THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 24, Issue of August 25, pp. 14342-14347, 1989
Printed in U.S.A.


# Control of Cytosolic Free Calcium in Rat and Chicken Osteoclasts

## THE ROLE OF EXTRACELLULAR CALCIUM AND CALCITONIN*

(Received for publication, February 16, 1989)

**Antonio Malgaroli‡§, Jacopo Meldolesi‡, Alberta Zambonin Zallone, and Anna Teti¶**

*From the Institute of Human Anatomy, University of Bari, and the ‡Department of Pharmacology, Consiglio Nazionale delle Ricerche Center of Cytopharmacology, San Raffaele Scientific Institute, University of Milan, Italy*

**Single cell $[Ca^{2+}]_i$ studies were performed in chicken and rat osteoclasts loaded with fura-2 and exposed to a variety of treatments. Under resting conditions, basal $[Ca^{2+}]_i$ was 79.2 ± 47.3 and 84.3 ± 65.7 nM (averages ± S.D.; $n$ = 141 and 126) in the osteoclasts of the two species, respectively. Basal $[Ca^{2+}]_i$ was stable in all rat and in ≈80% of chicken osteoclasts. In the remaining 20%, spontaneous, irregular $[Ca^{2+}]_i$ fluctuations were observed (amplitude range: 50–200 nM over basal values). Increase of $[Ca^{2+}]_o$ over the concentration of the Krebs-Ringer incubation medium (2 mM) induced rises of $[Ca^{2+}]_i$ in almost all cells investigated. $[Ca^{2+}]_i$ rises were already appreciable with 0.5 mM $[Ca^{2+}]_o$ additions and reached high values with 4 mM additions: 390 ± 113 and 364 ± 214 nM $[Ca^{2+}]_i$ in rat and chicken osteoclasts, respectively ($n$ = 122 and 101). Qualitatively, the responses to $[Ca^{2+}]_o$ additions consisted of discrete $[Ca^{2+}]_i$ transients, biphasic (an initial spike followed by a plateau), or monophasic (either the spike or the plateau). In a few chicken osteoclasts, the $[Ca^{2+}]_i$ increase occurring after $[Ca^{2+}]_o$ addition consisted of multiple, irregular fluctuations, similar to those observed in 20% of these cells under resting conditions. In individual osteoclasts subsequently exposed to multiple $[Ca^{2+}]_o$ increase pulses, the type of the $[Ca^{2+}]_i$ transient (mono- or biphasic) was maintained, and the size was dependent on the magnitude of the $[Ca^{2+}]_o$ additions. Effects similar to those of $[Ca^{2+}]_o$ were induced by the addition of $Cd^{2+}$ or $Ba^{2+}$ (but not $La^{3+}$ or $Mg^{2+}$) into the medium. The $Cd^{2+}$ effect was maintained in part even in a $Ca^{2+}$-free medium. Of various hormones and factors, parathormone, 1,25-dihydroxyvitamin $D_3$, and prostaglandin $E_2$ were inactive. In contrast, calcitonin was active in rat osteoclasts (which express numerous receptors). $[Ca^{2+}]_i$ increases were small (19 ± 17.9 nM; $n$ = 21) when the hormone was administered alone; they were synergistic (severalfold potentiation) when the hormone was administered before or after $[Ca^{2+}]_o$. The $[Ca^{2+}]_i$ effects of calcitonin were mimicked by 8Br-cAMP (31 ± 26 nM; $n$ = 12) when the nucleotide was administered alone; marked synergism when it was administered in combination with $[Ca^{2+}]_o$. This paper demonstrates for the first time that changes of $[Ca^{2+}]_i$ are induced in osteoclasts by treatments with $[Ca^{2+}]_o$ and calcitonin and can therefore be involved in intracellular mediation of the physiological effects of these two extracellular signals.**

Osteoclasts are multinucleated cells known to play a pivotal role in the control of extracellular $Ca^{2+}$ homeostasis (1). Specifically, osteoclasts are responsible for the resorption of the bone matrix, a complex multistep process which includes (i) recognition and adhesion to the bone surface, (ii) establishment of cell polarity, and eventually (iii) degradation of the bone matrix components.

Recent studies have provided considerable information on these sequential events. Thus, recognition is possibly mediated by receptors of the integrin superfamily expressed at the osteoclast plasma membrane (2). After recognition, osteoclasts adhere to the bone surface by means of podosomes, which are short cell protrusions containing specialized cytoskeletal structures (3, 4). Organization of the podosomes goes together with the development, at the adherent pole of the cell, of the clear zone, a plasmalemma area which rapidly becomes the seal of the resorbing compartment. The latter is an extracellular space delimited on the one side by the plasma membrane ruffled border, on the other by the bone surface to be resorbed. Accumulation of a proton pump in the ruffled membrane area causes the pH of the resorption compartment to drop and thus permits the solubilization of hydroxyapatite crystals as well as the activity of lysosomal enzymes secreted by the cell. The combination of these two processes is responsible for the bone resorption (5–7).

