and control kidneys. Because no statistically significant size differences were found, the counts were expressed as grains per cross-sectioned tubule (Tables II and III).

BONE: The proximal tibial and humeral epiphyseal plates were sectioned parallel to the long axes of the bones. For quantitation with the light microscope, two analyses were performed. First, grains were counted over the tissue at the epiphyseal surface of the growth plate in areas delimited by an ocular micrometer grid (Table IV). Second, grains were counted over clearly defined multinucleate osteoclasts of approximately equal size located at the epiphyseal and metaphyseal surfaces of the plate. Thus, osteoclasts in the zones of vascular invasion (resorption of calcified cartilage) and of mixed spicules (resorption of calcified cartilage and bone) were counted (Table V).

Quantitation of the silver grains related to osteoclasts in electron microscope radioautographs was done from micrographs at a magnification of × 30,000. Grains were scored as being within the osteoclast, outside the osteoclast, or directly on the plasma membrane (or within a radius of 230 nm from the grain center).

RESULTS

In Vivo Assessment of the Biological Activity of ^{125}I-sCT

A dose-related decrease in plasma calcium was achieved with both doses of ^{125}I-sCT injected. This depression was approximately equal in magnitude to the fall achieved by equivalent amounts of injected unlabeled hormone, attesting to the full biological potency of the iodinated preparation (Fig. 1).

TABLE I

Protocol of the Three In Vivo Experiments with ^{125}I-Salmon Calcitonin in Male Sherman Rats

Experiment	Animal weight	Amount injected per 100 μl	Specific activity	"Hot" sCT	"Cold" sCT
	g	*cpm* × 10^{-6}	*μCi/μg*	*μg*	*μg*
1					
Experimental	109	90	562.5	.144	—
Control	108	70		.112	50
2					
Experimental	100	82	645	.119	—
Control	110	81		.118	50
3					
Experimental*	96	255	712.5	.326	—
Control*	94	255		.326	125

* These animals received 125 μCi of [^{3}H]thymidine (methyl-^{3}H, New England Nuclear, specific activity 20 Ci/mmol) 1 h before injection of calcitonin.

TABLE II

Grain Counts per Cross-Sectioned Proximal Convoluted Tubule‡ in Experimental and Control Rats Injected with ^{125}I-Salmon Calcitonin*

Experiment	Total grains	Mean grains per tubule	± SD	± SEM	*P*
1					
Experimental	4,815	96	52	7.4	<0.2§
Control	5,509	110	62	9.0	
2					
Experimental	6,662	113	37	5.2	<.001§
Control	10,583	213	55	7.6	
3					
Experimental	7,947	159	59	8.2	<.001§
Control	13,332	261	99	14.1	

* Counted in light microscope radioautographs exposed for 7 d.

‡ 50 Tubules counted in each rat.

§ In each experiment, the experimental value never exceeded the control.

TABLE III

Grain Counts per Cross-Sectioned Distal Convoluted Tubule‡ in Experimental and Control Rats Injected with ^{125}I-Salmon Calcitonin*

Experiment	Total grains	Mean grains per tubule	± SD	± SEM	P
1					
Experimental	1,708	34	13.6	1.9	<.001§
Control	382	7	7.4	1.1	
2					
Experimental	2,055	41	19.5	2.8	<.001§
Control	1,202	24	13.6	1.9	
3					
Experimental	3,072	61	28	4.0	<.001§
Control	823	16	10	1.5	

* Counted in light microscope radioautographs exposed for 7 d.

‡ 50 Tubules counted in each rat.

§ The experimental value exceeded the control by factors of 4.5, 1.7, and 3.7, respectively, in the three experiments.

TABLE IV

Grain Counts over the Tissue on the Epiphyseal Surface of the Proximal Tibial Growth Plate in Experimental and Control Rats Injected with ^{125}I-Salmon Calcitonin*

Experiment	Total grains‡	Mean grains per 3,000 μm^2	± SD	± SEM	P
1					
Experimental	6,588	132	55.7	7.9	<.001§
Control	1,342	27	15.0	2.1	
2					
Experimental	4,581	92	41.7	5.9	<.001§
Control	2,513	50	20.9	3.0	
3					
Experimental	9,862	197	82.8	11.7	<.001§
Control	1,047	21	10.5	1.5	

* Counted in light microscope radioautographs exposed for 21, 28, and 7 d, respectively, in experiments 1, 2, and 3.

‡ Grains were counted in a strip composed of 50 rectangles laid across the epiphyseal surface of the plate. Each rectangle measured 100-μm long by 30-μm high. This value represents all the grains counted in the 50 rectangles.

§ The experimental value exceeded the control by factors of 4.9, 1.8, and 9.4, respectively, in the three experiments.

Radioautographic Localization of ^{125}I-sCT

SUSPECTED TARGETS IN NONCALCIFIED TISSUES

GASTROINTESTINAL TRACT: Several sites, including the body of the stomach, duodenum, and proximal jejunum, were sampled. In both experimental and control rats of all three experiments, no radioactivity was detected by radioautography in these tissues, indicating that under the present experimental conditions no receptors for calcitonin were demonstrable.

KIDNEY: Numerous silver grains were present over the proximal convoluted tubules in the cortex from both experimental (Fig. 2) and control (Fig. 3) rats. Within the tubules, the grains were located at the base of the brush border (Figs. 2 and 3), and with electron microscopy (Fig. 4) these reactions were seen at the periphery of large, nearly empty vacuoles and smaller vesicles at the junction between the brush border and the supranuclear cytoplasm.

