Abundant Calcitonin Receptors in Isolated Rat Osteoclasts

Biochemical and Autoradiographic Characterization

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Abstract

Calcitonin receptors have been characterized for the first time in isolated osteoclasts. These receptors have been demonstrated by autoradiographic and biochemical methods, and the cells have also been shown to respond to calcitonin with a dose-dependent increase in cyclic AMP. The receptors in rat osteoclasts are specific and of high affinity (dissociation constant, K_d, 1 to 6 $\times 10^{-10}$ M), and are present in greater numbers than in any cell previously studied ($>10^6$ per cell). Chemical cross-linking of ^{125}I-labeled salmon calcitonin to osteoclasts using disuccinimidyl suberate resulted in identification of a receptor component with a relative molecular weight of 80,000–90,000. By counting grains in autoradiographic experiments, we found that >80% of specifically bound radioactivity was associated with multinucleate osteoclasts and the remainder was associated with mononuclear cells that are not osteoblasts, but that may be osteoclast precursors.

Introduction

Calcitonin inhibits bone resorption (1) by acting directly on osteoclasts to reduce their number and activity (2), and in vivo autoradiographic studies have localized calcitonin binding to osteoclasts in bone (3). There is no evidence for a direct action of calcitonin upon osteoblasts or osteocytes, although osteocyte shrinkage has been observed to take place after calcitonin administration (4). Thus, the osteoclast is the principal physiological target for calcitonin, and this is the basis of its therapeutic use in, for example, Paget's disease of bone. It has previously been impossible to carry out direct in vitro studies of the calcitonin receptor in osteoclasts because of the difficulty of preparing a pure population of osteoclasts in sufficient numbers. For that reason most of the available information on calcitonin-receptor interactions was derived from studying the calcitonin receptor in certain cancer cell lines. We report an abundance of calcitonin receptors in osteoclasts isolated from newborn rat bone, as shown by autoradiographic and biochemical methods (5–7). The large number of receptors in these osteoclasts has made it possible, for the first time, to characterize the osteoclast calcitonin receptor on small numbers of cells using methods we have previously

applied to the identification and characterization of calcitonin receptors in certain cancer cell lines (8–12).

Methods

Chemicals and hormones. Culture media and fetal calf serum (FCS)[1] were obtained from Flow Laboratories Inc., McLean, VA and bovine serum albumin (BSA) was obtained from the Armour Pharmaceutical Co., Tarrytown, NY. Bacitracin and isobutylmethylxanthine were from Sigma Chemical Co., St. Louis, MO. Nucleotides were obtained from Boehringer (Australasia). Na[^{125}I] and ^{125}I-tyrosine methyl ester succinyl cyclic AMP (cAMP) were purchased from Radiochemical Centre, Amersham, UK. Rabbit antiserum to succinylated cAMP was provided by Dr. N. H. Hunt, Canberra, Australia. Synthetic salmon calcitonin (sCT) and Gly^8-human calcitonin (Gly^8-hCT) were provided by Dr. R. C. Orlowski, Armour Pharmaceutical Co., and synthetic human calcitonin (hCT) was provided by Dr. W. Rittel, Ciba Geigy, Basel, Switzerland. Disuccinimidyl suberate (DSS) was obtained from Pierce Chemical Co., Rockford, IL, and photographic emulsions were from Eastman Kodak Co., Rochester, NY. All other chemicals were reagent grade from standard suppliers.

Osteoclast isolation and culture. Osteoclasts were prepared from 1- or 2-d-old Sprague Dawley rats as previously described (5–7). Femora, tibiae, and humeri were cleaned of soft tissue, cut across at the diaphysis, split longitudinally, and curetted into Medium 199 with 5% FCS (199/FCS) (1 ml per six bones). Cell suspensions were pipetted into the wells of six-well Teflon-Perspex molds clamped to glass microscopy slides (for autoradiographic experiments) or onto 22-mm-diam glass coverslips in six-well Costar tissue culture plates (for binding studies or hormone responses). After incubation at 37°C for 20 min, surfaces were washed vigorously with medium to remove less adhesive cells, and incubated with fresh 199/FCS for 2 h before experiments were begun.