This complex activity of osteoclasts is regulated by extracellular signals. A variety of hormones and other factors are known to be involved. The effect of some of these agents (parathormone, 1,25-dihydroxycholecalciferol, interleukin 1), however, is believed to be indirect, mediated via additional factors produced by osteoblasts (8, 9). A direct effect has been identified only for retinoids (stimulation) (4, 10) and calcitonin (CT,[1] inhibition), the latter mediated by specific surface receptors coupled to the activation of adenylate cyclase (11–14). Another candidate for an important, direct role in osteoclast regulation is the extracellular $Ca^{2+}$ concentration, $[Ca^{2+}]_o$ (15). The mechanism by which increases of cAMP and $Ca^{2+}$ (within and outside the cell, respectively) affect osteoclast physiology are still obscure. A reasonable possibility is that these two signals modify the intracellular concentration of $Ca^{2+}$, $[Ca^{2+}]_i$. This possibility has been investigated in the

* This work was supported by grants from the Italian Ministry of Education (to A. Z. Z. and A. T.) and from the Strategic Project Molecular Biology of the National Research Copuncil (to J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Fellow of the S. Raffaele Foundation.
¶ To whom reprint requests should be addressed.

[1] The abbreviations used are: CT, calcitonin; 1-34-bPTH, 1-34-bovine parathyroid hormone; hCT, human calcitonin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $[Ca^{2+}]_o$, extracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH, Krebs-Ringer-HEPES medium; MEM, minimal essential medium; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

present work by studying osteoclasts from two different species (one avian, the chicken, the other mammalian, the rat). The technique used in our studies was the microscopic, single cell analysis of $[Ca^{2+}]_i$, with employment of fura-2 as the intracellular calcium-sensitive fluorescent probe.

## EXPERIMENTAL PROCEDURES

*Incubation Media*—Krebs-Ringer-HEPES medium (KRH) contained (mmol/liter): NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgSO_4$ and $KH_2PO_4$, 1.2; HEPES-NaOH, 25; pH 7.4; glucose, 6. $Ca^{2+}$-free medium was KRH without $CaCl_2$ added. $Mg^{2+}$ and phosphate-free media were KRH without $MgSO_4$ and $KH_2PO_4$ added, respectively.

*Preparation of Osteoclasts*—Chicken osteoclasts were prepared by a modification of a previously described method (16). Briefly, medullary bone from laying hens, fed 7 days with a hypocalcemic diet, was removed from tibias and femurs, washed in Joklik-modified minimum essential medium (MEM) at 4 °C and squeezed through a 100-μm nylon net. The cell suspensions obtained were sedimented at 4 °C at unit gravity on 75% fetal calf serum in Joklik-modified MEM for 45 min (1 to 3 times). The cells, collected from the serum preparation and filtered twice through 100-μm nylon meshes, were plated in 3.5-cm diameter dishes containing a 24 × 24-mm square coverslip (density: 50,000 cells/dish). After incubation at 37 °C under 95% $O_2$, 5% $CO_2$ (24 h) in MEM (supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 50 IU/ml mycostatin, 3 μg/ml cytosine-1-β-D-arabinofuranoside) the cells were extensively washed in order to remove the non-adherent fraction. The osteoclastic nature of the adherent cells obtained was estimated by (i) their content in tartrate-resistant acid phosphatase, assayed by using the histochemical kit of Sigma; (ii) their reaction with specific anti-osteoclast monoclonal antibodies (a kind gift of Dr. Philip Osdoby, Washington University, St. Louis, MO); and (iii) their ability of resorbing bone, estimated by the technique described in detail elsewhere (10). The purity of the osteoclast populations was estimated to range from 70 to 95%.

Rat osteoclasts were obtained from the endosteal surface of calvaria, femurs, and tibias of 1–2-day-old Sprague-Dawley rats. Fragments, obtained from the bones, were pipetted in a small volume of HEPES-buffered MEM, and the resulting cell suspensions were plated on glass coverslips and incubated at 37 °C for 2 h. The coverslips were subsequently washed and (after replacing the medium with MEM supplemented with 10% fetal calf serum and antibiotics) maintained at 37 °C under 95% $O_2$, 5% $CO_2$. Under these conditions, only osteoclasts, together with a few fibroblasts and macrophages, remain attached to coverslips. Of these cell types, only osteoclasts are multinucleated, and this was one of the criteria used for their recognition. The osteoclastic nature of these cells was further confirmed by the capability of the multinucleated cells (i) to respond to $10^{-6}$ M human CT (hCT) by cell retraction (17), (ii) to excavate typical osteoclastic pits when plated onto bone slices (18), (iii) to be tartrate-resistant phosphatase-positive. The recovery of osteoclasts was found to be about 20/animal. Rat osteoclasts were used for the experiments within the first 4 h of culture.

*Single Cell Measurement of Cytosolic $[Ca^{2+}]$*—Isolated chicken and rat osteoclasts were loaded with 10 μM fura-2/AM (19) in KRH for 1 h at 15 °C (20). Coverslips were then washed to remove the extracellular dye and positioned with the attached cells facing downward in an appropriate Perspex chamber thermostatted at 37 °C. The chamber was equipped with a ministirrer as well as with inlets from which medium flow could be established during the experiment. The chamber was inserted over the stage of a Zeiss Photomicroscope III equipped with a 40× objective. In this apparatus, the light from a 75-watt xenon lamp passes through either one of two alternating narrow band width filters (350 and 380 nm) that were moved up and down by a pressured air-driven system. The electronic control of the filter changer was interfaced to a personal computer. The emitted signal was filtered by a 490 nm cut-off filter, collected by a photomultiplier, digitized by an analog-to-digital converter, and finally fed into the computer. The ratio of subsequent signals recorded separately at the two excitation wavelengths was thus calculated and converted into $[Ca^{2+}]_i$ by appropriate calibration (19). Background values were calculated from each cell at the end of the recording after treatment with 10 μM digitonin and subtracted from experimental values. For additional details, see Ref. 20.