Because the distribution of grains over the proximal convoluted tubules was identical in experi-

TABLE V

Grain Counts over Osteoclasts‡ at the Metaphyseal Surface of the Proximal Tibial Growth Plate in Experimental and Control Rats Injected with ^{125}I-Salmon Calcitonin*

Experiment	Total grains§	Mean grains per osteoclast	± SD	± SEM	*P*
1					
Experimental	2,612	52	24.8	3.5	<.001‖
Control	966	19	12.2	1.7	
2					
Experimental	2,322	46	18.8	2.7	<.001‖
Control	968	19	8.4	1.2	
3					
Experimental	4,390	88	43.2	6.1	<.001‖
Control	651	13	9.4	1.3	

* Counted in light microscope radioautographs exposed for 21, 28, and 7 d, respectively, in experiments 1, 2, and 3.

‡ 50 Osteoclasts selected for equal size.

§ All the grains over the 50 cells counted in each rat.

‖ The experiment value exceeded the control by factors of 2.7, 2.4, and 6.8, respectively, in the three experiments.

mental and control animals, and because no quantitative decrease could be shown in the number of silver grains per cross-sectioned tubule in the control (Table II), it was concluded that the reactions represented nonspecific binding of labeled hormone to high capacity sites. These sites were probably related to resorption and subsequent degradation of labeled hormone from the urinary filtrate.

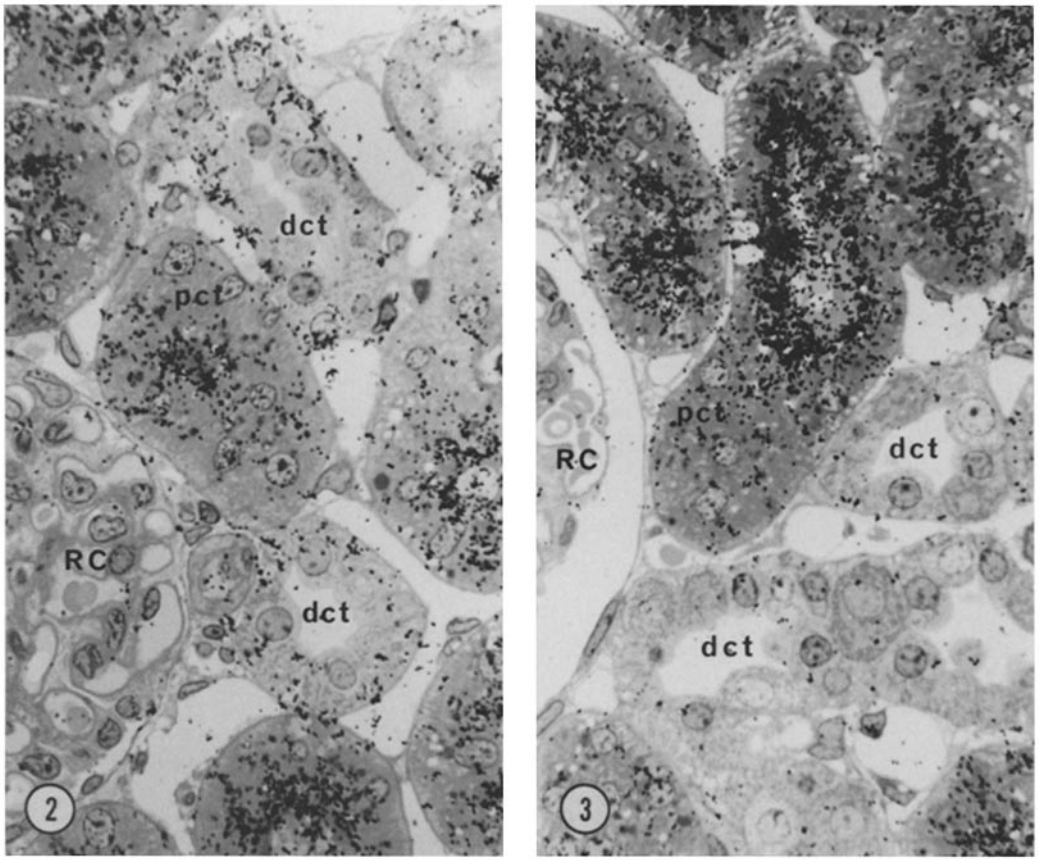
A moderate number of silver grains was present over the basal aspect of the distal convoluted tubules in the cortex of the kidneys from experimental animals (Fig. 2). With electron microscope radioautography, the silver grains were localized to the infolded basal cell membrane (Fig. 5). Grains were almost completely absent over the control kidneys (Fig. 3). Grain counts per cross-sectioned distal convoluted tubule (Table III) showed a decrease ranging from 1.7- to 4.5-fold. Thus, these basally disposed grains along the distal convoluted tubules represented specific, saturable receptor sites for calcitonin.

Suspected Targets in Calcified Tissues

BONE: The proximal tibial and humeral epiphyseal growth plates were examined. In the experimental animals (Figs. 6, 8, and 10), prominent labeling was present over the multinucleated osteoclasts. Labeling was also present over mononucleated cell fragments that could not be identified with the light microscope. However, clearly identifiable osteoblasts, osteocytes, chondrocytes, and bone marrow cells were unlabeled. In control animals, no grains were found over any cell types (Figs. 7, 9, and 11).

