Acid Extrusion Is Induced by Osteoclast Attachment to Bone

Inhibition by Alendronate and Calcitonin

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Abstract

Acid extrusion is essential for osteoclast (OC) activity. We examined Na^+ and HCO_3^--independent H^+ extrusion in rat- and mouse OCs by measuring intracellular pH (pH_i) changes, with the pH_i indicator BCECF (biscarboxyethyl-5-(6) carboxyfluorescein) after H^+ loading with an ammonium pulse. 90% of OCs attached to glass do not possess HCO_3^- and Na^+-independent H^+-extrusion (rate of pH_i recovery = 0.043±0.007 (SEM) pH U/min, $n = 26$). In contrast, in OCs attached to bone, the pH_i recovery rate is 0.228±0.011 pH_i U/min, $n = 25$. OCs on bone also possess a NH_4^+-permeable pathway not seen on glass. The bone-induced H^+ extrusion was inhibited by salmon calcitonin (10^{-8} M, for 2 h), and was not present after pretreating the bone slices with the aminobisphosphonate alendronate (ALN). At ALN levels of 0.22 nmol/mm^2 bone, H^+ extrusion was virtually absent 12 h after cell seeding (0.004±0.002 pH U/min) and ~ 50% inhibition was observed at 0.022 pmol ALN/mm^2 bone. The Na^+-independent H^+ extrusion was not inhibited by bafilomycin A_1 (up to 10^{-7} M), although a bafilomycin A_1 (10^{-8} M)-sensitive H^+ pump was present in membrane vesicles isolated from these osteoclasts. These findings indicate that Na^+-independent acid extrusion is stimulated by osteoclast attachment to bone and is virtually absent when bone is preincubated with ALN, or when osteoclasts are treated with salmon calcitonin. (*J. Clin. Invest.* 1995. 96:2277–2283.) Key words: bone resorption • H^+-ATPase • intracellular pH • bafilomycin • bisphosphonate

Introduction

Osteoclasts (OC)[1] alternate between resorptive and motile phases (1). The resorptive phase requires attachment to mineralized matrix and formation of a clear zone that separates the basolateral from the ruffled membrane. Osteoclasts rendered inactive by bisphosphonates (2, 3) or calcitonin (4, 5), as well as by the absence of c-*src* (6) lack ruffled membrane. During bone resorption, osteoclasts secrete H^+ into a resorption lacuna via a Na^+-independent vacuolar(V)-type proton pump (7–11). The V-ATPases are very sensitive to the macrolide antibiotic bafilomycin A_1 ($IC_{50} \approx 1$ nM[12]) shown to bind to the hydrophobic, 17-kD H^+ channel subunit (13, 14). Although (V)-H^+ pumps are highly conserved (15), the H^+-pumps in osteoclasts and osteoclastoma cells may have some unique characteristics (16, 17). The aminobisphosphonate alendronate (4-amino-1-hydroxybutylidene bisphosphonate [ALN]) is a potent inhibitor of osteoclastic bone resorption in vitro and in vivo (3, 18). The effects of bisphosphonates on bone have been known for over 20 yr (19), but their mode of action is still uncertain. More than one mechanism may be involved and several have been proposed: interference with osteoclastic differentiation (20–24), toxicity to osteoclasts (25, 26), and metabolic inhibition (27). ALN has an amphipatic structure and a high avidity for hydroxyapatite. It concentrates at sites of bone resorption and should be locally released within the resorption space, due to the acidification produced by osteoclasts (3). However, osteoblasts may also be the target cells for bisphosphonate action (28), and in turn affect osteoclast activity. The object of this study on single osteoclasts was to obtain direct physiologic evidence for acid extrusion and to examine the effects of ALN on this process.

We observed that osteoclasts plated on bone acquire the capacity for Na^+-independent H^+ extrusion. This property was inhibited in a dose-dependent manner by preincubating the bone with alendronate or by addition of salmon calcitonin to the culture media. This is a direct demonstration of a quantifiable pharmacological effect of a bisphosphonate on single osteoclasts which should enable future study of the bisphosphonate mechanism of action.