*Materials*—fura-2 $K^+$ salt and fura-2/AM were from Calbiochem. 1–34-Bovine parathormone (bPTH) from Sigma was dissolved in 0.01 N HCl; 1,25-dihydroxyvitamin $D_3$, kindly provided by Hoffmann-La Roche (Basel), was dissolved in absolute ethanol; hCT (Ciba, Basel) was dissolved in KRH at pH 7.4. All the other reagents (analaytical grade) were from either Sigma or Carlo Erba, Milan. The pH of the solutions to be added to the cells was adjusted to 7.4 shortly before use.

## RESULTS

*Effects of $Ca^{2+}$*—When osteoclasts were incubated in KRH ($[Ca^{2+}]_o$, 2 mM), the basal $[Ca^{2+}]_i$ was (means ± S.D.; $n$ = 126 and 141) 84.3 ± 65.7 nM for the chicken, 79.2 ± 47.3 nM for the rat, *i.e.* values comparable to those measured in a variety of other cell types, by both us and others. As indicated by the high S.D., the basal $[Ca^{2+}]_i$ values were, however, quite variable in the osteoclast population investigated. In all osteoclasts from the rat, and in 80% of those from the chicken, the resting $[Ca^{2+}]_i$ remained stable during incubations of at least 10 min. In contrast, approximately 20% of the chicken osteoclasts exhibited spontaneous, rapid $[Ca^{2+}]_i$ fluctuations. As can be seen in Fig. 1, these fluctuations were relatively small in size (amplitude range 50–200 nM over basal values) and occurred rather irregularly.

Figs. 2–6 illustrate events induced in osteoclasts of either species by the increase of the extracellular $Ca^{2+}$ concentration. The addition of 4 mM $CaCl_2$ to the medium (final $[Ca]_o$ = 6 mM) induced in a few minutes an appreciable cell retraction, as shown at phase contrast in Fig. 2. In the same experimental conditions, marked changes of $[Ca^{2+}]_i$ were observed in 86% of the osteoclasts investigated ($n$ = 223). Fig. 3 illustrates a group of responses that were observed after increasing

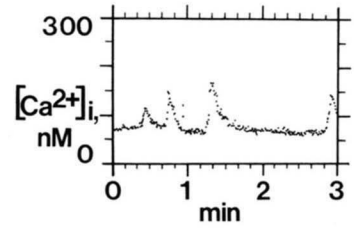


FIG. 1. **Unstimulated $[Ca^{2+}]_i$ fluctuations in a single chicken osteoclast incubated in complete KRH ($[Ca^{2+}]_o$, 2 mM) medium.** In this and the following traces, each *point* represents a 350–380 nm excitation fluorescence ratio recorded from a fura-2-loaded cell and converted in terms of $[Ca^{2+}]_i$ according to Grynkievicz *et al.* (19).

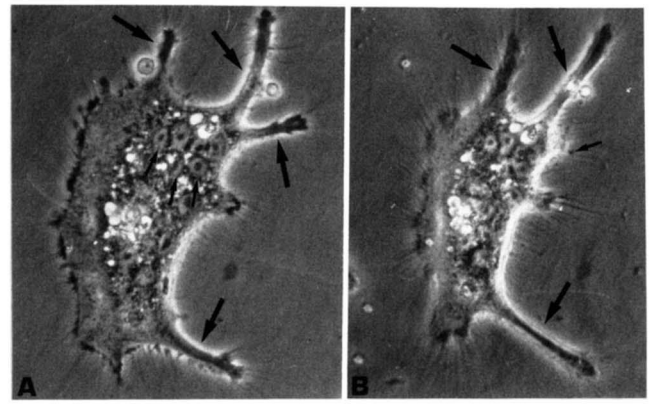


FIG. 2. **Phase contrast micrographs of a rat osteoclast showing the changes in shape after treatment with 6 mM $[Ca^{2+}]_o$.** *A*, before treatment, the cell shows multiple nuclei (*small arrows*) and prolongings (*big arrows*) radiating from the cell body, with several fillipodia. *B*, the same osteoclast, 15 min after treatment with high (6 mM) extracellular calcium appears retracted. One of the prolongings disappeared (*small arrows*), the other became longer (*large arrows*), apparently as a consequence of the retraction of the cell body. Fillipodia and nuclei are still evident. × 600.

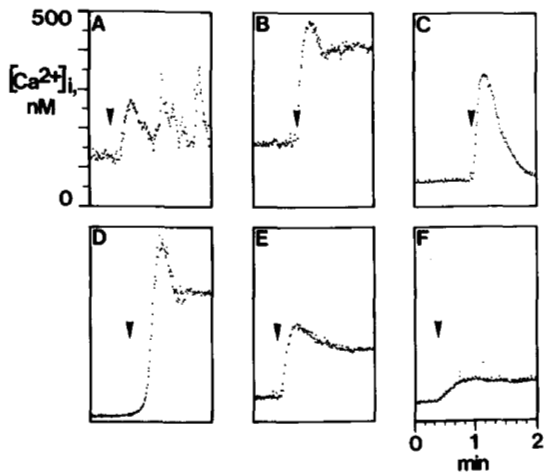


FIG. 3. **$[Ca^{2+}]_i$ transients induced in single chicken (*A–C*) and rat (*D–F*) osteoclasts by addition of $CaCl_2$ to the medium.** Additions of $CaCl_2$ (final concentration: 4 mM, in addition to the 2 mM contained in the KRH medium) are marked by *arrowheads*. Note the fluctuations triggered in a chicken osteoclast (*A*) and the variability in height and shape of the discrete transients in other osteoclasts of both species (*B–F*).

