Osteoclast Cytosolic Calcium, Regulated by Voltage-gated Calcium Channels and Extracellular Calcium, Controls Podosome Assembly and Bone Resorption

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Abstract. The mechanisms of Ca^{2+} entry and their effects on cell function were investigated in cultured chicken osteoclasts and putative osteoclasts produced by fusion of mononuclear cell precursors. Voltage-gated Ca^{2+} channels (VGCC) were detected by the effects of membrane depolarization with K^+, BAY K 8644, and dihydropyridine antagonists. K^+ produced dose-dependent increases of cytosolic calcium ($[Ca^{2+}]_i$) in osteoclasts on glass coverslips. Half-maximal effects were achieved at 70 mM K^+. The effects of K^+ were completely inhibited by dihydropyridine derivative Ca^{2+} channel blocking agents. BAY K 8644 (5×10^{-6} M), a VGCC agonist, stimulated Ca^{2+} entry which was inhibited by nicardipine. VGCCs were inactivated by the attachment of osteoclasts to bone, indicating a rapid phenotypic change in Ca^{2+} entry mechanisms associated with adhesion of osteoclasts to their resorption substrate. Increasing extracellular Ca^{2+} ($[Ca^{2+}]_e$) induced Ca^{2+} release from intracellular stores and Ca^{2+} influx. The Ca^{2+} release was blocked by dantrolene (10^{-5} M), and the influx by La^{3+}. The effects of $[Ca^{2+}]_e$ on $[Ca^{2+}]_i$ suggest the presence of a Ca^{2+} receptor on the osteoclast cell membrane that could be coupled to mechanisms regulating cell function. Expression of the $[Ca^{2+}]_e$ effect on $[Ca^{2+}]_i$ was similar in the presence or absence of bone matrix substrate. Each of the mechanisms producing increases in $[Ca^{2+}]_i$, (membrane depolarization, BAY K 8644, and $[Ca^{2+}]_e$) reduced expression of the osteoclast-specific adhesion structure, the podosome. The decrease in podosome expression was mirrored by a 50% decrease in bone resorptive activity. Thus, stimulated increases of osteoclast $[Ca^{2+}]_i$ lead to cytoskeletal changes affecting cell adhesion and decreasing bone resorptive activity.

THE cellular basis of bone remodeling is not completely understood. The osteoclast, the multinucleated cell involved in bone resorption, is a complex unit that develops a specialized apparatus for dissolving the bone matrix (King and Holtrop, 1975; Holtrop and King, 1977). Using cell culture systems, several advances have recently been made indicating the molecular events involved in osteoclast bone resorbing activity. For bone resorption to be initiated, the osteoclast polarizes (Baron et al., 1985) and directly attaches to the bone surface by a specialized area termed the clear zone (Holtrop and King, 1977), in which the contact with the substrate is established by specific adhesion structures called podosomes (Marchisio et al., 1984, 1987; Zambonin-Zallone et al., 1988). Morphologically, podosomes appear as short membrane protrusions with a core of microfilaments linked to the plasma membrane by talin and vinculin (Marchisio et al., 1984, 1987). Recent data suggest that podosomes play a pivotal role in substrate recognition by osteoclasts as a specific β_3 integrin of the RGD-superfamily of matrix receptors is expressed on their cell membrane surface (Davies et al., 1989; Zambonin-Zallone et al., 1989). Substrate recognition is a necessary early step in the initiation of bone resorption, and it may induce phenotypic differences in cellular responses as the osteoclast changes from a motile cell seeking bone substrate to an actively resorbing cell.

The organization of the podosome-containing clear zone allows tight sealing of the resorbing compartment between the osteoclast plasma membrane and the bone surface. The acidification of this extracellular microenvironment (Baron et al., 1985; Blair et al., 1989) produces hydroxyapatite solubilization. Lysosomal enzymes, secreted into this space by a mannose-6-P receptor driven mechanism (Baron et al., 1988; Blair et al., 1988), and activated by the acid pH, digest the organic components of the bone matrix (Blair et al., 1986). Tight sealing of the compartment is needed to maintain the pH of 5 and the Ca^{2+} concentrations of up to 40 mM (Silver et al., 1988).

While the mechanisms of osteoclast regulation are incompletely understood, we have recently reported that extracellular protons decrease cytosolic calcium ($[Ca^{2+}]_i$) and intracellular pH (pH_i) of osteoclasts attached to bone (Teti et al., 1989). Moreover, these changes in intracellular cation concentration directly stimulate podosome formation (Teti et al., 1989) leading to activation of bone resorption (Arnett and Dempster, 1986; Carano et al., 1990). In contrast, ionophore-induced enhancement in $[Ca^{2+}]_i$ decreased expression of podosomes (Teti et al., 1989). While these findings are provocative, the mechanisms by which changes in osteoclast $[Ca^{2+}]_i$ are mediated physiologically and their relevance to resorptive activity are unknown.

In the studies reported herein, we focused on the mechanisms of $[Ca^{2+}]_i$ regulation in osteoclasts and their relevance to the control of podosome organization and bone resorption activity. In our previous study of osteoclasts attached to bone (Teti et al., 1989), we noted that depolarization of the membrane potential by extracellular potassium chloride produced a prompt decrease in $[Ca^{2+}]_i$. However, Rizzoli, Schlegel, and Bonjour (personal communication) have noted that KCl addition to osteoclasts attached to glass resulted in increased $[Ca^{2+}]_i$. These conflicting studies prompted us to (*a*) identify and characterize voltage gated calcium channels (VGCCs)[1] in the osteoclast plasma membrane; (*b*) study modulation of VGCCs during changes in osteoclast activity from an inactive to an active resorbing state; (*c*) further characterize a mechanism recently reported (Malgaroli et al., 1989) whereby extracellular Ca^{2+} ($[Ca^{2+}]_e$) elicits changes in $[Ca^{2+}]_i$ for its action on cell function and (*d*) analyze the role of $[Ca^{2+}]_i$ regulation in podosome organization and osteoclast bone-resorbing activity.

Materials and Methods

Materials

The acetoxymethyl ester of fura-2 (fura-2 AM) was purchased from Molecular Probes (Eugene, OR). BAY K 8644 and nitrendipine were obtained from Miles Laboratories (West Haven, CT), and diltiazem hydrochloride from Marion Laboratories (Kansas City, MO). Ionomycin was from Calbiochem-Behring Corp. (La Jolla, CA), and all other reagents were of analytical grade from Sigma Chemical Co. (St. Louis, MO), Eurobio (Paris, France), and Carlo Erba (Milan, Italy).