Methods

Materials. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), or Fluka Chemical Corp. (Ronkonkoma, NY) and were of the finest grade available. Bovine collagen type I was from Collagen Corp. (Palo Alto, CA), salmon calcitonin was from Bachem California (Torrance, CA). BCECF/AM and H_2DIDS were from Molecular Probes, Inc. (Eugene, OR), valinomycin from Calbiochem Corp. (San Diego, CA), acridine orange from International Biotechnology Laboratories, Inc. (New Haven, CT). Collagenase was from Wako Bioproducts, Inc. (Richmond, VA) and dispase from Boehringer Mannheim (Mannheim, Germany). Bafilomycin A_1, etidronate, and alendronate were from Merck & Co., Inc. (Rahway, NJ) chemical supplies.

Cells and cell culture. Rat osteoclasts were isolated from the humeri and femora of 1–5-d-old Sprague-Dawley rats, being held without food the night before the experiment. The periosteum and cartilage were removed, the bones were split longitudinally, and the cells were scraped from the bone marrow cavity side, using a low magnification microscope. This procedure was carried out in 199 medium (GIBCO, Gaith-

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1. *Abbreviations used in this paper:* ALN, alendronate; BCECF, biscarboxyethyl-5-(6) carboxyfluorescein; OC, osteoclasts; pH_i, intracellular pH; TMACl, tetramethylammonium chloride; TRAP, tartrate-resistant acid phosphatase.

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ersburg, MD), containing 12 mM bicarbonate, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate, and 20 mM Hepes/Tris, pH = 7.1. The cells were plated either on flame sterilized glass coverslips or on bone slices glued to coverslips, incubated for 1 h at 37°C/5% CO_2 in the above medium containing 10% FBS JRH Biosciences, Lenexa, KS. Unless stated otherwise, the experiments were performed 12 h after cell attachment.

Mouse osteoclast-like cells were generated in vitro by coculturing 2.4×10^6 mouse neonatal calvaria-derived osteoblastic cells with 1.2×10^7 mouse bone marrow cells on collagen gels for 7 d in the presence of 10 nM 1,25-dihydroxyvitamin D3 (in a MEM [GIBCO] supplemented with 10% FBS, 100 U/ml penicillin G and 100 mg/ml streptomycin sulphate in 10 cm plastic dishes). Between 4 and 7 d of coculture, cells fuse to form multinucleated cells that exhibit osteoclastic features including calcitonin receptors, tartrate resistant acid phosphatase (TRAP), $\alpha_v\beta_3$ integrins and the formation of pits on bone slices (29, 30). Cells were released from collagen by 0.1% collagenase; 0.1% dispase treatment (15 min, 37°C, 5% CO_2). The multinucleated cells were larger and positive for TRAP. After their release, cells were cultured either on glass or on bone slices, as mentioned above.

Intracellular pH measurements. After cultivation, the cells were loaded with a H^+-sensitive fluorescent dye BCECF (biscarboxyethyl-5-(6) carboxyfluorescein; 5 mM) for 60 min at room temperature, conditions which minimize vesicular accumulation of the dye. Loaded cells were placed in a sealed, perfused (1 ml/min) microscope chamber (volume ≈ 100 μl), heated to 37°C. Fluorescence intensity in single cells was measured by adjusting the diaphragm in front of the photomultiplier tube. The cells were sequentially illuminated with two excitation wavelengths (λ_{ex} = 495 and 440 nm) using two monochromators. The ratio of the emission intensities (λ_{em} = 520–560 nm) was calculated using the software of PTI (Photon Technology International, New Brunswick, NJ) and readings were collected at 0.5-s intervals. Background correction was performed for each experiment. Conversion of BCECF fluorescence ratio to pH units was done by calibration with nigericin (31). The standard NaCl (or tetramethylammonium chloride [TMACl]) solution contained (mM): 130 NaCl (TMACl), 4 KCl, 1 $MgSO_4$, 1.7 $CaCl_2$, 1 NaH_2PO_4, 10 glucose, 20 Hepes/Tris; pH = 7.4. NH_4Cl solution contained 20 mM NH_4Cl and 110 mM (TMACl), which replaced NaCl. In Na^+-free solutions, tetramethylammonium phosphate was added instead of sodium phosphate. Tetramethylammonium phosphate was prepared by titration of tetramethylammonium hydroxide with phosphoric acid. H^+ and NH_4^+ flux rates were estimated by measurement of intracellular pH changes in a linear portion of pH changes within the range of 5.54–6.93 for H^+ extrusion and 7.53–5.54 for NH_4^+ influx. Intracellular buffering capacities could not be determined due to the lack of an effective direct inhibitor of the Na^+-independent H^+ extrusion described here. If not stated otherwise, data are expressed as mean±SEM with the indicated *n*.