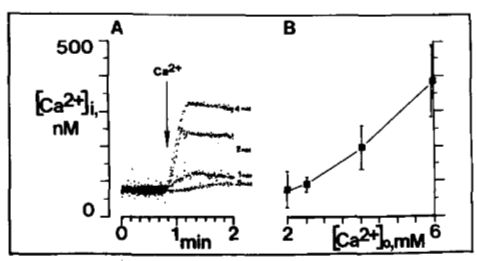


FIG. 4. **Concentration dependence of the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ responses in rat osteoclasts.** *Panel A* shows the superimposed $[Ca^{2+}]_i$ transients triggered in a single osteoclast by the successive administration (intermingled by washes) of different amounts of $CaCl_2$ to the indicated final concentrations (added to the 2 mM $Ca^{2+}$ of the KRH medium). *Panel B* illustrates the size of the maximal $[Ca^{2+}]_i$ increases (averages ± S.D.) induced in osteoclasts by switching the $[Ca^{2+}]_o$ from the 2 mM KRH to the values indicated on the *abscissa*. Results are from at least six measurements.

$[Ca^{2+}]_o$ to 6 mM. Only a few chicken osteoclasts (5%) that exhibited a stable basal $[Ca^{2+}]_i$ rapidly started fluctuating after the $[Ca^{2+}]_o$ increase, and this activity persisted for many (>10) min thereafter (Fig. 3*A*). In the other osteoclasts, both of chicken and rat origin, single $[Ca^{2+}]_i$ transients were observed after $[Ca^{2+}]_o$ increase. In some cells (36%), the $[Ca^{2+}]_i$ transient was clearly biphasic: an initial peak (to and sometimes above 500 nM) was followed by a plateau phase at a lower level, lasting longer than 10 min (Fig. 3, *B* and *D*). Other cells (64%) exhibited either the initial peak only (Fig. 3*C*) or monophasic increases of variable size and long persistence (>10 min), without the initial peak (Fig. 3, *E* and *F*).

The $[Ca^{2+}]_i$ responses induced by increases of $[Ca]_o$ were found to be concentration-dependent. Fig. 4*A* illustrates the responses triggered in a single rat osteoclast by multiple administrations of $CaCl_2$ (intermingled by washes) to final concentrations from 0.5 to 4 mM (in addition to the 2 mM $CaCl_2$ contained in the medium). Stepwise increases of the $[Ca^{2+}]_o$ often induced cumulative $[Ca^{2+}]_i$ increases (not shown). Quantitative data for the rat cell population investigated are shown in Fig. 4*B*. The averages of $[Ca^{2+}]_i$ maxima ± S.D. in chicken and rat osteoclasts were: 96 ± 33 and 87.6 ± 21 for 0.5 mM; 285 ± 203 and 199 ± 64 for 2 mM; 364 ± 214 and 390 ± 113 for 4 mM $[Ca^{2+}]_o$ increases. In individual cells, the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ responses were reproducible. In fact, the state of successive transients was similar, even if the plateau phases often exhibited the tendency to a progressive increase (Fig. 5*A* for the chicken). On the other hand, decrease of the $[Ca^{2+}]_o$ to values lower than 2 mM (the concentration in KRH) was found to elicit only small decreases of $[Ca^{2+}]_i$. Fig. 5*B* shows that, in a chicken osteoclast incubated in KRH, three successive administrations of 1 mM EGTA induced step decreases of $[Ca^{2+}]_i$ of increasing magnitude. A large decrement of $[Ca^{2+}]_i$ was observed only when, after the third EGTA administration, $[Ca^{2+}]_o$ reached a value around 100 nM. Similar results were obtained in 37 chicken and 41 rat osteoclasts.

*Effects of Other Cations*—In a subsequent series of experiments, the $[Ca^{2+}]_i$ effect of a group of cations other than $Ca^{2+}$ was investigated. In principle, these cations could affect fura-2 fluorescence either by causing $[Ca^{2+}]_i$ changes or by binding directly to the intracellular probe. Two criteria were employed to exclude direct intracellular binding. First (for cations, $Cd^{2+}$ and $La^{3+}$, that induce drastic changes of the 350/380 fluorescence ratio), the coordinate evolution of the signals at the two wavelengths which is typical of the $Ca^{2+}$-fura-2 interaction; second, the rapid disappearance of the cation-induced $[Ca^{2+}]_i$ signal after cell washing. Trivial effects of the added cation, for example, cell injury, were excluded by the criteria discussed in Ref. 20. In both chicken and rat osteoclasts, addition of either $LaCl_3$ (200 μM) or $MgCl_2$ (1.8 mM, added to a $Mg^{2+}$-free medium) failed to induce any $[Ca^{2+}]_i$ changes (Fig. 6, *A* and *B*). In contrast, clear effects were observed with $BaCl_2$ and, especially, $CdCl_2$ (Fig. 6, *C* and *F*). The increases of the fura-2 ratio signal after addition of 2 mM $BaCl_2$ were slow (Fig. 6, *C* and *F*). At least part of these responses was due to the entry of $Ba^{2+}$ into the cells, as indicated by the lack of disappearance after washing. In contrast, with $CdCl_2$ (200 μM), the responses were rapidly dissipated by cell washing. Although variable from cell to cell, the $Cd^{2+}$-induced $[Ca^{2+}]_i$ increases (mean ± S.D. = 462 ± 221 nM; $n$ = 26) were always rapid and of considerable size. Compared to the $[Ca^{2+}]_o$ responses, the initial $[Ca^{2+}]_i$ spikes were usually steeper, and the delayed plateau phases were only rarely observed (Fig. 6, *D*, *E*, and *F*). Responses to $Ba^{2+}$, $Ca^{2+}$, and $Cd^{2+}$ (2, 2, and 0.2 mM) elicited in the same cell are superimposed in Fig. 6*F*.