Autoradiography. Osteoclasts were grown on glass slides within wells made from Teflon-Perspex molds. 24 h previously, these wells had been lightly seeded with either calvarial osteoblasts (13) or UMR 106-01 osteogenic sarcoma cells (14), which are phenotypically osteoblast and have no calcitonin receptors or responses. sCT was labeled with ^{125}I as previously described to preserve biological activity (8, 9), using 1 μg chloramine T for 10 s as oxidant, and purification with QUSO G32, elution and storage at −20°C in 20% acetone–1% acetic acid. Specific activities ranged from 150 to 200 μCi/μg. Cells were incubated for 1 h at 22°C in 0.5 ml Medium 199 with 0.1% BSA and 0.1% bacitracin (199/BSA) containing 0.2 nM ^{125}I-salmon calcitonin. Nonspecific binding was assessed on each slide in the presence of an excess (300 nM) of unlabeled sCT. After incubation, cells were washed three times with cold phosphate-buffered saline (PBS), then fixed for 10 min at room temperature in 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4. After rinsing in the same buffer and drying in air at room temperature, slides were dipped in NTB2 emulsion (Eastman Kodak Co.), dried, and incubated in the dark at −20°C for 2 wk. After developing, slides were stained with Giemsa, mounted, and photographed.

^{125}I-salmon calcitonin binding. Replicate osteoclast cultures were

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1. *Abbreviations used in this paper:* DSS, disuccinimidyl suberate; FCS, fetal calf serum; 199/FCS, Medium 199 with 5% FCS; Gly^8-hCT, Gly^8-human calcitonin; hCT, synthetic human calcitonin; sCT, salmon calcitonin.

prepared on 22-mm-diam glass coverslips and randomized into six-well tissue culture plates. Coverslips were incubated for 1 h at 22°C (or otherwise as indicated) in 1 ml 199/BSA. For competition experiments, incubations were carried out with a fixed amount of ^{125}I-sCT and increasing amounts of unlabeled sCT or other peptides. For saturation analysis, increasing amounts of ^{125}I-sCT were incubated in the absence and presence of excess unlabeled sCT. At the end of incubations, coverslips were washed three times in cold PBS, dried in air, and counted in an autogamma counter (Packard Instrument Co., Inc., Downers Grove, IL) with 70% efficiency. In all such experiments three replicate coverslips were retained to count osteoclasts after fixing in formaldehyde and staining with Giemsa.

Cross-linking of ^{125}I-salmon calcitonin with DSS. Osteoclast cultures were prepared in 20-cm^2 glass petri dishes (60 bones/dish) and incubated with 0.2 nM ^{125}I-sCT in the absence or presence of excess unlabeled ligand for 1 h at 22°C. After being washed in cold PBS, the cells were incubated in DSS (1 mg/ml) in PBS for 10 min at 22°C followed by 10 min at 4°C, washed in cold PBS, and solubilized in Triton (0.1% vol/vol), 0.05 M Tris, 0.15 M NaCl, 10 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 0.06% N-ethylmaleimide, pH 8.0. Extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (11).

Cyclic AMP production by osteoclasts. Osteoclasts were plated on 22-mm-diam glass coverslips in six-well tissue culture plates. Coverslips were incubated in 199/BSA and 1 mM isobutylmethylxanthine for 20 min, then treated with increasing amounts of sCT together with 10^{-6} M forskolin for 12 min. Coverslips were washed in cold PBS and covered with 1 mM HCl–95% ethanol (pH 3) at 4°C for 2 h. Samples were dried in a boiling water bath for 5 min, dissolved in assay buffer, acetylated, and assayed by radioimmunoassay as described (15). Cross-reactivity of the antiserum has been reported (16).