Osteoclast Preparation

In our previous report (Teti et al., 1989) adherence of osteoclasts to bone particles was used as a purification technique. The objectives of the studies reported here required cells detached from bone, thereby excluding the use of the bone particle purification technique. Thus, osteoclasts were isolated by a modification of a previously described method (Zambonin-Zallone et al., 1982). Briefly, the medullary bone from femurs and tibias of calcium-deficient laying hens was removed and pressed through a 100-μm nylon sieve. The filtrate was centrifuged for 5 min at 300 *g* and then suspended for two minutes in a hypotonic solution (0.2% NaCl) to lyse erythrocytes. The osteoclast-rich suspension was centrifuged at 235 *g* for 20 min at 4°C on Ficoll-Hypaque in PBS (density 1.077), and the osteoclast-containing pellet was collected. Centrifugation of the cell suspension was repeated to increase osteoclast enrichment. Because isolated osteoclasts did not express podosomes as they attached to the glass coverslips, they were cultured at a density of 30,000 osteoclasts/3.5 cm in Petri dishes containing glass coverslips. The culture medium was α-MEM plus 5% FCS plus 5% chicken serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 3 μg/ml cytosine 1β-D arabino furanoside. Temperature was maintained at 37°C in a water-saturated atmosphere containing 5% CO_2. Cultures were washed after 24 h to remove the non-adhering contaminating cells. This produced a significant increase in the purity of the preparation, as the nonadhering cells were mononuclear. After this step, the enrichment of the preparation for osteoclasts was increased to 80–90% of the counted nuclei. Thereafter, the number of multinucleated cells in the culture declined and the percent of nuclei in the preparation that were from mononuclear cells increased. Thus, there was no evidence for new formation of multinucleated cells in culture. Culture was necessary because freshly prepared cells did not express podosomes. Podosome expression in osteoclasts isolated on bone develops rapidly, but on the glass coverslips podosome expression began between 48 and 72 h. Thus, experiments were performed between 3 and 5 d in culture. The osteoclastic features of these multinucleated cells have been previously described (Zambonon-Zallone et al., 1982). In addition, the osteoclasts added to bone slices produced well formed resorption pits assessed by scanning electron microscopy. In some experiments, 100 μg of devitalized rat bone particles, 38–63 μm diameter, were added to osteoclasts cultured on glass coverslips and incubated for 5 h. During this time, changes in phenotypic expression of Ca^{2+} entry mechanisms were assessed.

Because the purity of the osteoclast preparations could not be increased above 80–90%, there was a possibility that contaminating cells were regulating osteoclast responses to experimental protocols. Thus, we also used a technique recently developed in our laboratory whereby virtually pure preparations of multinucleated putative osteoclasts were generated in culture from mononuclear cell precursors. Putative osteoclast precursors were obtained from bone marrow of laying hens maintained on calcium deficient diets by recovering the cells from the interfaces of the Ficoll/Hypaque gradient centrifugation described above. These mononuclear cells were cultured in the presence of cytosine 1β-D arabino furanoside (5 μg/ml) to prevent proliferation of contaminating cells. After 3 d, highly purified precursor preparations were obtained by removing nonadherent cells (lymphocytes, granulocytes, and erythrocytes). Between day 3 and 6 of culture, pure preparations (>98% of cell number) of multinucleated cells were formed that expressed the following osteoclast characteristics: ruffled membranes apposed to bone surfaces, formation of resorption pits in bone slices, resorption of ^{3}H-proline labeled bone particles (Blair et al., 1986), tartrate-resistant acid phosphatase and reactivity with osteoclast-specific mAbs, 121F (Oursler et al., 1985) and 23C6 (Davies et al., 1989). For $[Ca^{2+}]_i$ measurements, 4×10^6 precursors were cultured in 35 mm petri dishes containing glass coverslips for 3–5 d before use. We found that regardless of the method of osteoclast preparation, the results obtained in our studies were the same. Because pure populations were obtainable with the cell fusion preparation, the influences of contaminating cells were eliminated. The data presented in the figures of this report were obtained using both osteoclast preparations.

Measurement of Cytosolic Ca^{2+} $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in single cells using the fluorescent calcium indicator fura-2. Osteoclasts cultured on coverslips were loaded for 1 h at 25°C in a buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes, 6 mM glucose (Krebs-Ringer Hepes [KRH]) and 10 μM fura-2-AM. In these loading conditions, resulting fura-2 fluorescence was diffuse and no punctate distribution was observed, as described in other cells (Malgaroli et al., 1987). Cells were then washed three times with KRH and used for the experiments. Fluorescence was measured in single cells excited with 340 and 380 nm light selected by two monochromators and directed through the stage of a Nikon inverted microscope equipped with a 100× fluor objective. Emitted light was collected at 505 nm after being filtered through a cut-off filter (490 nm) and monitored photometrically (SPEX Industries, Edison, NJ). Coverslips were mounted at the bottom of a Sykes-Moore open chamber with 1 ml KRH within a climate box maintained at 37°C.

Fura-2 fluorescence was calibrated to $[Ca^{2+}]_i$ at the end of each experiment, exposing the cells to 5 μM ionomycin to assess the Ca-saturated fluorescence (F_{max}), followed by 5 mM EGTA to determine fluorescence at nominally Ca^{2+}-free condition (F_{min}). 2 mM $MnCl_2$ was finally added to estimate autofluorescence which was subtracted from the experimental values. $[Ca^{2+}]_i$ was calculated using the formula published by Grynkiewicz et al. (1985).

Activation of VGCCs

VGCCs were activated in osteoclasts by depolarizing the cells with high K^+

1. *Abbreviation used in this paper*: VGCC, voltage-gated calcium channels.

KRH. Experiments were performed in isotonic conditions by substituting from 6.2 to 100 mM KCl for corresponding amounts of NaCl in the KRH buffer. Activation of VGCCs was also obtained using BAY K 8644, a specific dihydropyridine VGCC agonist. The effects of several VGCC antagonists were evaluated, including diltiazem, verapamil, nicardipine, or nitrendipine (10^{-10}–10^{-5} M) before depolarization with high K^+ KRH (100 mM) or addition of BAY K 8644. A complete series of parallel experiments were performed using osteoclast cultures in which devitalized bone particles (38–63-μm-diam) were added 5 h before the experiments were performed.

Activation of Calcium-operated Regulation of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were observed during changes in extracellular calcium concentrations. Osteoclasts, previously incubated in KRH containing 2 mM Ca^{2+}, were treated with increasing extracellular calcium concentrations. To block release of calcium from intracellular stores, a series of experiments was performed using cells pretreated with dantrolene (10^{-5} M) 10 min before $CaCl_2$ additions. To block Ca^{2+} entry, $LaCl_3$ and Ca^{2+} channel antagonists were studied for their effects on the changes in $[Ca^{2+}]_i$ stimulated by adjustments in $[Ca^{2+}]_e$. Parallel experiments in the presence of bone particles were also performed to evaluate the effects of increasing extracellular calcium concentration on $[Ca^{2+}]_i$ during bone resorption.

Cytoskeletal Studies

The effect of membrane depolarization on podosome expression was evaluated in osteoclasts incubated for 90 min in MEM in which 30 or 50 mM NaCl was isotonically substituted with KCl. Control osteoclasts were incubated in regular MEM containing 5.0 mM KCl. The effect of extracellular calcium on podosome expression was evaluated by incubating osteoclasts in nominally calcium-free MEM and adding $CaCl_2$ from 0 to 4 mM. Incubation times were 90 min unless otherwise indicated.