*Origin of the Cation-induced $[Ca^{2+}]_i$ Transients*—The osteoclast responsiveness to $Cd^{2+}$ permitted us to investigate whether, and to what extent, the $[Ca^{2+}]_i$ increases induced by

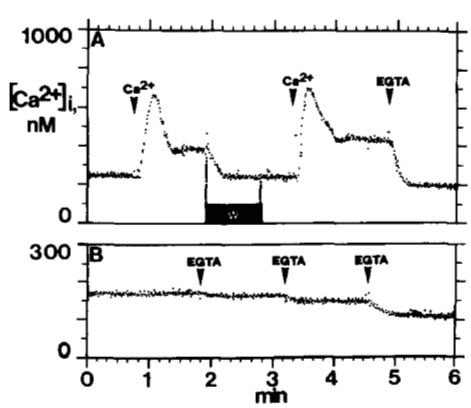


FIG. 5. **$[Ca^{2+}]_i$ changes induced by either the increase or the decrease of $[Ca^{2+}]_o$ in two chicken osteoclasts.** *Panel A* illustrates two biphasic transients induced in the same osteoclast by two successive applications of $CaCl_2$ (final $[Ca^{2+}]_o$, 6 mM) and the fading out of the transients after washing (*W*) or addition to the medium of 7 mM EGTA (final concentration). *Panel B* shows the effects of three successive additions of 1 mM EGTA (without washes) to an osteoclast bathed in complete KRH medium (initial $[Ca^{2+}]_o$, 2 mM).

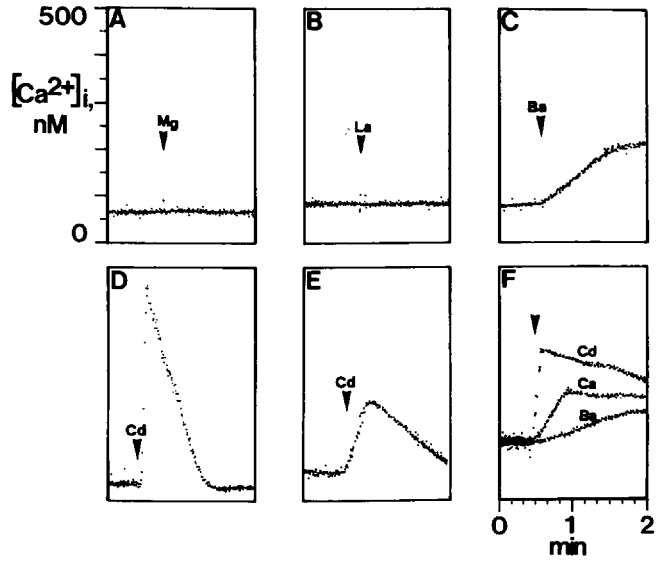


FIG. 6. **$[Ca^{2+}]_i$ measurements in rat osteoclasts treated with different cations.** The osteoclast in *A* was incubated in $Mg^{2+}$-free medium and treated with 1.8 mM $MgCl_2$ ($Mg^{2+}$); those in *B*, *D*, *E*, and *F* ($Ca^{2+}$ and $Cd^{2+}$) were incubated in KRH and treated with $LaCl_3$ (200 $\mu$M, $La^{3+}$), $CdCl_2$ (200 $\mu$M, $Cd^{2+}$), or $CaCl_2$ (2 mM, $Ca^{2+}$), where indicated; the osteoclast in *C* and *F* ($Ba^{2+}$) were incubated in phosphate-free medium and treated with $BaCl_2$ (2 mM, $Ba^{2+}$). The *traces* shown in *F* are superimposed records from the same osteoclast successively stimulated with $Ca^{2+}$, $Cd^{2+}$, and finally $Ba^{2+}$, with washes in between. The $[Ca^{2+}]_i$ and time scales drawn on the *y* and *x* axes of *A* and *F*, respectively, are valid for all *panels* of the figure.

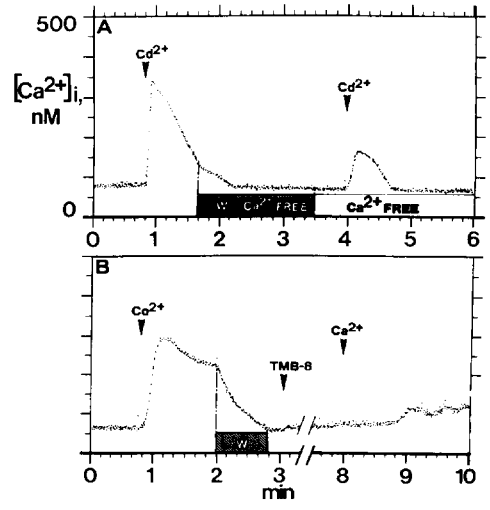


FIG. 7. **$[Ca^{2+}]_i$ transients in rat osteoclasts: effect of $Ca^{2+}$-free medium and TMB-8.** *Panel A* illustrates two $[Ca^{2+}]_i$ transients induced by $Cd^{2+}$ (200 $\mu$M) added first in the $Ca^{2+}$-containing, then (after washing, *W*) in the $Ca^{2+}$-free KRH medium. *Panel B* illustrates two $[Ca^{2+}]_i$ transients induced by $CaCl_2$ additions (2 mM + 2 mM KRH, final concentrations) before and after treatment with TMB-8 (200 $\mu$M).