Results

Osteoclast cultures. Osteoclasts isolated from newborn rat long bones responded to calcitonin as described by Chambers et al. in several recent reports (5–7), with rapid cessation of mobility and a marked decrease in cell size which was reproducible and dose-dependent (results not shown). ~40–45% of cells were osteoclasts, with an equal number of mononuclear cells that bind calcitonin specifically (see below). The remainder, ~10%, consisted mainly of osteoblasts based on alkaline phosphatase histochemistry.

Autoradiography of ^{125}I-salmon calcitonin binding to isolated rat osteoclasts. Fig. 1 shows the distribution of silver grains due to radioactivity after incubation of cultures with ^{125}I-sCT. Specific binding of ^{125}I-sCT was confined to multinucleate osteoclasts and to mononuclear cells which on morphological grounds are not osteoblasts. Furthermore, these mononuclear cells do not have morphological features of macrophages, but it is not yet possible to be certain of their identity. In all such experiments, silver grains were located over 100% of visualized osteoclasts. When excess unlabeled calcitonin was included in incubations, no radioactivity was detected in association with any cells. Thus, ~90% of isolated cells were either multinucleated osteoclasts or mononuclear cells that bound labeled calcitonin specifically. Cultures were sparsely contaminated (<10%) with cells that were osteoblastic in appearance and staining characteristics (13), and no radioactivity was associated with these cells. The autoradiographs shown in Fig. 1 A, B, and C are from experiments in which osteoclasts were co-cultured with osteoblast-like cells in order to highlight the fact that specific radioactivity localizes only to osteoclasts and to certain mononuclear cells. The latter are not derived from the added osteoblast-like cells. By counting cell-associated grains in four separate autoradiography experi-
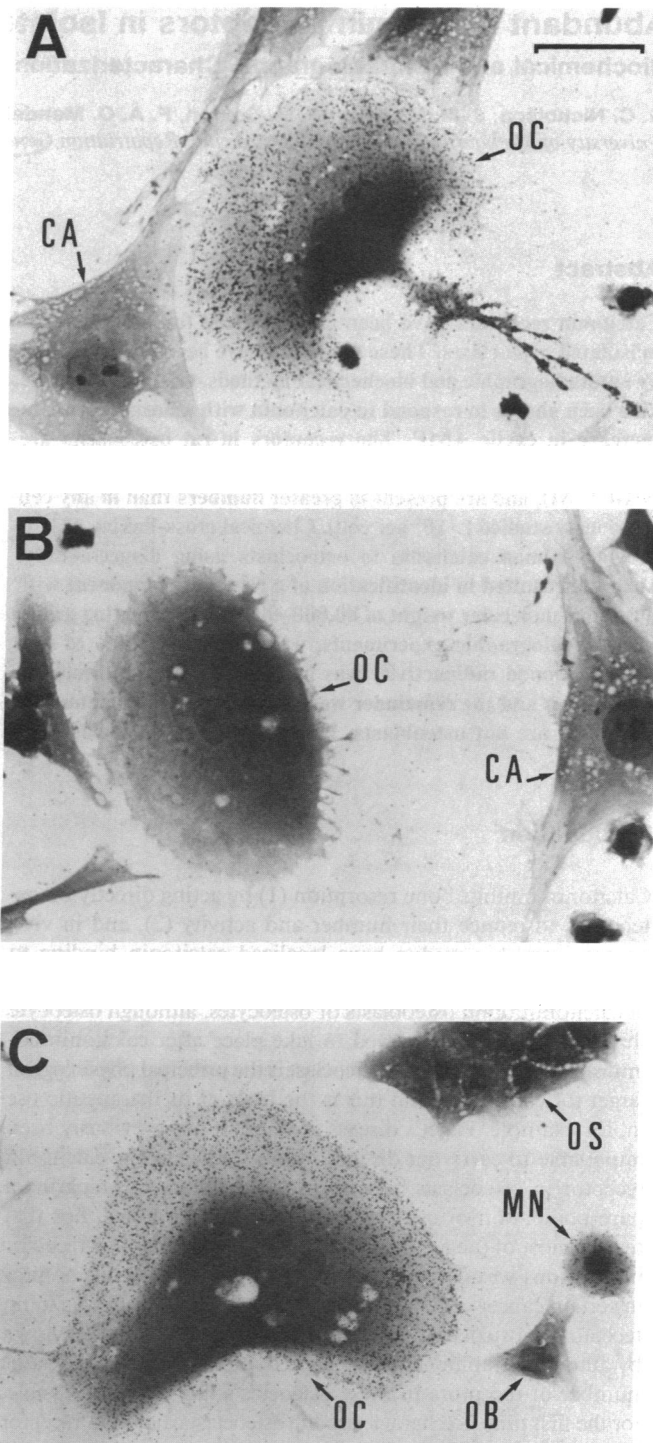