OSTEOCLASTS: On the metaphyseal aspect of the growth plate, large osteoclasts were associated with the zone of vascular invasion and resorption of calcified cartilage. In experimental animals, these osteoclasts, in both tibia (Fig. 8) and humerus (Fig. 10), were heavily labeled predominantly at their periphery. Osteoclasts in control animals showed no silver grains (Figs. 9 and 11). Similarly, osteoclasts located on the epiphyseal surface of the growth plate were heavily labeled (Fig. 6). Cells of the control animals showed no labeling (Fig. 7). Osteoclasts were also labeled on resorption surfaces of the alveolar bone surrounding the persistently erupting incisors.

OTHER LABELED CELL FRAGMENTS: Other cells or cell fragments associated with bone showed specific, peripherally located silver grains, which were displaced in the control situation. These nonnucleated, or mononucleated, cell fragments were usually located in the connective tissue space between blood vessels and fully differentiated osteoblasts (Figs. 6–11). Nuclear labeling with [^{3}H]-thymidine at 1 h after injection was invariably confined to cells similarly located (Figs. 6, 8–11). With [^{3}H]thymidine, the only cells labeled 1 h after injection in bone populations were classically described as osteoprogenitor cells (17, 19). Osteoclast nuclei were never labeled at that time (17,

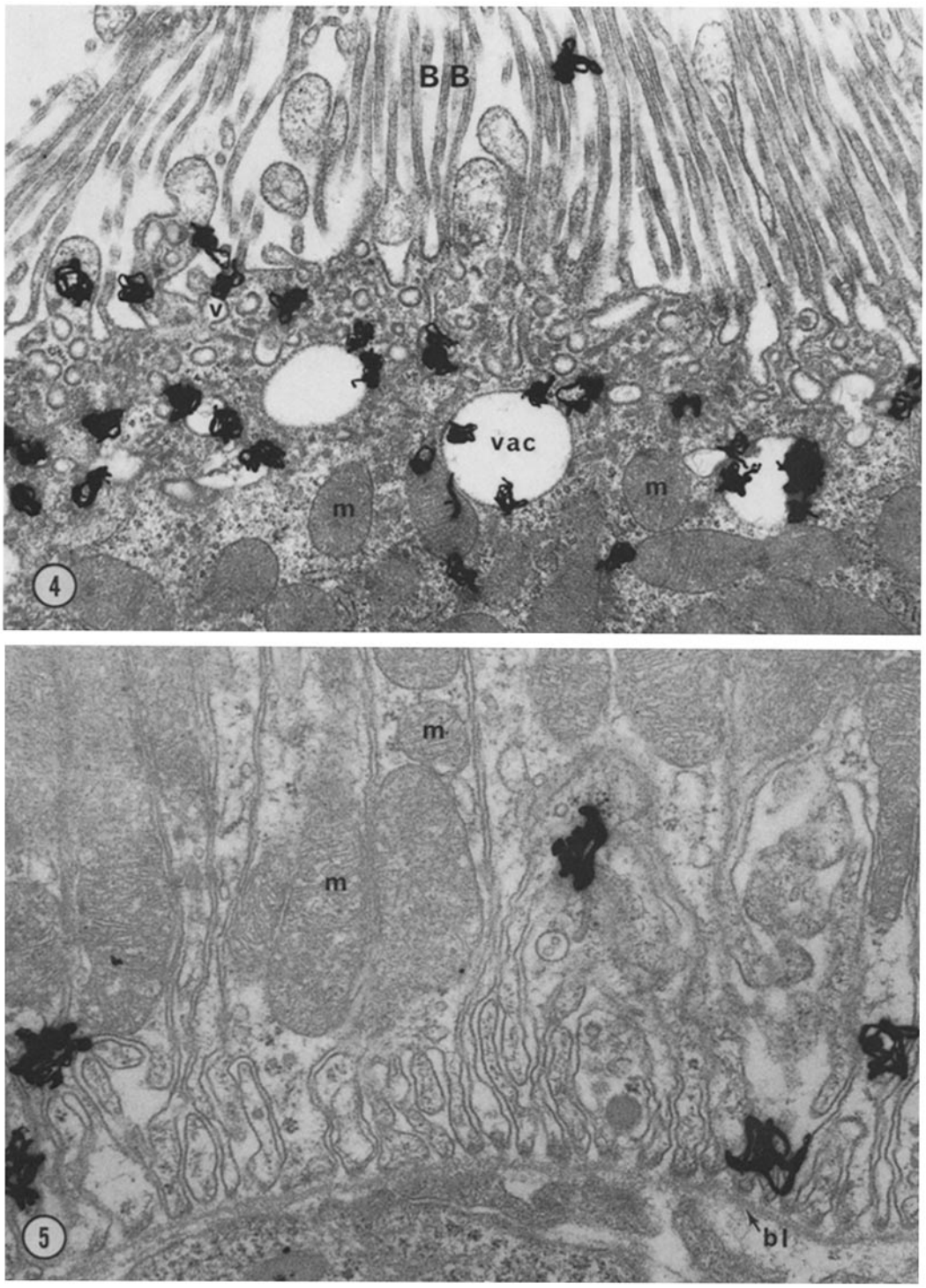


FIGURES 2 and 3 Kidney cortex from an experimental (Fig. 2) and a control animal (Fig. 3). Radioautographs of 0.5-μm-thick Epon sections exposed for 14 days. × 680. In Fig. 2, numerous silver grains are present over the cytoplasm of proximal convoluted tubules (*pct*). These grains are localized at the junction between the brush border and the apical cytoplasm. Distal convoluted tubules (*dct*) are labeled preferentially over their basal surface. Renal corpuscles (*RC*) are unlabeled. In Fig. 3, silver grains are present over identical locations within the proximal convoluted tubules (*pct*) of the control, thus indicating that the labeling is nonspecific. These heavy accumulations of silver grains presumably represent pinocytic uptake of labeled hormone from the urinary filtrate. Distal convoluted tubules (*dct*) are almost completely unlabeled, indicating that the basal surfaces of these tubules possess specific binding sites for calcitonin. *RC*, renal corpuscle.