Microfilaments were detected by decoration of F-actin with rhodamine-conjugated phalloidin (R-PHD). Cells were fixed for 5 min at room temperature with 3% formaldehyde, 2% sucrose in PBS. They were then permeabilized for 3 min at 0°C with 50 mM NaCl, 300 mM sucrose, 20 mM Hepes (pH 7.4), 3 mM $MgCl_2$, 0.5% Triton-X 100, and then they were incubated for 45 min at 37°C with 5 μg/ml R-PHD. Coverslips were then washed, mounted in 10% mowiol, and observed with a Leitz Diavert fluorescence microscope. Due to the presence of a core of microfilaments perpendicularly oriented with respect to the substrate, podosomes were recognized as fluorescent dot-like structures distributed at the level of the ventral surface of the cell.

To quantitate the results, the numbers of osteoclasts presenting podosomes in each treatment expressed as the percent of the total number of osteoclasts present. Data were expressed as mean percentage ± SE. The statistical significance was calculated by the *t* test. For each treatment, an average of 300 osteoclasts was counted. A decrease in podosome expression did not cause the cells to lift off the coverslip since the osteoclast has other mechanisms of substrate adhesion besides podosome expression (Zamboni-Zallone et al., 1989).

Bone Resorption

The effects of increasing $[Ca^{2+}]_i$, by activation of VGCC or changing $[Ca^{2+}]_e$, on the bone-resorbing activity of osteoclast cultures was determined. For this purpose, 2-d-old osteoclast cultures were incubated for 48 h with [^{3}H]proline prelabeled devitalized rat or chicken bone particles (Blair et al., 1986) at a density of 400 μg of bone/50,000 osteoclasts in 1.5 cm φ culture dishes. The cells were cultured in serum-free MEM in the presence of varying calcium concentrations, varying K^+ concentrations or by the addition of BAY K 8644. The experiments were carried out by (*a*) preincubating the cells for 30 min with the experimental medium, then adding the bone particles, and (*b*) preincubating the cells for 24 h with the bone particles, then substituting the control medium with the experimental medium.

Bone resorption was evaluated by measuring the radioactivity present in the medium, which derived from osteoclast-mediated release of tritiated products of bone collagen degradation (Blair et al., 1986). Parallel samples, in absence of cells, were evaluated to measure the nonspecific ^{3}H release, which was subtracted from the corresponding experimental values. The total radioactivity present in the 400 μg of bone added to the cultures was measured after demineralization and solubilization of the organic matrix, as previously described (Blair et al., 1986), and the micrograms of bone resorbed were calculated. Data were expressed as mean micrograms of bone resorbed ± SE. The statistical significance was evaluated by the *t* test. Each experiment was performed with at least three different osteoclast preparations in quadruplicate.

Results

Effect of Membrane Depolarization on Osteoclast $[Ca^{2+}]_i$

Basal $[Ca^{2+}]_i$ of osteoclasts on glass coverslips in KRH was 123 ± 9 (n = 41) (mean ± SE). The response of osteoclasts $[Ca^{2+}]_i$ to 100 m K^+ is shown in Fig. 1. The effect of K^+ on $[Ca^{2+}]_i$ was directionally opposite to the response we previously reported in osteoclasts attached to bone particles (Teti et al., 1989). High K^+ induced an immediate increase in $[Ca^{2+}]_i$ and a second slower rise to a new steady state of 267 nM within 300 s. High K^+ increased $[Ca^{2+}]_i$ in a similar manner in 83% of examined cells attached to glass coverslips (n = 18). The other 17% of cells demonstrated a decrease in $[Ca^{2+}]$ upon depolarization with K^+. The increase in $[Ca^{2+}]_i$ from basal to peak levels achieved by various concentrations of K^+ in high K^+ KRH was dose-dependent with a half-maximal effect achieved by 66 mM (Fig. 2). When cells were placed in a Ca-free KRH ($CaCl_2$ omitted from the buffer and 1 mM EGTA added), KCl additions failed to elevate $[Ca^{2+}]_i$ (not shown). This indicated that the increases in $[Ca^{2+}]_i$ stimulated by high K^+ were due to calcium entry from the extracellular fluid. The effects of high K^+ were almost completely inhibited by the dihydropyridine, nicardipine (Fig. 1 *b*).

The Effect of BAY K 8644 on $[Ca^{2+}]_i$

BAY K 8644 is a dihydropyridine-derivative Ca^{2+} channel agonist that increases opening frequency of dihydropyridine-sensitive Ca^{2+} channels (Duncan and Misler, 1989; Guggino et al., 1989; Meier et al., 1988). After the addition of BAY K 8644 (5×10^{-6} M) in calcium containing KRH a

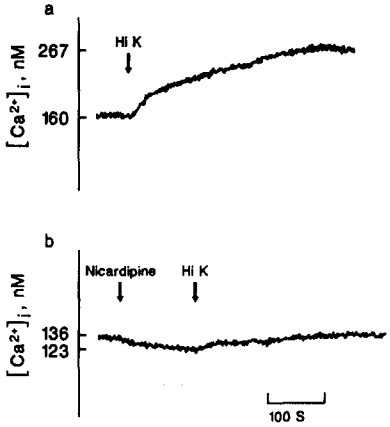

Figure 1. Effect of K^+ on osteoclast $[Ca^{2+}]_i$. (*a*) A fura-2 loaded single osteoclast, bathed in KRH, responded to substitution with high K^+ (100 mM) KRH with a sustained increase in $[Ca^{2+}]_i$. (*b*) The effect of pretreatment with nicardipine on high K^+ (100 mM) KRH induced increase in $[Ca^{2+}]_i$. Nicardipine (10^{-7} M) inhibited the rise in $[Ca^{2+}]_i$ after membrane depolarization.

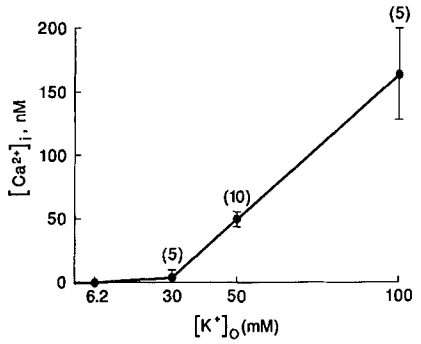

Figure 2. Concentration dependence of the K^+-induced changes in $[Ca^{2+}]_i$ of single osteoclasts. The maximal increase in $[Ca^{2+}]_i$ produced with increasing K^+ concentrations is plotted against K^+_o. The number of experiments is indicated in parentheses. Results are the mean ± SE.