Figure 1. Autoradiography of ^{125}I-salmon calcitonin binding to isolated rat osteoclasts. Osteoclasts were prepared and plated onto slides that had previously been lightly seeded with either rat calvarial osteoblasts or UMR 106-01 cells and processed for autoradiography as described in Methods. Incubations contained 0.2 nM ^{125}I-sCT. All photographs are at magnification × 1,000. (A) Osteoclasts co-cultured with rat calvarial cells. Dense grains associated with multinucleate osteoclasts, *OC;* no grains over calvarial cells, *CA.* Bar = 30 μm. (B) Nonspecific binding, showing virtual absence of grains associated with osteoclasts. (C) Osteoclasts co-cultured with UMR 106-01 rat osteogenic sarcoma cells. Dense grains associated with osteoclast and a mononuclear cell, *MN;* no grains over osteogenic sarcoma cells, *OS* or neonatal rat long-bone osteoblast, *OB.*

Table I. Distribution of Radioactivity between Multinucleate Osteoclasts and Mononuclear Cells in Autoradiography Experiments

Experiment	Osteoclasts				Mononuclear cells			
	Total number per slide	Mean area	Grain density	Percent of total ratioactivity	Total number per slide	Mean area	Grain density	Percent of total ratioactivity
		μm^2	per μm^2			μm^2	per μm^2	
1	185	3,269±326	26.8±1.5	92	134	538±23	18.6±1.6	8
2	175	3,137±299	40.3±2.2	83	222	701±58	27.9±2.3	17
3	231	2,962±312	22.3±1.3	91	119	666±37	15.3±1.4	9
4	227	2,249±236	51.9±2.4	84	296	623±60	29.4±2.9	16

Values are mean±SEM. In sample slides from four separate autoradiography experiments the percentage radioactivity associated with osteoclasts and with mononuclear cells was calculated. Osteoclast areas (50 cells per slide) were measured by digitizing morphometry (5), and grains were counted in 10 randomly selected areas (42.5 μm^2) of each osteoclast. For the mononuclear cells grains were counted over the total cell areas; grains were not counted in mononuclear cells with <20 grains per cell.

ments without added osteoblasts, we determined that >80% of the total specifically cell-bound radioactivity in these experiments was associated with multinucleate osteoclasts; the remainder was associated with the mononuclear (nonosteoblast) cells (Table I). Note also that the grains per unit area in association with the mononuclear cells were less than with osteoclasts ($P < 0.05$, paired t test), and were more variable, consistent with heterogeneity of numbers of binding sites per cell within the mononuclear population.