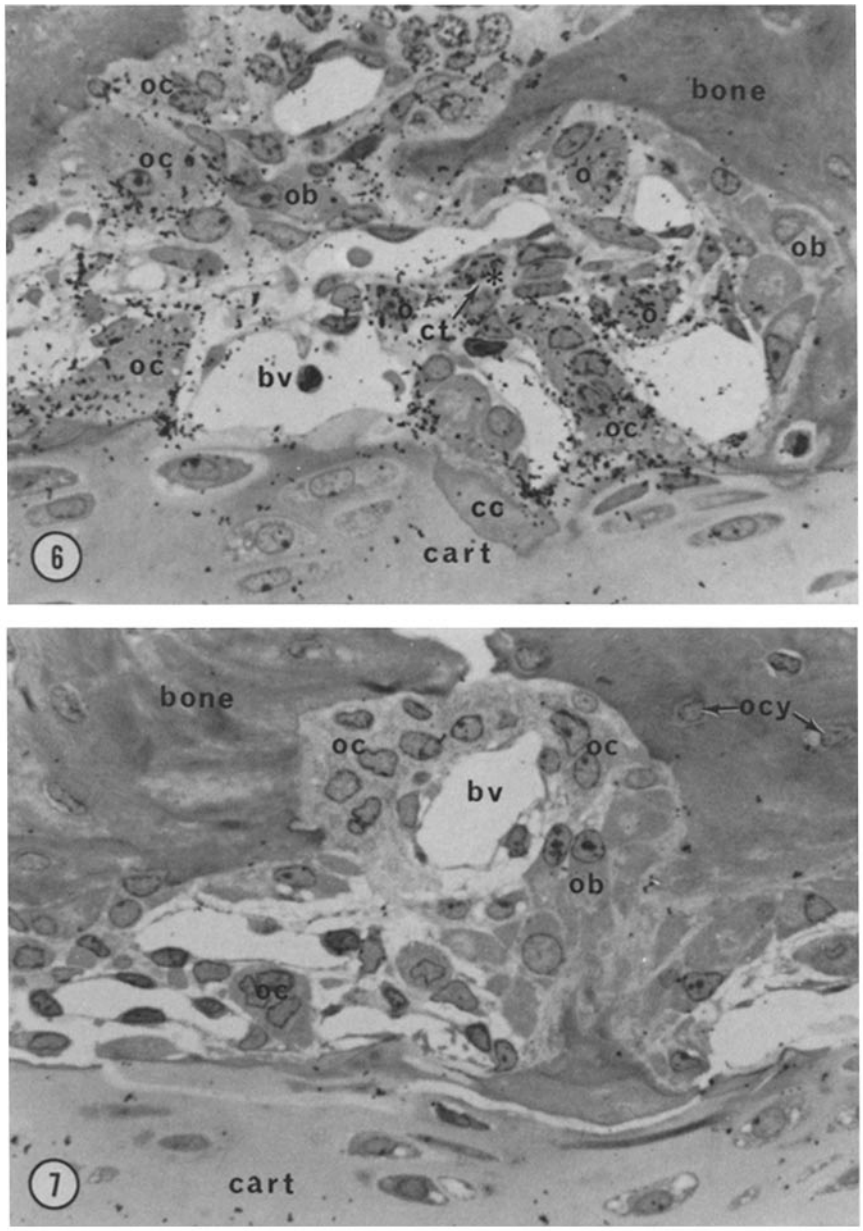
19). Therefore, it was assumed that these calcitonin-labeled cells could either be osteoprogenitor cells because of their morphology and location, or portions of osteoclasts that appeared mononucleated because of the plane of section. The latter interpretation was strengthened by the lack of nuclear labeling of these cells with [^{3}H]thymidine.

Electron microscope radioautography confirmed that the peripheral localization of silver grains seen in the light microscope resulted from reactions at the cell membrane of osteoclasts (Figs. 12–14). In addition, the labeled cell fragments of questionable identity at light microscope resolution could, in all cases, be attributed to osteoclasts on the basis of organelle morphology and distribution.