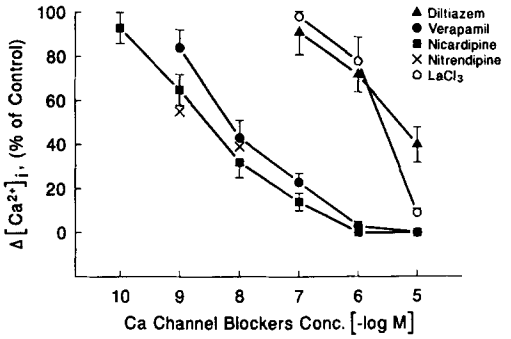

Figure 4. Effects of Ca^{2+} channel antagonists on K^+-induced elevations in $[Ca^{2+}]_i$. Dose-response curves are illustrated for osteoclasts treated with nicardipine, nitrendipine (dihydropyridine derivatives), verapamil (phenylalkylamine), diltiazem (benzodiazepine), or $LaCl_3$ before the addition of 100 mM K^+. The data represent the size of the maximal high K^+ (100 mM) induced elevation of $[Ca^{2+}]_i$ in osteoclasts pretreated for 3 min with the corresponding VGCC antagonist. The data are expressed as mean ± SE of three or four separate experiments.

rise in $[Ca^{2+}]_i$ of 78 ± 8 nM (n = 3) was observed, reaching plateau levels in 7–8 min (Fig. 3 *a*). BAY K 8644 (5×10^{-6} M) had no effect on $[Ca^{2+}]_i$ in a Ca^{2+}-free medium (not shown), and the increase of $[Ca^{2+}]_i$ induced by BAY K 8644 was completely abolished by nicardipine (10^{-6} M) (Fig. 3 *b*). The effects of BAY K 8644 were slower in onset than high K^+ KRH probably due to time for incorporation into plasma membranes in our system. The increase in $[Ca^{2+}]_i$ produced by 5×10^{-6} M BAY K was similar to that of 70 mM K^+. These findings suggest that BAY K 8644 stimulated Ca^{2+} influx through dihydropyridine-sensitive voltage-gated Ca^{2+} channels.

Effects of Ca^{2+} Channel Blocking Agent on the K^+-induced Increase in $[Ca^{2+}]_i$

The effects of the dihydropyridine Ca^{2+} channel blocker, nicardipine (10^{-7} M), on the K^+-stimulated changes in $[Ca^{2+}]_i$ are shown in Fig. 1 *b*. KRH with nicardipine (10^{-7} M) was added for 2 min before high K^+ with nicardipine. K^+ had no significant effect on $[Ca^{2+}]_i$ in the presence of nicardipine. The K^+-induced increase in $[Ca^{2+}]_i$, was restored after transfer to nicardipine-free KRH and a second addition of K^+.

The effects of various Ca^{2+} channel blockers on high K^+ KRH- (100 mM) induced elevations of $[Ca^{2+}]_i$ were compared, as shown in Fig. 4. Dihydropyridine Ca^{2+} channel blockers (nicardipine, nitrendipine at concentrations of 10^{-10}–10^{-5} M (n = 3 or 4 of each concentration) inhibited the K^+-induced $[Ca^{2+}]_i$ increases in a dose dependent manner. The half-maximal inhibition was observed at 5×10^{-9} M. Verapamil was less potent than dihydropyridines, and diltiazem was less effective than verapamil.

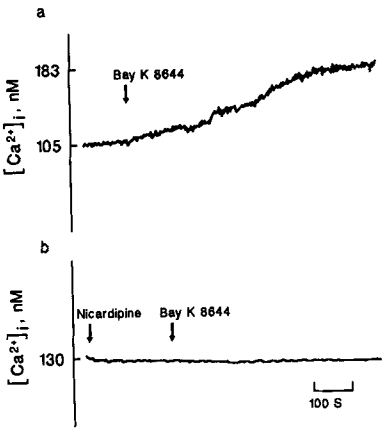

Figure 3. Effect of the dihydropyridine VGCC agonist BAY K 8644 on osteoclast $[Ca^{2+}]_i$. *a* illustrates the rise in $[Ca^{2+}]_i$ in a single osteoclast treated with 5×10^{-6} M BAY K 8644 (marked by an arrow). The increase in $[Ca^{2+}]_i$ reached a new steady state in 7–8 min. *b* illustrates the effect of pretreatment with 10^{-6} M nicardipine, which prevented BAY K 8644-induced increases in $[Ca^{2+}]_i$. Experiments shown in *A* and *B* are a pair of four similar experiments.

Effects of Changing Extracellular Calcium on $[Ca^{2+}]_i$

As we have previously reported (Malgaroli et al., 1989), increasing extracellular Ca^{2+} concentrations induced rapid dose-dependent increases in $[Ca^{2+}]_i$. As shown in Fig. 5, basal $[Ca^{2+}]_i$ of 108 nM was increased by addition of 2 mM

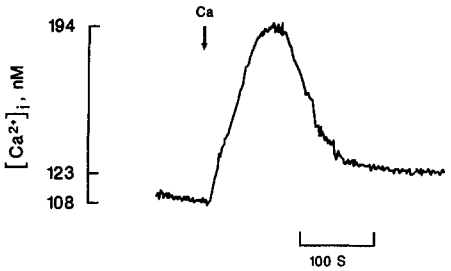

Figure 5. Transient elevation in $[Ca^{2+}]_i$ induced in a single osteoclast by addition of $CaCl_2$ to the medium. Addition of 2 mM $CaCl_2$ to KRH containing 2 mM Ca^{2+} (final concentration: 4 mM) induced a biphasic transient involving a rapid increase in $[Ca^{2+}]_i$ followed by a return towards baseline which was incomplete resulting in a sustained elevation.

Ca^{2+} to a peak of 194 nM in 80 s, and then decreased to a steady state at 123 nM (Fig. 5). The threshold dose of $CaCl_2$ needed to increase $[Ca^{2+}]_i$ was 0.25 mM. Half-maximal increases in $[Ca^{2+}]_i$, 182 ± 16 nM, n = 7, were observed at addition of 4 mM $CaCl_2$ (final concentration 6 mM), and the increases in $[Ca^{2+}]_i$ were maximal with addition of 8 mM $CaCl_2$. The Ca^{2+} channel blockers (10^{-5} M nicardipine, nitrendipine, verapamil, and diltiazem) failed to inhibit the $CaCl_2$-induced changes, which, however, were partially blocked by $LaCl_3$ (10^{-4}–10^{-5} M). As shown in Fig. 6 *a*, $LaCl_3$ (10^{-4} M) did not alter basal $[Ca^{2+}]_i$ levels, but reduced the $CaCl_2$-prompted increase in $[Ca^{2+}]_i$ by 43% (40 ± 4% in three similar experiments). The increases in $[Ca^{2+}]_i$ stimulated by external Ca^{2+} were not affected by removing extracellular Na (Fig. 6 *b*). This and similar experiments also demonstrated the reproducible elevations in $[Ca^{2+}]_i$ observed with repeated additions of $CaCl_2$ after washes. Dantrolene (10^{-5} M), which inhibits mobilization of Ca^{2+} from intracellular stores (Kojima et al., 1985; Reid et al., 1987), dampened the $CaCl_2$ induced rise in $[Ca^{2+}]_i$ by 80% (55.4 ± 17% in three experiments) and eliminated the spike phase of the increases stimulated by $CaCl_2$ (Fig. 6 *c*). Together, Dantrolene (10^{-6} M) and $LaCl_3$ (10^{-4} M) were sufficient to completely inhibit stimulation of $[Ca^{2+}]_i$ by additions of $CaCl_2$ (Fig. 6 *d*).