Experiments were performed in nominally CO_2/HCO_3^--free conditions, only on viable multinucleated cells which were attached to glass or bone and capable of hydrolyzing and retaining the pH-sensitive dye. Cells selected for experiments were visualized by BCECF fluorescence and had diameters > 3× of mononuclear cells. In situ, visible bone resorption pits were observed underneath many untreated cells and such cells were preferentially chosen for the experiments. Postexperimental TRAP staining (32) was used after some experiments when resorption pits were not visible or the nature of the cells was uncertain.

Isolation of membrane vesicles from osteoclastic cells and measurements of H^+ pump activity. After cocultivation of 1.6×10^6 calvaria derived osteoblasts and 4×10^6 bone marrow cells in 15-cm plastic dishes (similar as above) the osteoclasts were partially purified by collagenase–dispase treatment for 20 min at 37°C, followed by vigorous pipetting to remove the osteoblastic cells. Thereafter, the enriched osteoclast cell population was harvested and homogenized in a homogenizing buffer (300 mM mannitol; 12 mM Hepes/Tris; pH = 7.4) with a glass/teflon Potter homogenizer (4°C; 20 strokes; high speed). The homogenate was centrifuged at 1,500 *g* for 15 min at 4°C in a desktop centrifuge with a swinging rotor (GPR centrifuge; Beckman Instruments, Inc. Fullerton, CA). The supernatant was centrifuged at 27,000 *g* for 25 min at 4°C (JA-20 fixed angle rotor, centrifuge J2-21; Beckman Instruments, Inc.) and the pellet was resuspended in a buffer (100 mM mannitol, 100 mM KCl, 3 Hepes/Tris; pH = 7.0) using a Potter homogenizer (20 strokes, high speed at 4°C). The homogenate was centrifuged (27,000 *g* for 25 min at 4°C in JA-20 rotor) and the pellet was resuspended in the same buffer. Membrane vesicles were additionally dispersed with an insulin syringe (20×) and left to equilibrate for 2 h at room temperature. Protein was determined using the BCA assay (Pierce Chemical Co., Rockford, IL).

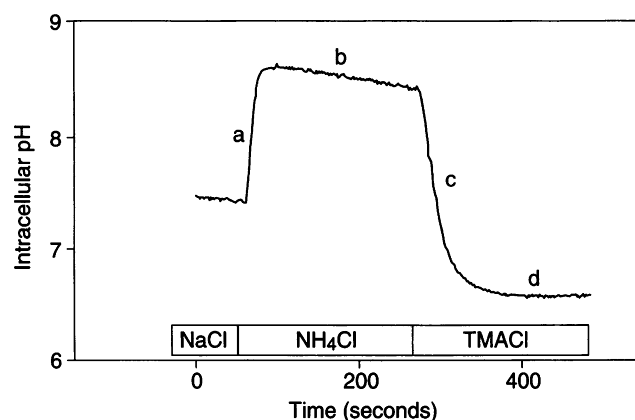

Figure 1. Absence of Na^+-independent acid extrusion in rat osteoclasts plated on glass. Osteoclast perfusion with a NH_4Cl solution (see Methods) induces a rapid intracellular alkalinization (*trace a*), followed by a slow acidification (*trace b;* 0.03±0.01 pH U/min, *n* = 60). Upon removal of NH_4Cl, a further rapid acidification occurs (*trace c*) with very limited or no pH_i recovery in a Na^+-free medium (*TMACl; trace d;* 0.043±0.007; *n* = 26). In 90% of osteoclasts on glass the H^+ extrusion rate was under these conditions below 0.020 pH U/min.

Formation and dissipation of H^+ gradients was visualized by acridine orange fluorescence. Fluorometric experiments (PTI Deltascan; λ_{ex} = 493; λ_{em} = 525) were performed in a cuvette heated at 37°C equipped with a magnetic stirrer. An aliquot of membrane vesicles was diluted in 2 ml of measuring buffer: 100 mM mannitol, 100 mM KCl, 1 mM ATP, 20 Hepes/Tris; pH = 7.0, 5 mM valinomycin, 6 mM acridine orange, and 5 mM $MgCl_2$ which was used to initiate the H^+ pumping. The data were collected by a computer at 1–3-s intervals using PTI software.