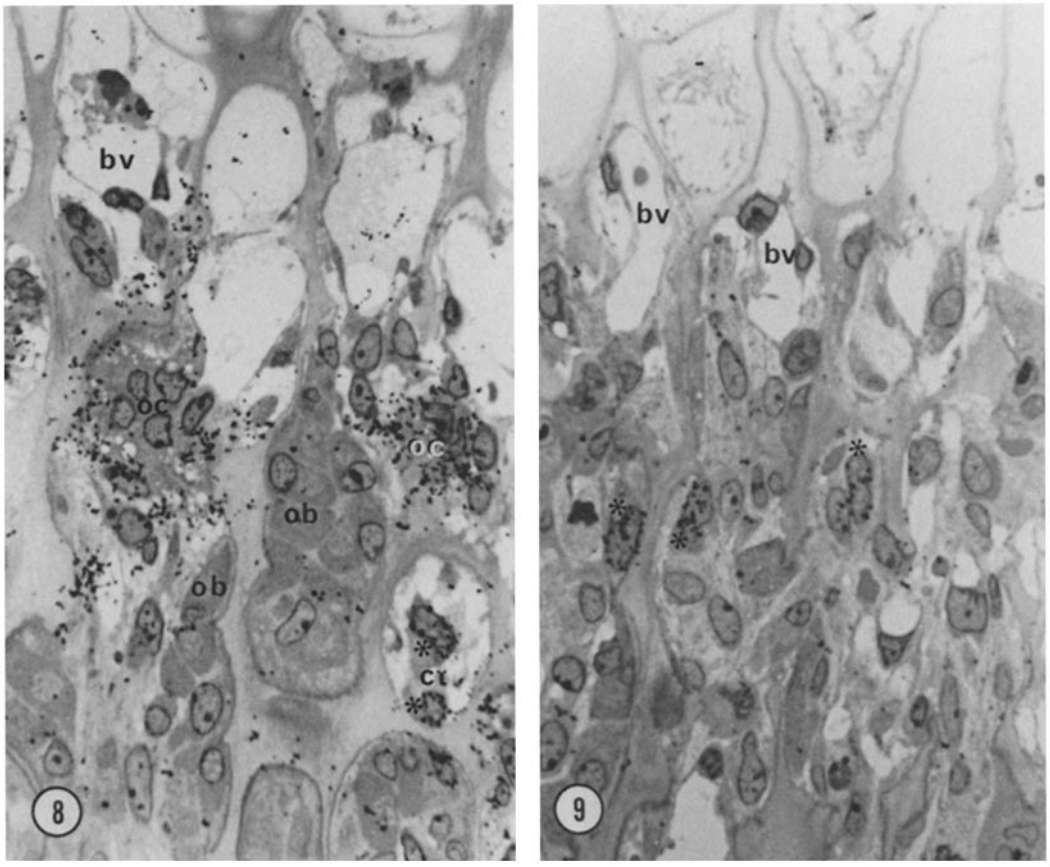
Quantitative analysis was done on two regions of the tibia in light microscope radioautographs. Grain counts per unit area of the tissue on the epiphyseal surface of the growth plate showed that the counts were significantly higher in the experimental rats than in the controls (Table IV). Also, the controls were reduced by factors of 1.8–9.4 from the experimental values. Grain counts over



FIGURES 4 and 5 Electron microscope radioautographs from the kidney cortex of experimental animals. Fig. 4 shows a proximal convoluted tubule in which the location of the nonspecific labeling is seen at the periphery of large vacuoles (*vac*) and small pinocytic vesicles (*v*). Exposed for 30 d. *BB*, brush border; *m*, mitochondria. × 15,000. Fig. 5 shows the basal aspect of a distal convoluted tubule in which specific binding is associated with the deeply invaginated cell membrane. Exposed for 80 d. *bl*, Basal lamina; *m*, mitochondria. × 30,000.



FIGURES 6 and 7 Epiphyseal surface of the cartilage growth plate in experimental (Fig. 6) and control animals (Fig. 7). Light microscope radioautographs of 0.5-μm-thick Epon sections exposed for 14 d. × 680. In Fig. 6, the narrow space between the bone of the epiphyseal cavity and the cartilage of the growth plate (*cart*) contains capillaries (*bv*), osteoclasts (*oc*), osteoblasts (*ob*), and connective tissue cells (*ct*), some of which are presumed to be osteoprogenitor cells. In the experimental rat, silver grains are found over osteoclasts (*oc*) and cells of uncertain identity (*o*). Electron microscopy revealed these to be fragments of labeled osteoclasts. Osteocytes seen within the bone, osteoblasts on the bone surfaces, and chondrocytes within the cartilage plate are not labeled. The asterisk indicates cells labeled with [^{3}H]thymidine injected 1 h before labeled calcitonin. These cells, showing thymidine labeling, are not labeled by calcitonin and are presumed to be connective tissue cells (*ct*) with osteoprogenitor potential. *cc*, Calcified cartilage. In Fig. 7, a similar region in the control animal shows a scattering of silver grains that are not localized to any of the cell types. *bv*, Blood vessel; *cart*, cartilage; *ob*, osteoblast; *oc*, osteoclast; *ocy*, osteocyte.



FIGURES 8 and 9 The zone of vascular invasion of the proximal tibial epiphyseal growth plate in experimental (Fig. 8) and control rats (Fig. 9). Light microscope radioautographs of 0.5-μm-thick Epon sections exposed for 14 d. × 680. In Fig. 8, below the zone of hypertrophic chondrocytes, osteoclasts (*oc*) are often seen along the remnants of calcified cartilage. These cells show numerous silver grains over the periphery of their cytoplasm. Connective tissue cells (*ct*), labeled with [^{3}H]thymidine (asterisks) indicating their osteoprogenitor potential, are not labeled with ^{125}I-calcitonin. Fully differentiated osteoblasts (*ob*) are not labeled. *bv*, Blood vessel associated with vascular invasion of chondrocyte lacunae. In Fig. 9, silver grains are absent over all structures in the section, except for [^{3}H]thymidine-labeled nuclei of connective tissue cells presumed to be osteoprogenitor cells (asterisks). *bv*, Blood vessel.