Binding of ^{125}I-salmon calcitonin to isolated rat osteoclasts. Fig. 2 illustrates specific binding of ^{125}I-sCT to osteoclasts, with competition by unlabeled sCT. The relative efficacies of hCT and Gly[8]-hCT reflect their relative abilities to compete for binding to the calcitonin receptor in human cancer cells (8–10, 18) and to lower serum calcium in the rat (17). Several unrelated peptides (insulin, parathyroid hormone, glucagon, and ACTH) to concentrations of 1 $\mu g/ml$ had no effect on ^{125}I-sCT binding to osteoclasts (data not shown). Based on Scatchard analysis of separate competition binding experiments similar to that of Fig. 2 the mean calcitonin receptor numbers were 7.3±1.7 ($n = 3$, mean±SEM) million per cell, with dissociation constants (K_d) of 5.6±0.8 × 10^{-10} M. Similar values were obtained by Scatchard analysis of saturation binding data (Fig. 3), in which receptor numbers were 4.5±0.1 ($n = 3$) million per cell, with K_d of 1.6±0.1 × 10^{-10} M. The r value for the Scatchard line in Fig. 3 is 0.89; mean±SEM of r values for six separate experiments was 0.92±0.012.

The binding of labeled sCT was very poorly reversible, as has been shown to be the case in other systems (12, 18–23). After ^{125}I-sCT had been bound to osteoclasts on coverslips at 22°C, they were washed and incubations continued at 22°C for up to 5 h in medium with or without excess unlabeled sCT at the same temperature. No significant loss of cell-bound radioactivity occurred under these conditions (Fig. 4), but at 37°C there was progressive dissociation resulting in removal of 55% of the bound radioactivity by 5 h (data not shown).

Cross-linking of ^{125}I-salmon calcitonin to osteoclasts. SDS-PAGE analysis of solubilized cells following cross-linking of ^{125}I-sCT revealed a broad band of radioactivity of approximate molecular weight 80,000–90,000 (Fig. 5).

Cyclic AMP response to salmon calcitonin in osteoclasts. A sensitive, dose-dependent increase in cyclic AMP was obtained in response to sCT treatment of isolated osteoclasts (Fig. 6).

Discussion

The development of methods to isolate viable osteoclasts provides the means, for the first time, to investigate calcitonin interaction with its major target cell in bone. From the present data it is clear that osteoclasts isolated from newborn rat long bone contain large numbers of calcitonin receptors, and that apart from osteoclasts, the only other cells in these isolates which are capable of detectable binding of calcitonin are mononuclear cells, which on morphological and histochemical grounds are not osteoblasts. This latter group of cells could consist of mononuclear precursors of osteoclasts, some of which are in the process of expressing calcitonin receptors as part of their development. As such, they are of considerable interest and will warrant very careful study with a number of techniques, to obtain further information about pathways of osteoclast development. In their in vivo autoradiographic study of ^{125}I-labeled salmon calcitonin localization in rats, Warshawsky et al. (3) drew attention to specific radioactivity in association with mononuclear cells of uncertain identity, which, on the basis of their electron microscopic studies, they felt to be "cell fragments" of osteoclasts. Such an explanation could not apply to those in the present experiments. It is generally considered that osteoclasts develop from mononuclear precursors of the monocyte/macrophage series (24–27), although it has also been suggested that they might derive from a different type of marrow stem cell (28). In either case the origin
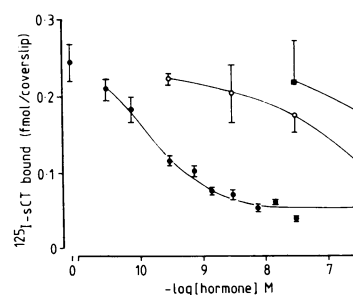
Figure 2. ^{125}I-salmon calcitonin binding to osteoclasts. Incubations of replicate osteoclast cultures on coverslips contained 0.1 nM ^{125}I-salmon calcitonin and increasing amounts of unlabeled sCT (●), hCT (o), or Gly[8]-hCT (■). Conditions were as in Methods. No significant binding competition was observed with a range of unrelated peptides up to a concentration of 10^{-7} M (parathyroid hormone, insulin, ACTH, Glucagon, EGF) (results not shown). Means±SEM, $n = 3$. Osteoclast number 125±14 per coverslip. Scatchard analysis of salmon calcitonin binding indicated K_d of 3.7 × 10^{-10} M and receptor number of 3.2 × 10^6 per cell.
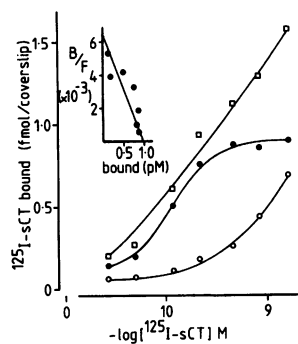
Figure 3. Saturation analysis of ^{125}I-salmon calcitonin binding to osteoclasts. Replicate osteoclast cultures on coverslips were incubated with increasing amounts of ^{125}I-sCT in the absence and presence of excess unlabeled sCT. Conditions were as in Methods. □, total binding; ○, nonspecific binding; ●, specific binding. Points are means of triplicates. Osteoclast number 134±15 per coverslip. Scatchard analysis of specific binding (inset) indicated K_d of 1.6×10^{-10} M and receptor number of 4.8×10^6 per cell.