Results

Individual osteoclasts, loaded with the pH-sensitive fluorescent dye BCECF, were examined for their ability to extrude acid after NH_4^+-induced acid loading (33). In the presence of extracellular Na^+, most cells throughout the organism have the capacity to restore their pH_i after such treatment via the almost ubiquitous Na^+/H^+ exchanger (34). However, only cells that have an additional mechanism for acid extrusion or HCO_3^- intake, such as macrophages, kidney, or gastric parietal cells, can restore their physiological pH_i in the absence of Na^+. Since osteoclasts are thought to secrete acid during bone resorption via a Na^+-independent H^+ pump (7), we tested this capacity for acid extrusion in the nominal absence of extracellular HCO_3^- and Na^+.

In cells plated on glass the addition of Na^+-free NH_4^+ solution causes an elevation in pH_i (Fig. 1, *trace a*), attributed to the high NH_3:NH_4^+ permeability ratio (33). This response occurs in most cell types because of limited entry pathways for

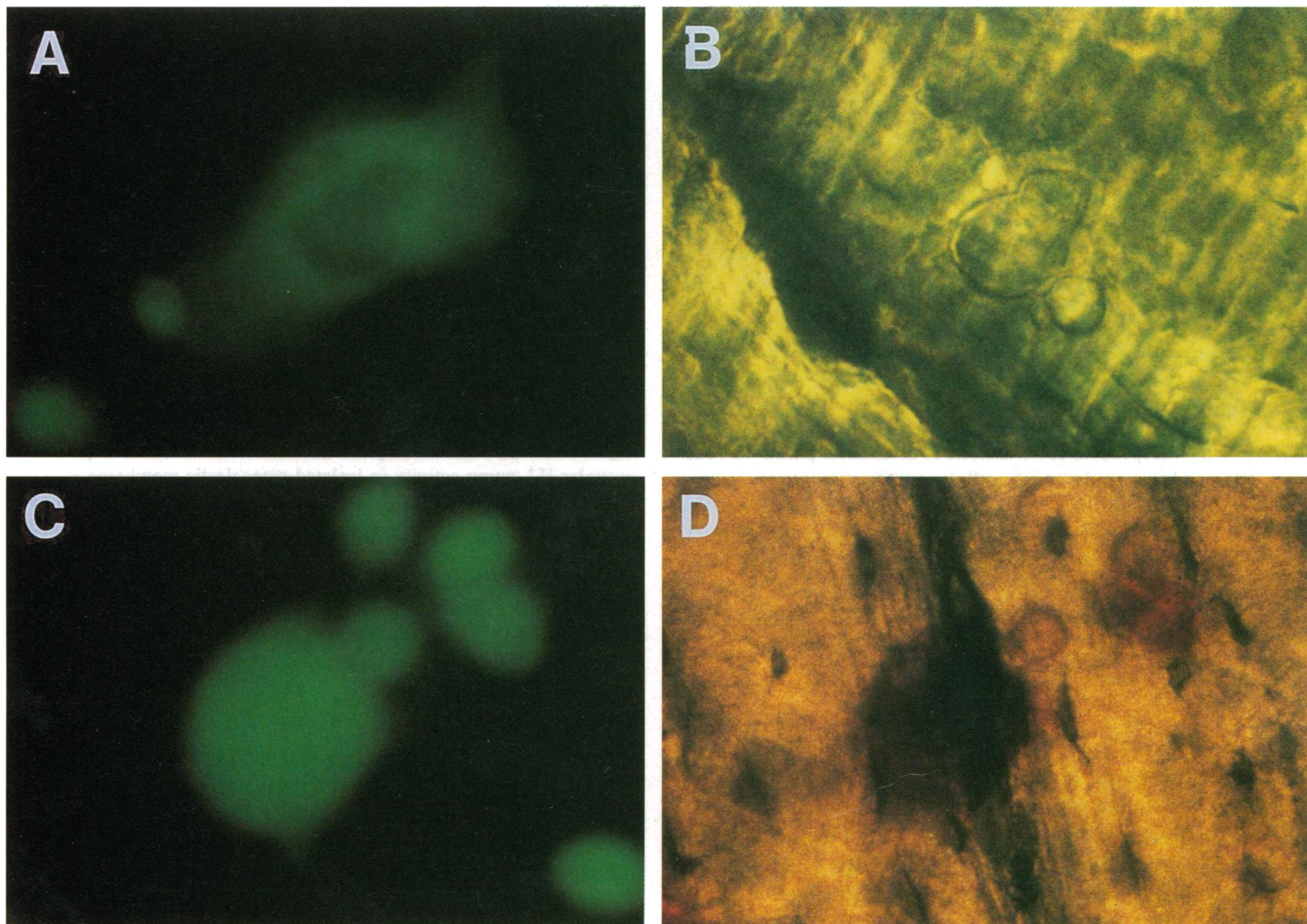

Figure 2. Visualization of osteoclasts on bone slices, loaded with BCECF and stained for TRAP. (*A* and *C*) Cells attached to bone slices and loaded with 5 mM BCECF (λ_{ex} = 495 and 440 nm; λ_{em} = 520 nm) were used for measurements in this study. With monochromator generated excitation no significant background fluorescence from bone was observed; the signal to background ratio > 10 (*B*) A resorption pit underneath the cell in *A*. (*D*) Same cell as in *B* is stained for TRAP.

NH_4^+. After rapid alkalinization in osteoclasts, a slower acidification occurs (0.030±0.010 pH U/min, n = 60), due to NH_4^+ influx (Fig. 1, *trace b*). This slow NH_4^+ influx was attenuated by a mixture of K^+ channel blockers (1 mM 4-aminopyridine and 10 mM tetraethylammonium; Zimolo, G.A. Rodan, unpublished observation). Upon removal of extracellular NH_4^+, the pH_i drops below the resting pH_i (Fig. 2, *trace c*) due to a rapid NH_3 exit. 90% of osteoclasts examined could not efficiently recover their pH_i in the absence of Na^+ (< 0.020 pH_i U/min), indicating that active Na^+-independent acid extrusion was generally absent in the plasma membrane of these cells. Since