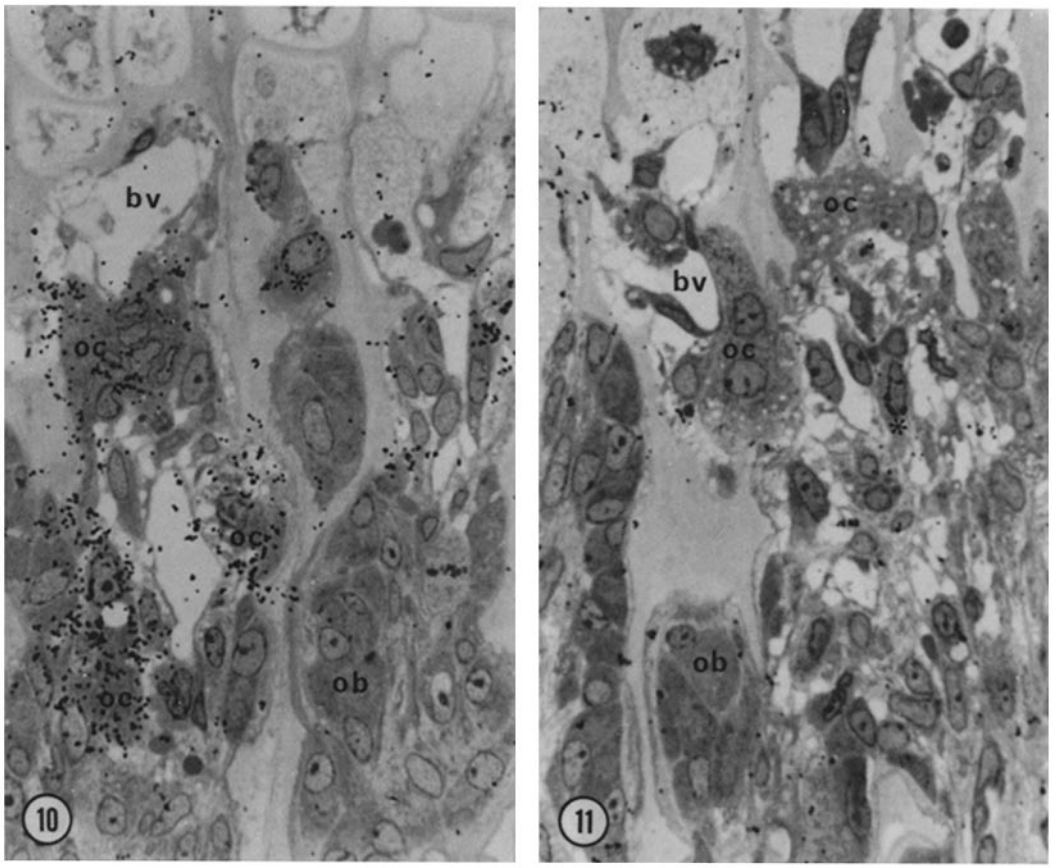
individual osteoclasts of the tibia confirmed that the cells were significantly more labeled in the experimental rats than in the controls (Table V). The control counts were reduced from experimental values by factors of 2.4–6.8.

Grain counts in the electron micrographs were made by placing a 230-nm radius resolution boundary circle around each grain and recording the structure within that circle. Of the 532 grains counted, 67% were related to the cell membrane of the osteoclasts. This value is similar to that previously shown for insulin receptors on hepatocytes (4).

RADIOAUTOGRAPHIC CONTROLS OF BINDING SPECIFICITY

When ^{125}I-sCT was injected with a large excess of unlabeled and unrelated hormone, such as bPTH(1–34), no change in distribution or number of silver grains was observed over any tissue as compared with radioautographs from rats injected with ^{125}I-sCT alone.

Radioautographs from the tissues of rats injected with either ^{125}I-insulin or Na^{125}I showed no silver grains over bone cells or the basal aspect of the distal convoluted tubules.