of osteoclasts is believed to be through a lineage quite distinct from that of osteoblasts. The development of a detectable calcitonin receptor might be a valuable marker in osteoclast ontogeny, and for that reason further efforts are being made to characterize the osteoclast calcitonin receptor.

Previous biochemical studies of the calcitonin receptor have been confined to nonbone cells. Thus, calcitonin receptors have been identified in kidney (23, 29, 30), brain (20, 31), testis (32), pig lung (22), fish gill (21), certain lymphoid cell lines (33), and human cancer cell lines (8–12, 18, 34–37). The specificity of calcitonin binding to the cells in the present experiments was demonstrated by the lack of competition for binding by nonrelated peptides, and by the relative efficacies of human calcitonin and Gly^8-human calcitonin, which are similar to those obtained in other well-characterized calcitonin responsive systems (9, 10, 17, 18). Furthermore, we have identified in breast cancer and lung cancer cells an 85,000 molecular weight receptor component, using photoaffinity labeling (11, 36). Until now, the most abundant source of calcitonin receptors has been the human breast and lung cancer cell lines, with calculated receptor numbers of 5,000–30,000 per cell (9, 10, 37). Although only small numbers of osteoclasts can be obtained with the present approach, substantial specific binding can be measured readily, with very low nonspecific binding. From our data it is clear that the rat osteoclast is extremely rich in calcitonin receptors that are specific and saturable. It must be stressed that the calculations of receptor numbers and binding constants provide only approximate values, since the calcitonin binding is poorly reversible. This has been a constant feature of salmon calcitonin binding in all systems in which it has been studied (12, 18–23), and for that reason we have emphasized the limitations of calculations of binding constants and receptor numbers (9, 12, 18). Nevertheless, it is clear from the present experiments that the number of calcitonin receptors per cell in rat osteoclasts is of the order
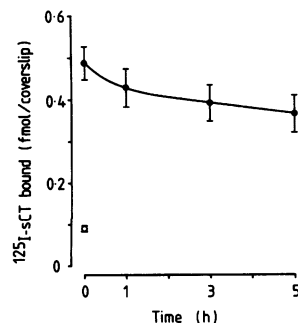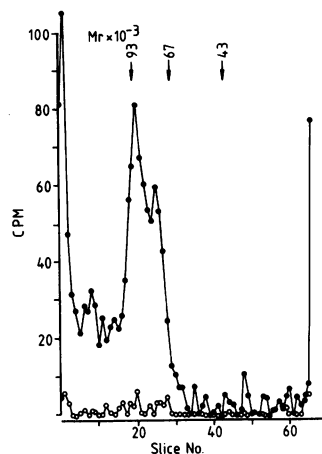