cation addition originate from extra- and/or intracellular origin. Experiments of $CdCl_2$ stimulation in the $Ca^{2+}$-free medium were carried out. As can be seen, part of the response elicited by the divalent cation when administered in the $Ca^{2+}$-containing medium was maintained (mean = 49%; $n = 6$; Fig. 7*A*). Moreover, when osteoclasts were treated with TMB-8 (200 $\mu$M, 5 min), a drug with various effects including the blockade of receptor-stimulated intracellular $Ca^{2+}$ release, the responses to increased $[Ca^{2+}]_o$ were markedly inhibited (mean inhibition = 93%; $n = 4$; Fig. 7*B*). Taken together, these results indicate that release from intracellular stores is involved in the $[Ca^{2+}]_i$ effects induced by divalent cations, particularly in the early peak phase of the response, the rest being due to influx from the extracellular medium. In order to obtain information on this influx process, experiments were carried out in which the $[Ca^{2+}]_o$ increase was preceded or followed (1 min in both cases) by the addition of either $LaCl_3$ (200 $\mu$M) (a blocker of many $Ca^{2+}$ transport systems), verapamil (20 $\mu$M), or nifedipine (10 $\mu$M) (two blockers of voltage-gated $Ca^{2+}$ channels). In these experiments, no inhibition of the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ transients was ever observed. Likewise, substitution of NaCl from the medium with *N*-methylglucamine remained without effect on the same transients (data not shown).

*Effect of Hormones and Other Factors*—A number of hormones and local factors are known to play important roles in the regulation of extracellular $Ca^{2+}$ homeostasis and, in particular, in bone resorption. The effect of these agents on $[Ca^{2+}]_i$ of both chicken and rat osteoclasts was thus investigated. Among these factors, 1–34-bPTH (1 $\mu$M), 1,25-dihydroxyvitamin $D_3$ (1 $\mu$M), and prostaglandin $E_2$ (1 $\mu$M) failed to elicit any $[Ca^{2+}]_i$ changes in the osteoclasts isolated from either rat or chicken. In chicken osteoclasts also, hCT (1 $\mu$M) was without effect (not shown). In contrast, in rat osteoclasts, the application of the hormone often elicited a slow, very modest $[Ca^{2+}]_i$ increase (mean ± S.D.: 19 ± 17.9 nM; $n = 21$, Fig. 8), which failed to become visible when the hormone was applied in a $Ca^{2+}$-free medium (not shown). Interestingly, an effect very similar to hCT (mean ± S.D.: 31 ± 26.5 nM; $n = 12$) was elicited by 8Br-cAMP, (500 $\mu$M, Fig. 8*C*), the analog

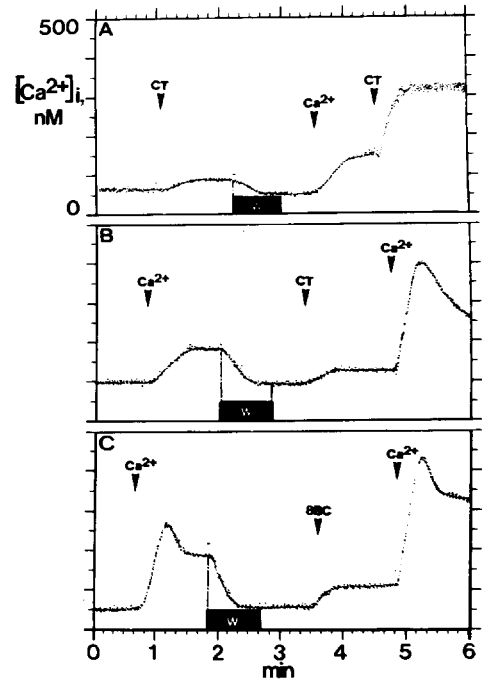


FIG. 8. **Effects of calcitonin and 8Br-cAMP, with or without $[Ca^{2+}]_o$ increase, on the $[Ca^{2+}]_i$ of rat osteoclasts.** *Panel A* illustrates first a small transient by calcitonin (CT, 1 $\mu$M), then (after washing, *W*) the transient induced by a $[Ca^{2+}]_o$ addition (4 mM) and finally the markedly potentiated transient induced by a second administration of the hormone, in a medium however containing 6 mM $Ca^{2+}$. In *panel B*, the order of additions is different: first $CaCl_2$, then (after washing) calcitonin, finally $CaCl_2$ again. Notice the marked potentiation of the $[Ca^{2+}]_o$-induced transient elicited in the presence of the hormone. In *panel C*, 8Br-cAMP (500 $\mu$M, *8BC*) is used instead of calcitonin, with a potentiation of the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ transient similar to that observed in the hormone-treated cell of *panel B*.

of cAMP, *i.e.* the second messenger known to be generated by the activation of CT receptors (11–14). Taken together, the results with hCT and 8Br-cAMP indicate that the $[Ca^{2+}]_i$ effects of the hormone are mediated by cAMP-dependent mechanism(s).