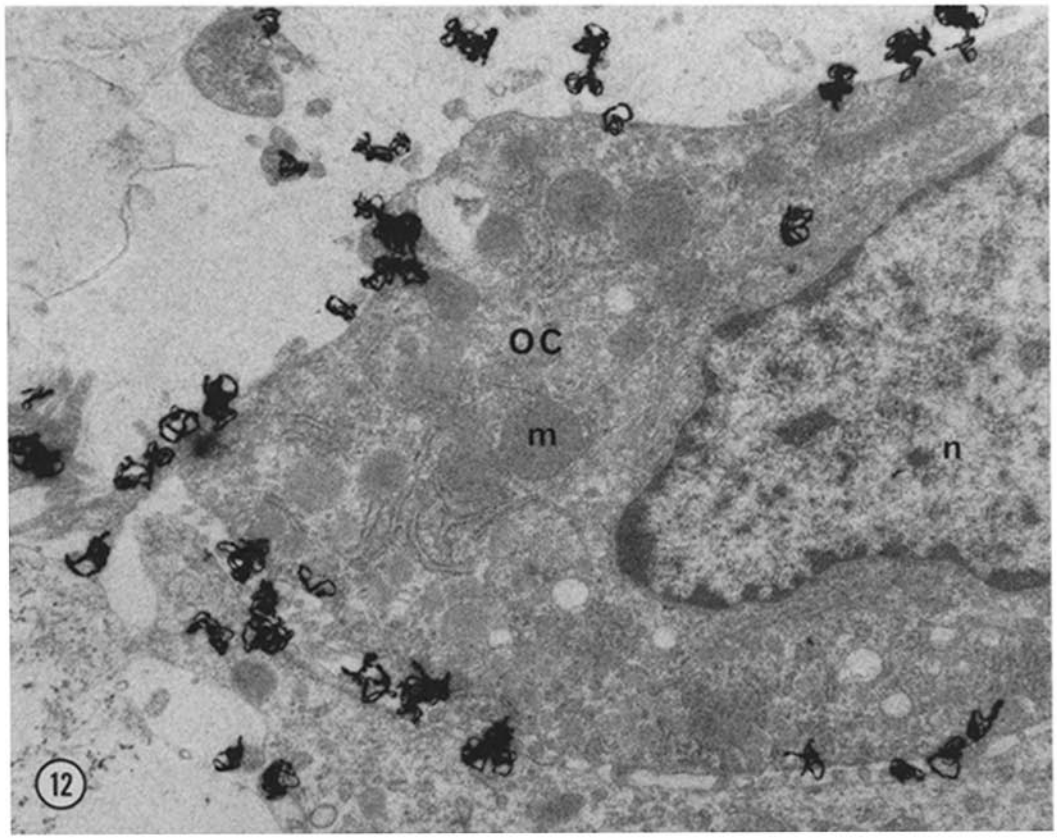


FIGURES 10 and 11 The zone of vascular invasion in the proximal humerus of experimental (Fig. 10) and control animals (Fig. 11). Light microscope radioautographs of 0.5-μm-thick Epon sections exposed for 14 d. × 680. In Fig. 10, labeled osteoclasts (*oc*) are seen at the surfaces of calcified cartilage and mixed spicules. Osteoblasts (*ob*) are unlabeled. *bv*, Blood vessel; asterisk, [^{3}H]thymidine-labeled cells. In Fig. 11, similarly located osteoclasts (*oc*) in the control animals are not labeled. Again, only [^{3}H]thymidine-labeled cells (asterisk) are seen. *bv*, Blood vessel; *ob*, osteoblast.

DISCUSSION

Two major hormones, parathyroid hormone and calcitonin, are known to have antagonistic effects on the level of serum calcium (9, 16) and consequently on the regulation of bone homeostasis. In this study we have prepared ^{125}I-labeled salmon calcitonin by a method known to preserve its biological activity (8, 14, 15). Indeed, intravenous injection of this labeled hormone depressed the plasma calcium levels in a dose-dependent manner identical to that given by the unlabeled hormone (Fig. 1). This labeled, biologically active hormone was used in an in vivo assay for binding sites in suspected target tissues involving calcium regulation by calcitonin. In control animals, the concomitant administration of an excess of unlabeled calcitonin, but not of unrelated hormone, produced a competitive inhibition of labeling on the specific, saturable sites. Consequently, a comparison of experimental and control tissues disclosed specific binding sites similar to those found in in vitro studies of receptor binding. The outstanding advantage in the present method is the use of radioautography to identify the location of radioactivity. This technique allows for resolution of labeling to individual, histologically identifiable cell types in any target tissue, including mineralized bone.

Identical results were obtained with ^{125}I-sCT in three separate procedures involving three experi-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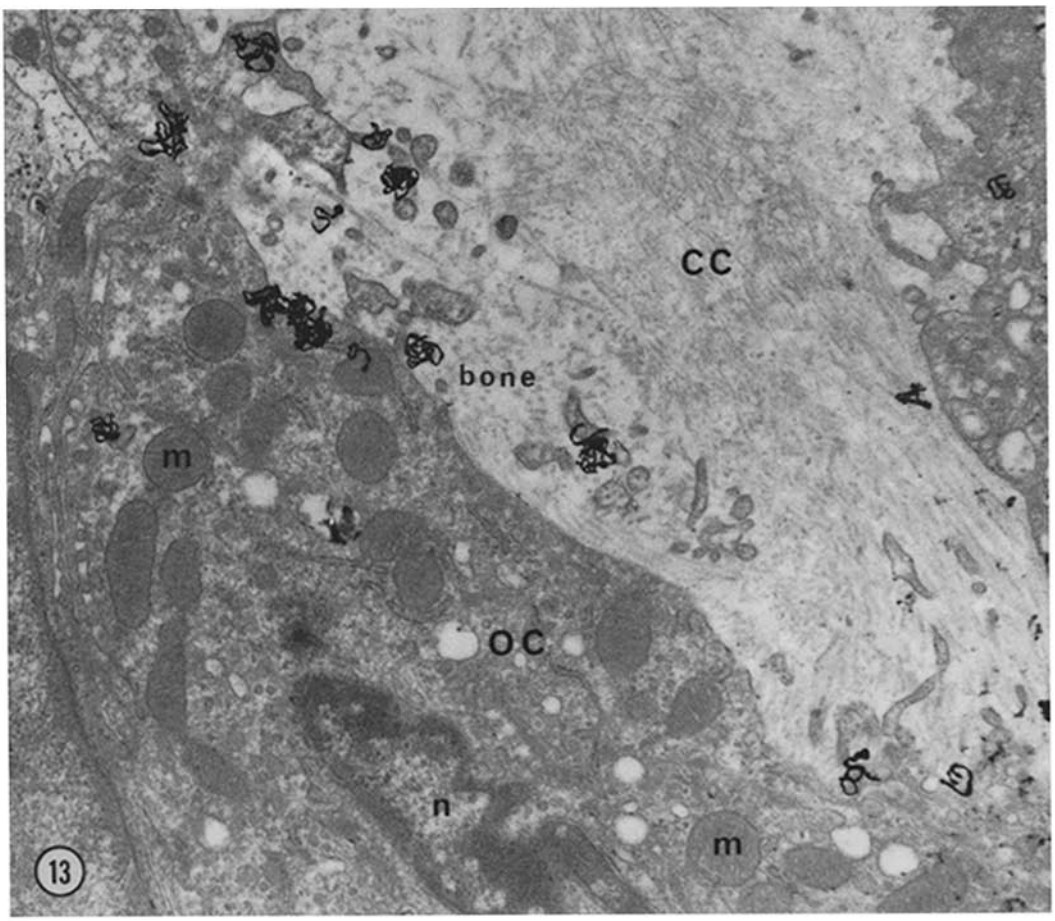
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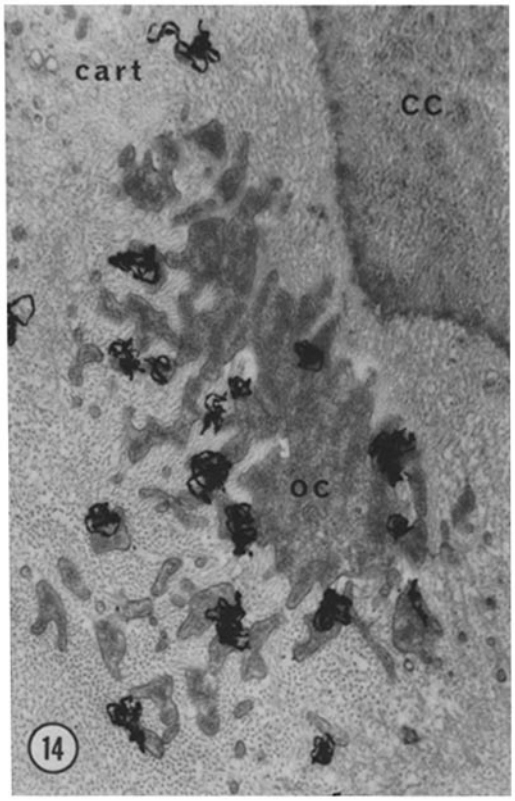