Figure 5. Cross-linking of ^{125}I-salmon calcitonin to osteoclasts using disuccinimidyl suberate. Incubation, cross-linking, and PAGE were carried out as described in Methods. ●, 0.2 nM ^{125}I-sCT alone; ○, plus 300 nM sCT. Specifically bound ^{125}I radioactivity in the solubilized extract was 9,700 cpm.

of millions per cell, some two to three orders of magnitude greater than that in any cell previously studied. On the other hand, the surface areas of these cells (breast, lung cancer) in culture are only approximately one order of magnitude less than the osteoclast surface area. Furthermore, such a large number of receptors for a peptide hormone has not been found in normal mammalian cells; the A431 epidermal carcinoma cell line has been shown to have ~2 million epidermal growth factor receptors per cell (38). Thus, the rat osteoclast is enriched in calcitonin receptors which possess the recognition functions to be expected of a physiological calcitonin receptor. Furthermore, the cross-linking experiments identified a specific receptor component, the molecular weight of which is consistent with that of a major calcitonin receptor component identified in other studies in which photoactive labeled calcitonin has been cross-linked to receptors in human breast cancer cells (11).

It is thought that in mammalian cells the initial action of calcitonin upon osteoclasts requires activation of adenylate cyclase. This is based on activation of adenylate cyclase by calcitonin in bone membrane preparations (23), increase of cAMP in "osteoclast-rich" cultures from rodent calvaria (39), and the lack of evidence for an effect of calcitonin on cyclic AMP production in osteoblast-like cells (13, 39). In the present experiments a sensitive dose-dependent increase in cAMP was found with calcitonin treatment of the cultures. In other work, using immunocytochemical methods, we have shown that calcitonin treatment of rat osteoclasts is associated with an increase in cy-

Figure 4. Dissociation of ^{125}I-salmon calcitonin bound to osteoclasts. After ^{125}I-sCT (0.1 nM) had been bound to osteoclasts growing as replicate cultures on coverslips (1 h at 22°C), coverslips were washed, and incubations continued in medium containing 300 nM unlabeled sCT for the indicated times, when coverslips were washed, dried, and counted. Means±SEM, $n = 3$.
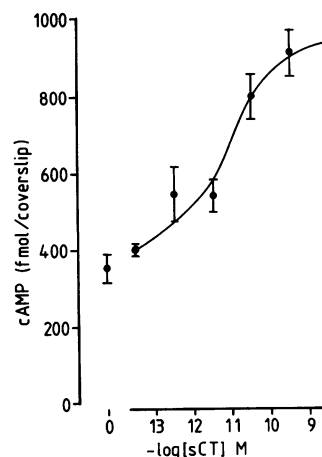

Figure 6. Cyclic AMP production by osteoclast cultures in response to salmon calcitonin. Osteoclasts growing as replicate cultures on coverslips were incubated with increasing amounts of sCT together with 10^{-6} M forskolin for 12 min, and cyclic AMP in individual incubations assayed as described in Methods. Osteoclast number, 108±9 per coverslip. Means±SEM, $n = 6$.

toplasmic cAMP (40). The same is not true of chicken osteoclasts either in our experiments (40, 41) or in those of others (42). It is likely that cAMP is an important initial messenger in calcitonin action on rat osteoclasts, although we cannot be certain that it is the only molecular messenger. Experiments are in progress to investigate the effects of calcitonin on components of the cAMP pathway, using methods applicable to the study of single cells.

It is concluded that the most abundant cellular source of calcitonin receptors yet recognized is the rat osteoclast. The physiological significance of such a large number of receptors needs to be investigated, but it is likely that the expression of calcitonin receptors signals an important stage of osteoclast development. The present work provides a basis for studying its significance, besides offering a means of studying directly in osteoclasts some particular features of calcitonin action which might be related to its therapeutic use. These include the phenomena of calcitonin-induced receptor loss (43, 44), "escape" (45), persistent activation of adenylate cyclase (12, 46), and homologous desensitization (12, 43).

Acknowledgments

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