*Potentiation of the Cation-induced $[Ca^{2+}]_i$ Transients by Calcitonin and 8Br-cAMP*—Further interesting results were obtained by combining hCT with high $[Ca^{2+}]_o$ treatments. In 5 out of 7 rat osteoclasts first stimulated by the addition of 4 mM $CaCl_2$ to the medium (total $[Ca^{2+}]$, 6 mM), the response to a subsequent application of hCT was amplified 5–10-fold with respect to the hormone alone (Fig. 8*A*). In addition, pretreatment of the osteoclasts with either hCT (1 μM, Fig. 8*A*) or 8Br-cAMP (500 μM, Fig. 8*C*) usually resulted in a marked (2–3-fold) potentiation of the $[Ca^{2+}]_i$ transients triggered by the subsequent addition of $CaCl_2$ to the medium ($n$ = 8 and 5, respectively). As can be seen in Fig. 8*B*, the potentiation by hCT of the $[Ca^{2+}]_o$ effect concerned both the size and the rate of the $[Ca^{2+}]_i$ increase. The general morphology of the $[Ca^{2+}]_i$ transient was either unchanged (Fig. 8*C*) or modified, with a more rapid rising phase (Fig. 8*B*) by the hCT or 8Br-cAMP pretreatment.

## DISCUSSION

Osteoclast physiology is still little known, due primarily to fragility and isolation problems. Another problem concerns the identification of the cells to be investigated. Osteoclast cells employed in this work were identified on the basis of firmly established criteria: overall morphology at the phase contrast, immunocytochemistry and expression of tartrate-resistant acid phosphatase, and bone resorption ability. Moreover, the rat cells were found to respond to CT with a retraction and to excavate single or multiple pits when cultured over bone slices, two properties of *bona fide* osteoclasts. In the osteoclasts from both species, $[Ca^{2+}]_i$ was measured by fura-2 microscopy, a technique that has already been employed in a variety of cell types. The microscopic approach has proven to be highly appropriate to the study of minority cell populations. In our studies, this approach was mandatory, especially with rat osteoclasts, which are prepared with very low recovery (≈20 cells/animal). Another big advantage of the microscopic approach is the possibility of investigating in one individual cell the basal $[Ca^{2+}]_i$ activity as well as the responses elicited by various treatments. Our studies provide information about the maintenance of the resting and stimulated $[Ca^{2+}]_i$ activities in individual cells (a property that has been recently named fingerprint, Ref. 21); the species specificity of the responses, their variability in the cell population, and their properties following various stimulations.

*$[Ca^{2+}]_i$ Effects of Extracellular $Ca^{2+}$ and Calcitonin*—Chicken and rat octeoclasts were found to differ in some basal $[Ca^{2+}]_i$ control mechanism(s) inasmuch as a fraction of the chicken cells exhibited $[Ca^{2+}]_i$ fluctuations (at rest as well as after $[Ca^{2+}]_o$ increase), which in contrast were never observed in the rat cells. $[Ca^{2+}]_i$ fluctuations have recently been reported (usually after receptor stimulation) in a variety of cell types (for review, see Ref. 22), including macrophages, which resemble osteoclasts in many respects. The underlying mechanisms, as well as the physiological significance of these events, are still open to question (22).

Most osteoclasts from both rat and chicken responded to $[Ca^{2+}]_o$ increase with $[Ca^{2+}]_i$ transients, either biphasic (an initial peak followed by a plateau phase) or monophasic (the rapid peak or the slower plateau only). This property had been reported previously only in a few cell types, the best example being the PTH-producing cells of the parathyroid gland (23). The demonstration that the $[Ca^{2+}]_i$ increase in parathyroid cells (i) is sustained by both stimulated $Ca^{2+}$ influx across the plasma membrane and $Ca^{2+}$ release from intracellular stores, and (ii) can be elicited not only by $Ca^{2+}$, but also by other cations (such as $Mg^{2+}$ and $Sr^{2+}$) added to the medium, indicated the existence in these cells of a receptor mechanism triggered by extracellular cations (23). The $[Ca^{2+}]_o$-triggered $[Ca^{2+}]_i$ responses we have observed in osteoclasts resemble in many respects those previously described in parathyroid cells. Thus, a cation-sensitive receptor appears to exist in osteoclasts as well. A number of differences, in particular the lower sensitivity to $[Ca^{2+}]_o$ and the lack of response to $La^{3+}$ and $Mg^{2+}$ observed in osteoclasts, indicate, however, that the machinery in the two cell types may not be identical. The fact that osteoclasts require larger $[Ca^{2+}]_o$ changes in order to become stimulated, with respect to PTH-producing cells, could be related to their homing in the bones, where $[Ca^{2+}]_i$ in the extracellular fluid is expected to change more extensively than elsewhere. In fact, it has been reported that at the level of ruffled border of active osteoclasts $[Ca^{2+}]_o$ can be as high as 26 mM (25).

In addition to $[Ca^{2+}]_o$, a number of hormones and factors known to be active, as stimulators or inhibitors, on bone resorption have been investigated. Most of these agents were found to be without effect on $[Ca^{2+}]_i$. This was the case of PTH and 1,25-dihydroxyvitamin $D_3$, two stimulators of osteoclast function, which, however, are believed to act indirectly on those bone cells (1, 8, 9). Negative results were also obtained with prostaglandin $E_2$. As far as CT is concerned, its function is known to be very important to mammalian osteoclasts, which express large number of receptors (11), and are blocked by the hormone *in vitro* (17). In contrast, no receptors could be detected in avian osteoclasts (24). Accordingly, we found that chicken osteoclasts fail to respond to hCT, whereas rat osteoclasts often increase their $[Ca^{2+}]_i$. The most exciting result we have obtained with CT is however the marked potentiation of the $[Ca^{2+}]_o$ effects, observed no matter whether the hormone was administered before or after $CaCl_2$. This potentiation is highly interesting because in the intact animal high $[Ca^{2+}]_o$ stimulates the secretion of the hormone, which therefore is expected to interact with the target cells in a high $[Ca^{2+}]_o$ environment. The $[Ca^{2+}]_i$ effect of CT on rat osteoclasts was mimicked by 8Br-cAMP and appears therefore to be mediated by cAMP, the known second messenger generated at the CT receptor (11–14). The site of cAMP-induced action could be the cation receptor or its associated transmembrane signaling molecules (G protein, phosphodiesterase). Additional actions at the intracellular $Ca^{2+}$ store and/or at the $Ca^{2+}$ channel in the plasma membrane might also be envisaged.