FIGURE 12–14 Electron microscope radioautographs of portions of osteoclasts from the tibia of experimental rats, showing silver grains related to their surface membrane. Fig. 12 shows an osteoclast (*oc*) from the zone of vascular invasion. Exposed for 50 d. *m*, Mitochondria; *n*, nucleus. × 15,000. Fig. 13 shows the osteoclast (*oc*) surface adjacent to bone near the zone of vascular invasion. Exposed for 30 d. *cc*, Calcified cartilage; *m*, mitochondria; *n*, nucleus. × 15,000. Fig. 14 shows cytoplasmic processes of an osteoclast (*oc*) in the tissue above the epiphyseal plate. The cell is in cartilage (*cart*) next to an isolated island of calcified cartilage (*cc*). Exposed for 80 d. × 15,000.

mental and three control rats. The presence of radioactivity in all animals was confirmed by the nonspecific binding seen in the kidneys of all experimental and control rats. Thus, the absence of radioactivity from specific binding sites in the controls could be attributed only to competitive inhibition. In the experimental animals, the presence of labeling on identical cell types in locations as remotely separated as the alveolar bone of the incisors, the humerus, and the tibia, confirmed the reliability of the tracer methodology for determination of specific binding sites. Parallel studies were designed to further confirm the specificity of the method. The injection of unlabeled parathyroid hormone with the labeled calcitonin did not reduce the number of silver grains over the specifically labeled cells of bone and kidney. The injection of labeled insulin did not result in silver grains over osteoclasts or the basal aspect of distal convoluted tubules. However, a heavy grain accumulation was seen over the luminal aspect of the proximal convoluted tubules, similar to the nonspecific binding shown by labeled calcitonin. Finally, labeled iodide alone showed no radioautographic silver grains over the specific calcitonin-binding sites in bone and kidney.

Because of the known effects of calcitonin, presumed sites of action in gut, kidney, and bone were examined. In the experiments performed, no labeling was seen in the gastrointestinal tissues examined. Consequently, a direct correlation of hormone binding with ion transport in the gut could not be demonstrated.

In the kidney, the large number of radioautographic silver grains over proximal convoluted tubule cells of experimental and control animals was caused by ingestion of labeled hormone by vesicles and vacuoles at the base of the brush border. This intense but nonspecific binding was presumed to be related to degradation of the labeled hormone derived from the urinary filtrate. However, specific sites were located on the infolded basal cell membrane of the distal convoluted tubules, which suggests that these cells are involved in the action of calcitonin, presumably enhancing renal excretion of electrolytes (1).

In bone, the finding of specific calcitonin receptor sites on the membrane of osteoclasts provides strong evidence to support the contention that direct inhibition of osteoclastic bone resorption is primarily involved in the hypocalcemic action of the hormone (11). The absence of demonstrable receptor sites on osteoblasts suggests that secretion of new bone, the mineralization of which could contribute to the hypocalcemic effect of the hormone, is not caused by direct stimulation of this cell. The absence of receptors on osteocytes argues against calcitonin regulation of bone resorption by osteocytic osteolysis (2).

In future studies, this methodology should also be useful in localizing the target cells for parathyroid hormone, as well as for detecting modifications of the binding sites for both calcium-regulating hormones that may occur under conditions of altered calcium homeostasis or skeletal pathology.

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