Effects of K^+ and $[Ca^{2+}]_e$ on Osteoclasts Attached to Bone Particles

Osteoclasts cultured on glass coverslips attached to devitalized bone particles (38–63 μm diameter) as they settled. Basal $[Ca^{2+}]_i$ levels in the particle-attached cells were 136 ± 7, n = 28, similar to osteoclasts not exposed to bone. Addition of 2 mM $CaCl_2$ increased $[Ca^{2+}]_i$ by 100 ± 22 nM (mean ± SE), n = 7 (Fig. 7 *a*). In contrast to the effects of K^+ on osteoclasts not attached to bone, high K^+ KRH prompted a decrease in $[Ca^{2+}]_i$ after 5 h of osteoclasts attaching to bone. As shown in Fig. 7 *b*, $[Ca^{2+}]_i$ decreased from 125 to 75 nM in such a representative osteoclast. This 5-h contact with bone reduced the percentage of osteoclasts expressing VGCC from 83% without bone to 10%. Furthermore, osteoclasts attached to bone particles were insensitive to BAY K 8644, and this agent produced no change in $[Ca^{2+}]_i$ in these cells. These findings indicate that, with osteoclastic bone attachment, the voltage-gated Ca^{2+} channel is functionally lost, but the Ca^{2+}-induced Ca^{2+} release mechanism was not inactivated. Furthermore, the effect of K^+ to decrease $[Ca^{2+}]_i$ in osteoclasts attached to bone appeared to be a stimulation of Ca^{2+} efflux since inhibition of Ca^{2+} entry had no significant effect on the decrease stimulated by K^+ (Fig. 7 *c*).

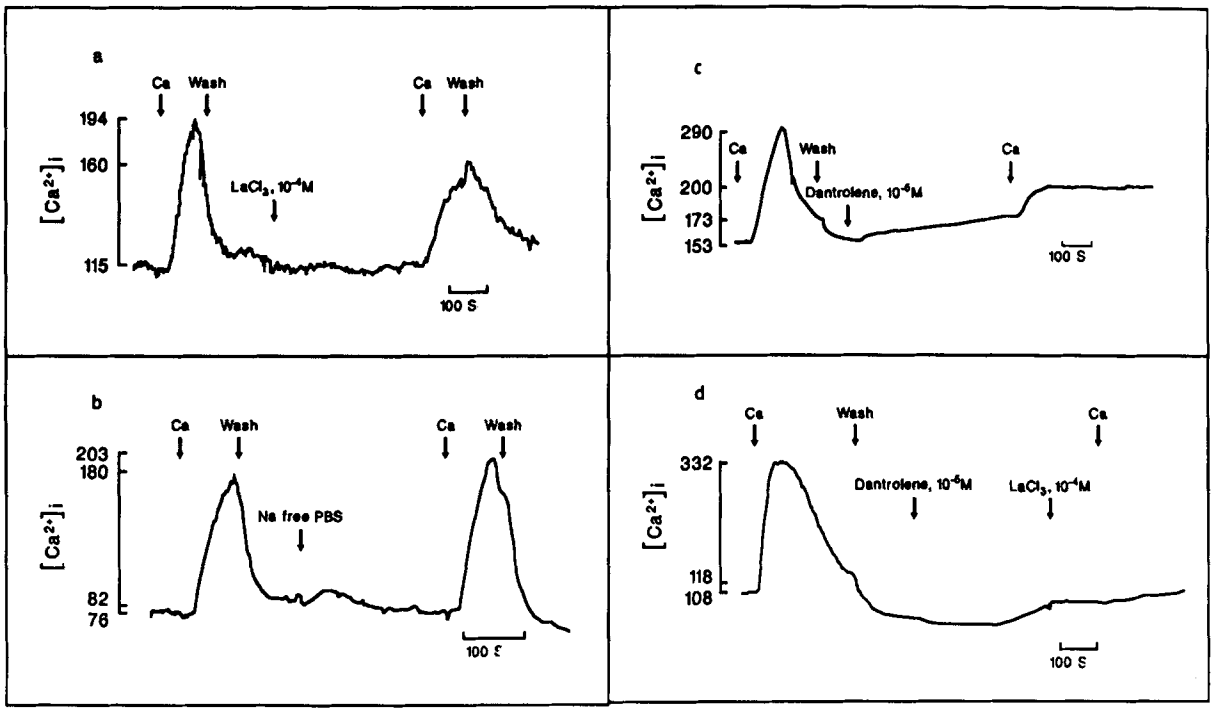

Figure 6. Effects of La^{3+} and dantrolene on external Ca^{2+}-elicited increases in $[Ca^{2+}]_i$. *a* illustrates the effect of $CaCl_2$ (2 mM) on $[Ca^{2+}]_i$-induced biphasic in a single osteoclast followed (after washings) by treatment with $LaCl_3$ (10^{-4} M). La^{3+} had no effect itself, but reduced the height of the Ca^{2+} spike after stimulation with $CaCl_2$. In *b*, a control experiment is shown to demonstrate that a second addition of 2 mM $CaCl_2$, after removal of the first addition by washing, produces the same increment in $[Ca^{2+}]_i$. We have previously demonstrated (Teti et al., 1989; Malgaroli et al., 1989) that removal of Na^+ had no effect on osteoclast stimulated $[Ca^{2+}]_i$. *c* illustrates the effect of dantrolene (10^{-5} M) on a 4 mM $[Ca^{2+}]_e$-elicited increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ spike was abolished, while the sustained phase still occurred. *d* illustrates that together, $LaCl_3$ and dentrolene were sufficient to completely inhibit the effect of $CaCl_2$ (4 mM).