*Possible Role of $[Ca^{2+}]_i$ in Osteoclast Physiology*—The physiological role of the osteoclast $[Ca^{2+}]_i$ increase induced by high $[Ca^{2+}]_o$ and (in the rat) by application of CT remains to be evaluated. Bone resorption requires the adherence of osteoclasts to the bone surface and the establishment of cellular polarity, with the basolateral surface receiving regulatory signals, and the ruffled border involved in the secretion of $H^+$ and lysosomal enzymes to the sealed resorbing compartment (3–7). A reasonable possibility is that the $[Ca^{2+}]_i$ increase, as it is the case in a variety of other cells, causes contraction of the cytoskeleton, which is documented also by the cell retraction visible by phase contrast microscopy (Fig. 2). Contraction could in turn affect the adhesion properties of the cells and thus result in the arrest of bone resorption (15). $[Ca^{2+}]_i$ increases, particularly those synergistically activated by high $[Ca^{2+}]_o$ and CT that we have identified in the rat, might

therefore represent a key intracellular signal in the control of osteoclast activity. Although still partially hypothetical, our proposal appears entirely consistent with the present knowledge in the field of osteoclast cell physiology. Clearly, however, further experimental work is needed in order to define in more detail the role of $[Ca^{2+}]_i$ and, possibly, to identify additional intracellular signals which might also contribute to the control of osteoclast functions.

*Acknowledgments*—The contribution of Dr. M. Grano for chicken osteoclast culture investigation and L. Di Giorgio for the secretarial assistance are gratefully acknowledged.

REFERENCES

1. Vaes, G. (1988) *Clin. Orthop. Relat. Res.* **231,** 239–271
2. Zambonin Zallone, A., Teti, A., Rubinacci, A., Abbadini, M., Gaboli, M., and Marchisio, P. C. (1989) *Exp. Cell Res.* **182,** 645–652
3. Marchisio, P. C., Cirillo, D., Naldini, L., Primavera, M. V., Teti, A., and Zambonin Zallone, A. (1984) *J. Cell Biol.* **99,** 1696–1705
4. Zambonin Zallone, A., Teti, A., Carano, A., and Marchisio, P. C. (1988) *J. Bone Miner. Res.* **3,** 517–523
5. Baron, R., Neff, L., Louvard, D., and Courtoy, P. J. (1985) *J. Cell Biol.* **101,** 2210–2222
6. Baron, R., Neff, L., Brown, W., Courtoy, P. J., Louvard, D., and Farquhar, M. G. (1988) *J. Cell Biol.* **106,** 1863–1872
7. Blair, H. C., Kahn, A. J., Crough, E. C., Jeffrey, J. J., and Teitelbaum, S. L. (1986) *J. Cell Biol.* **102,** 1164–1172
8. Rodan, G. A., and Martin, T. J. (1981) *Calcif. Tissue Int.* **33,** 349–351
9. McSheedy, P. M. J., and Chambers, T. J. (1986) *Endocrinology* **118,** 824–828
10. Oreffo, R. C., Teti, A., Triffitt, J. T., Francis, M. J. O., Carano, A., and Zambonin Zallone, A. (1988) *J. Bone Min. Res.* **3,** 203–209
11. Nicholson, G. C., Moseley, J. M., Sexton, P. M., Mendelson, F. A. H., and Martin, T. J. (1986) *J. Clin. Invest.* **77,** 312–314
12. Nicholson, G. C., Livesey, S. A., Moseley, J. M., and Martin, T. J. (1986) *J. Cell Biochem.* **31,** 229–238
13. Nicholson, G. C., Moseley, J. M., Sexton, P. M., Mendelsohn, F. A. O., and Martin, T. J. (1986) *J. Clin. Invest.* **78,** 356–360
14. Nicholson, G. C., Moseley, J. M., Yates, A. J. P., and Martin, T. J. (1987) *Endocrinology* **120,** 1902–1908
15. Teti, A., and Zambonin Zallone, A. (1986) *Excerpta Med. Int. Congr. Ser.* **9,** 358–362
16. Zambonin Zallone, A., Teti, A., and Primavera, M. V. (1982) *Anat. Embryol.* **165,** 405–413
17. Chambers, T. J., Athanasou, N. A., and Fuller, K. (1984) *J. Endocrinol.* **102,** 281–286
18. Arnett, T. R., and Dempster, D. W. (1987) *Endocrinology* **120,** 602–608
19. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
20. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) *J. Cell Biol.* **105,** 2145–2155
21. Prentki, M., Glennon, M. C., Thomas, A. P., Morris, R. L., Matschinsky, F. M., and Corkey, B. E. (1988) *J. Biol. Chem.* **263,** 11044–11047
22. Berridge, M. J., and Gallione, A. (1988) *FASEB J.* **2,** 3074–3082
23. Nemeth, E. F., and Scarpa, A. (1987) *J. Biol. Chem.* **262,** 5188–5196
24. Nicholson, G. C., Moseley, J. M., and Martin, T. J. (1987) *J. Bone Miner. Res.* **2,** 53–59
25. Silver, A., Murrills, R. J., and Etherington, D. J. (1988) *Exp. Cell Res.* **175,** 226–276