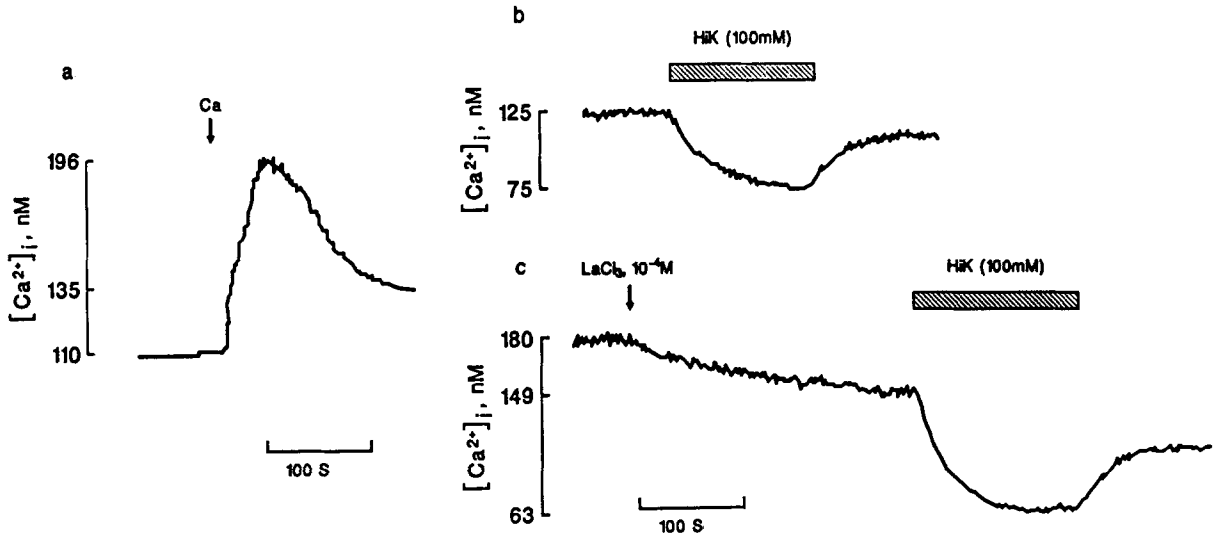

Figure 7. Effects of increasing extracellular Ca^{2+} and K^+ on $[Ca^{2+}]_i$ of osteoclasts attached to bone particles. (*a*) Increasing Ca^{2+} of KRH from 2 to 4 mM elicited a transient increase in $[Ca^{2+}]_i$ similar to the effect observed in osteoclasts on glass coverslips (Fig. 5). (*b*) High K^+ (100 mM) KRH decreased $[Ca^{2+}]_i$. The mean decrease of $[Ca^{2+}]_i$ from baseline by high K^+ (100 mM) KRH was 73 ± 12 nM (n = 9). (*c*) High K^+ (100 mM) KRH decreased $[Ca^{2+}]_i$ in a $LaCl_3$-treated cell. The mean decrease of $[Ca^{2+}]_i$ of $LaCl_3$-treated cells was 65 ± 11 nM (n = 5). This was not significantly different from the effect of high K^+ (100 mM) in cells not treated with $LaCl_3$.

Cytoskeletal Studies

The distribution of microfilaments in osteoclasts at different times of culture was evaluated by decoration of F-actin with R-PHD and observation by fluorescent microscopy. Podosomes, due to the perpendicular orientation of F-actin-containing microfilaments with respect to the substrate, appear as fluorescent dot-like structures located at the level of the ventral surface of the cell (Fig. 8). In osteoclasts cultured in standard conditions on glass coverslips, podosome number increased with time, and they were spontaneously organized into several concentric rows located at the cell edge, in an area resembling the actin-rich clear zone (Fig. 8, *a* and *b*). Membrane depolarization with K^+ was a potent inhibitor of podosome expression. A $[K^+]$ of 50 mM reduced podosome expression by 65%. However, this dose of K^+ increased $[Ca^{2+}]_i$ by only ~25% of maximal. Thus K^+ may affect podosome expression by mechanisms other than opening VGCC. Further evidence for VGCC-regulated podosome expression came from the fact that as BAY K 8644 increased $[Ca^{2+}]_i$, it inhibited appearance of these attachment structures (Fig. 9 *a*). Similarly, increasing $[Ca^{2+}]_e$ from 0 to 4 mM led to a clear dose-dependent decrease in podosome expression (Figs. 8 *c* and 10 *a*). The specificity of the $[Ca^{2+}]_e$ effect on osteoclast microfilaments was demonstrated by failure of the cation to impact on fibroblast focal adhesions and stress fibers (Fig. 8, *d* and *e*).

Bone Resorption

We examined the effects of depolarization by K^+ or increases in $[Ca^{2+}]_e$ on osteoclastic bone resorption and found that K^+-KRH (50 mM) elicited a 60% inhibition of ^3H release from bone particles at 48 h of culture. However, as for podosome expression, this effect may represent actions other than opening VGCC. BAY K 8644 added prior to the addition of the labeled bone particles inhibited bone resorption in a dose dependent manner (Fig. 9 *b*). Similarly, increasing extracellular calcium reduced osteoclastic bone resorption. An increase in $[Ca^{2+}]_e$ from 2 to 4 mM reduced resorption by 50% (Fig. 10 *b*). BAY K 8644 had no effect on bone resorption if the osteoclasts were allowed to adhere to bone particles for 24 h before its addition. On the other hand, increasing $[Ca^{2+}]_e$ continued to inhibit resorptive activity of osteoclasts similarly preincubated with bone. Furthermore, the addition of bone particles to osteoclasts in 4 mM $[Ca^{2+}]_e$ for 5 h before a return to 2 mM restored resorptive activity to that of cells maintained in 2 mM Ca^{2+} for the full 48 h, documenting reversibility of the inhibitory effects of $[Ca^{2+}]_e$.

Discussion

Osteoclasts are cells involved in the manipulation of high calcium concentrations. This is due to their peculiar physiology which produces dissolution of the mineralized bone matrix and the release of calcium ions to the extracellular fluid. Silver et al. (1988) have shown that the calcium concentration achieved in the resorbing compartment during bone mineral dissolution is severalfold higher than in the extracellular fluid. This is possible because of the low pH (5.0) actively created by an outwardly directed H^+-pump mechanism located on the osteoclast ruffled border membrane (Baron et al., 1985; Blair et al., 1989). The low pH of the resorption space, on one hand, causes hydroxyapatite solubilization and, on the other hand, prevents calcium precipitation.

Very little is known of the mechanisms involved in calcium transport and storage by osteoclasts. A Ca^{2+} ATPase has been immunolocalized on the membrane opposite the ruffled border (Akisaka et al., 1988), and we have shown that this transport mechanism is responsible for the decrease in $[Ca^{2+}]_i$ stimulated by metabolic acids (Teti et al., 1989).

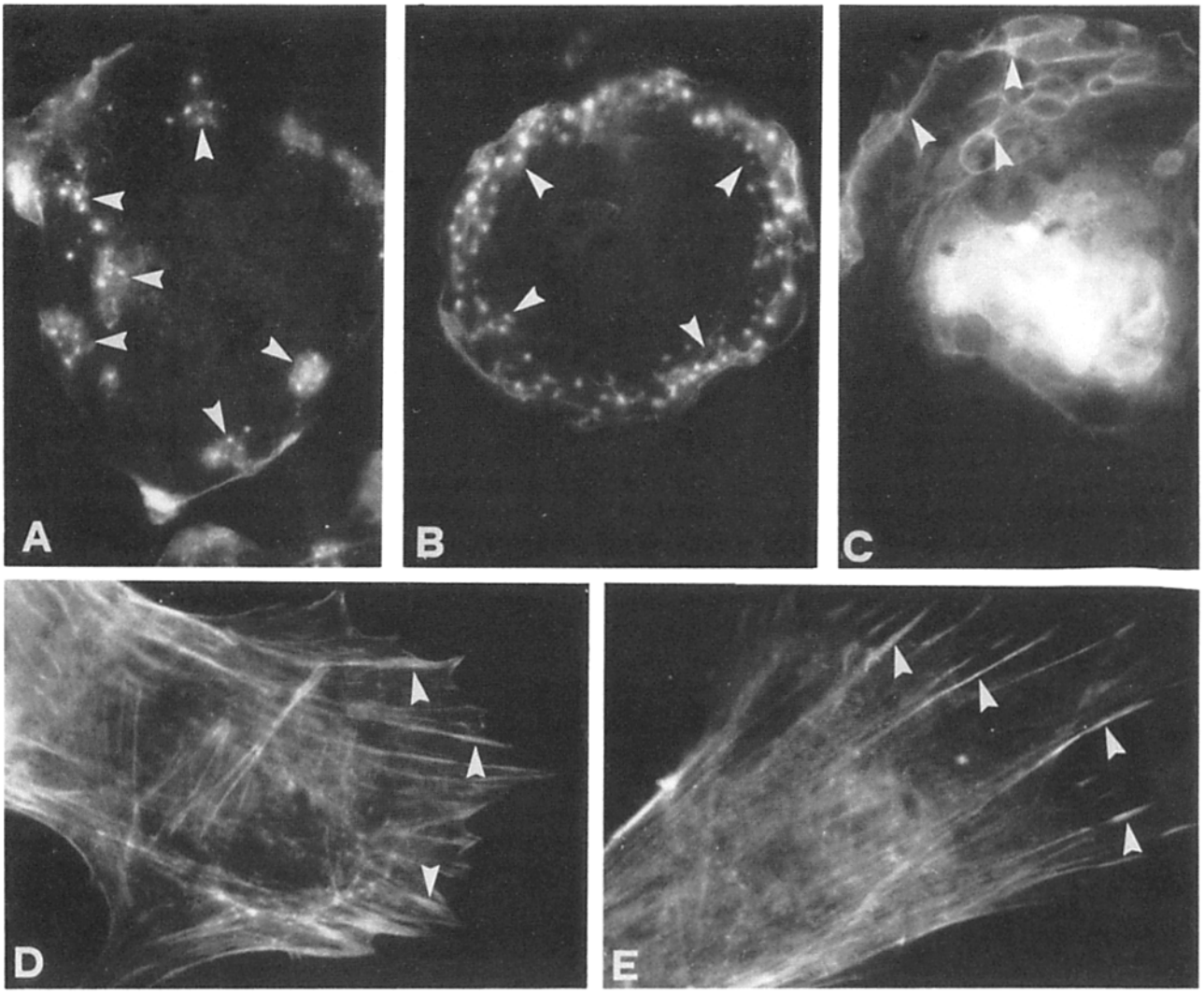

Figure 8. Fluorescence micrograph of osteoclasts and fibroblasts, cultured on glass coverslips, reacted with rhodamine-conjugated phalloidin to detect microfilaments. (*a*) 3-d-old control osteoclast microfilaments were organized in dot-like structures, corresponding to podosomes, clustered in small groups at the cell periphery (*arrows*). (*b*) 5-d-old control osteoclast: podosomes were numerous and organized in a multilayer ring, resembling the clear zone, at the cell edge (*arrows*). (*c*) 5-d-old osteoclast incubated for 90 min in the presence of 4 mM extracellular calcium; the fluorescent dots disappeared and microfilaments became organized at the level of membrane ruffles (*arrows*). (*d*) A control fibroblast, and (*e*) a fibroblast incubated in the presence of 4 mM extracellular calcium did not show differences in the distribution of microfilaments, which were organized in typical stress fibers connected to the ventral membrane at the level of focal adhesions (*arrows*). Magnification of *a–e* are the same. Bar: (*e*) 10 μm.

Furthermore, we found that a divalent cation-operated mechanism present in both chicken and rat osteoclasts (Malgaroli et al., 1989) stimulates $[Ca^{2+}]_i$ transients through both calcium entry and the release of calcium from intracellular stores. In rat osteoclasts, this system is potentiated by treatment with calcitonin, and we hypothesized that it could have an inhibitory role on osteoclast activity. In the present report, we demonstrate that inhibition of Ca^{2+} entry by La^{3+}, but not dihydropyridine Ca^{2+} channel antagonists, partially blocked enhancement of $[Ca^{2+}]_i$ by increasing $[Ca^{2+}]_e$. Importantly, the early spike phase of the change in $[Ca^{2+}]_i$, which is derived from intracellular stores, was not affected by La^{3+} treatment, indicating that in the presence of Ca^{2+} entry blockade, the effect of increasing $[Ca^{2+}]_e$ was derived from Ca^{2+} release. This was also supported by the observation that dantrolene, a more specific blocker of Ca^{2+} release from intracellular stores than TMB8 previously used (Malgaroli et al., 1989), diminished the response to changes in $[Ca^{2+}]_e$ and removed the early spike phase of the transient. The resultant tonic elevation in $[Ca^{2+}]_i$ to a higher plateau is consistent with stimulated Ca^{2+} entry. Altogether, our data indicate that increases in $[Ca^{2+}]_e$ stimulate both Ca^{2+} entry and Ca^{2+} release. Moreover we have shown that Cd^{2+} stimulates transient elevations in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Malgaroli et al., 1989). Similar to the parathyroid glands, our data indicate that Ca^{2+} may act through a receptor-operated mechanism stimulating Ca^{2+} release from intracellular stores (Nemeth et al., 1986).

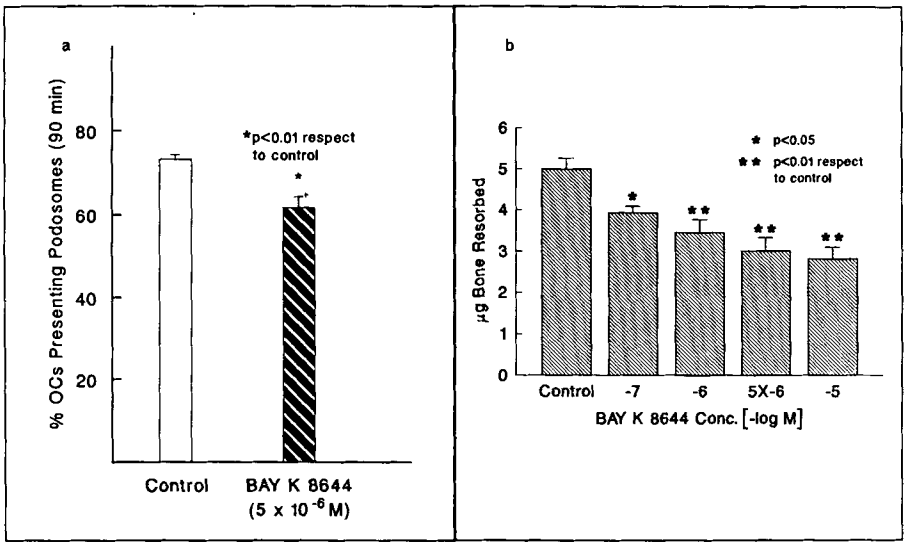

Figure 9. Effect of BAY K 8644 on podosome expression and bone resorption. (*a*) 5×10^{-6} M BAY K 8644 decreased podosome expression. (*b*) Dose-dependent inhibition of bone resorption in osteoclasts treated with increasing dose of BAY K 8644. Cells were preincubated for 30 min with BAY K 8644, then the bone particles were added. Bone resorption was evaluated as described in Materials and Methods. The data are expressed as mean ± SE in quadruplicate.

In this report, we also demonstrate that osteoclasts transiently express VGCC. Depolarization of the membrane potential elevated $[Ca^{2+}]_i$ in osteoclasts cultured without bone. The addition of bone produced a time-dependent disappearance of VGCC. Specifically, whereas 83% of the bone-free cells studied had increased levels of $[Ca^{2+}]_i$, after 5 h in the presence of bone, depolarization prompted a decrease in $[Ca^{2+}]_i$ as previously reported (Teti et al., 1989). The decrease in $[Ca^{2+}]_i$ is compatible with an increase of Ca^{2+} efflux produced by depolarization.

The VGCCs of the osteoclast exhibit the functional properties of those described in other cell types. They are more sensitive to dihydropyridine agonists (BAY K 8644) and antagonists (nicardipine and nitrendipine) than to phenilalchilamine (verapamil) and benzodiazepine (diltiazem) antagonists. These data are consistent with the presence of an L-type VGCC found in the plasma membrane of excitable and nonexcitable cells (Nowycky et al., 1985; Schramm and Towart, 1985; Miller, 1987; Meier et al., 1988).

The next phase of this study involved assessing the effects of increasing $[Ca^{2+}]_i$ on osteoclast activity. First, we studied the effects of membrane depolarization, BAY K 8644 and increasing external Ca^{2+} on podosome expression. We found that each mechanism of increasing $[Ca^{2+}]_i$ decreased podosome expression. The effect of K^+ on podosome expression appeared more effective than other means of increasing $[Ca^{2+}]_i$. This suggested that K^+ additions were affecting osteoclastic functions besides stimulating VGCC. However, BAY K 8644 is a specific VGCC agonist and thus it indicates that VGCC stimulation decreases podosome expression. These results are consistent with the effects of Ca^{2+} ionophores on podosome expression previously reported (Teti et

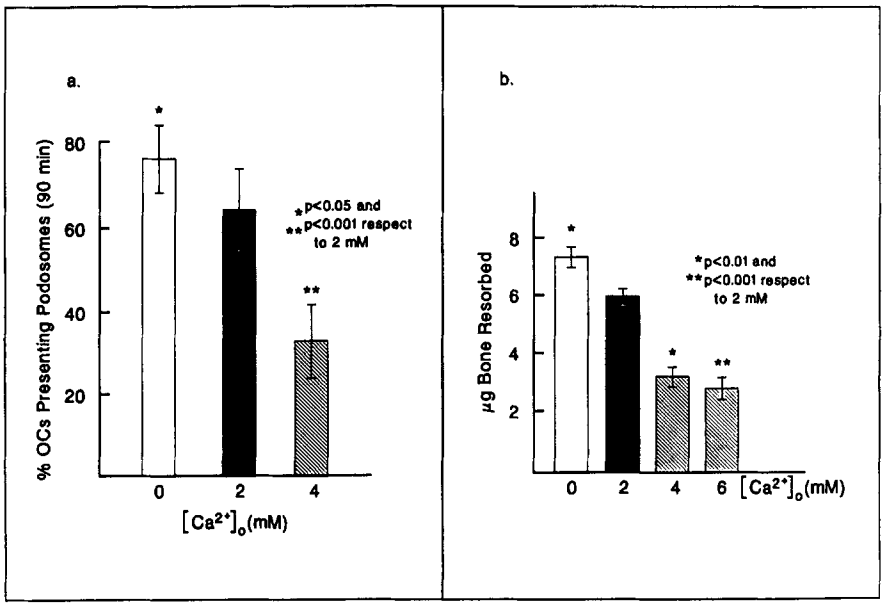

Figure 10. Effects of extracellular Ca^{2+} on podosome expression and bone resorption (*a*) increasing $[Ca^{2+}]_e$ from nominally absent to 4 mM resulted in dose-dependent inhibition of podosome expression in osteoclasts. 4 mM extracellular Ca^{2+} (final concentration) induced 42% inhibition of osteoclast presenting podosomes with respect to the cultures treated with 2 mM Ca^{2+}. Data are mean ± SE of three experiments performed in triplicate. (*b*) Dose-dependent inhibition of bone resorption in osteoclasts treated with increasing doses of extracellular calcium. 4 mM extracellular calcium (final concentration) reduced osteoclast resorbing activity by 50% of the value obtained at 2 mM. Data are mean ± SE of at least three experiments performed in triplicate.

al., 1989), and indicate that podosomes are a calcium-regulated adhesion structure.

The main candidate for mediating the effect of $[Ca^{2+}]_i$ on podosomes is gelsolin, a calcium-dependent actin regulating protein present in the osteoclast podosomes (Marchisio et al., 1987). Gelsolin, after complexing calcium, is capable of fragmenting pre-existing microfilaments and nucleating new ones, thereby contributing to the rearrangement of the microfilament network of the cell (Yin and Stossel, 1979). Furthermore, gelsolin presents a site for binding membrane phosphatidylinositol 4,5-bisphosphate, which inhibits its activity (Janmey and Matsudaira, 1988; Yin et al., 1988). We have only preliminary information concerning the metabolism of polyphosphoinositides in osteoclasts, suggesting that the PIP_2–gelsolin–calcium complex could contribute to their cytoskeletal organization and adhesion properties.

Finally, we studied the effect of VGCC-dependent and $[Ca^{2+}]_e$-elicited $[Ca^{2+}]_i$ increase on osteoclastic bone resorption. We found a reduction of bone collagen degradation by osteoclasts exposed to BAY K 8644 or increases in $[Ca^{2+}]_e$. These results suggest that increases in $[Ca^{2+}]_i$ negatively regulate osteoclast resorptive activity. They also demonstrated a direct correlation between reduced podosome expression and decreased bone resorption.

It therefore appears that $[Ca^{2+}]_e$-stimulated increase in cytosolic calcium may be a mechanism for inhibiting avian osteoclasts, an event reminiscent of the cation's ability to block parathyroid hormone secretion (Schoback et al., 1983; Nemeth and Scarpa, 1986; Wallfelt et al., 1988). Calcitonin synergistically augments $[Ca^{2+}]_e$ enhanced $[Ca^{2+}]_i$ (Margaroli et al., 1989), further indicating that the Ca^{2+} signal may be a regulated osteoclast function. The inhibition of osteoclast adhesion and bone resorption by $[Ca^{2+}]_e$, through podosome disassembly is a potential feedback mechanism for signaling to the cell that the products of bone resorption have accumulated at the cell–matrix interface. Inhibition of adhesion, leading to leakage of the resorptive microenvironment could lead to release of these products into the extracellular fluid.

The role of the VGCC in bone resorption is more complex at this point than the modulation by $[Ca^{2+}]_e$. The decrease in bone resorptive activity with BAY K 8644 pretreatment can be explained by the inhibition of podosome formation. However, osteoclasts allowed to attach to bone and begin resorption were resistant to BAY K 8644, consistent with the observed disappearance of functional VGCC after addition of bone particles. The possibility exists that, during the cell's motile phase, VGCC are expressed on the plasma membrane of osteoclasts and promote movement. Activation of these cells by BAY K 8644 would decrease their ability to promote their adhesive capacity, express podosomes, and resorb bone.

Addendum

While this manuscript was in review, Zaidi et al. (1989) reported that extracellular Ca^{2+} increases $[Ca^{2+}]_i$ in neonatal rat long bone osteoclasts and inhibits bone resorption in agreement with the results reported herein and our previous report (Malgaroli et al., 1989).

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