extracellular H^+ inhibits Na^+/H^+ exchange (34, 35), that process is not likely to be responsible for the acidification of the resorption pit. It was, therefore, surprising that in these cells, which form erosion pits when seeded on bone, we could not detect Na^+-independent H^+ extrusion. In an effort to explain this apparent paradox we repeated the same experiments in osteoclasts plated on bone slices. With appropriate monochromatic excitation and restricted emission, no significant bone autofluorescence was observed (Fig. 2, *A* and *C*), a frequently encountered obstacle with broader excitation/emmission spectra (i.e., with a mercury lamp and a dichroic mirror). After 12-h attachment to bone and BCECF loading, the largest cells in the field were chosen for examination. Pits were frequently observed by phase contrast microscopy underneath such cells and selection was always aimed at those cells (Fig. 2 *B*). In some experiments, the pits were not visible and the identity of these cells was confirmed by postexperimental TRAP staining in situ (Fig. 2 *D*). For osteoclasts generated in culture, every cell that had a diameter three times that of the mononucleated cell or larger was TRAP positive. About one-third of the data were collected on primary rat osteoclasts and the remainder on mouse bone marrow–derived osteoclasts. No significant qualitative or quantitative differences were observed between the two species.

The pH_i changes produced by an Na^+-free NH_4^+ solution in OCs attached to bone differed from those in OCs attached to glass. In OCs on bone, there was no or very little alkalinization (Fig. 3, *trace a*) followed by an immediate acidification (Fig. 3, *trace b*), at the mean rate of 0.550±0.130 pH U/min, n = 27, indicating a significant influx pathway for NH_4^+. After removal of NH_4^+ and intracellular acidification (Fig. 3, *trace c*) most cells on bone had the ability to restore their pH_i in the absence of Na^+ (fig. 3, *trace d*) at a mean rate of 0.228±0.011 pH U/min, n = 25. This Na^+-independent H^+-recovery observed in nominally HCO_3^--free medium is attributed to H^+ pumping. This pH_i recovery was not inhibited by H_2DIDS (100 μM; not shown), thereby excluding the possibility that intracel-

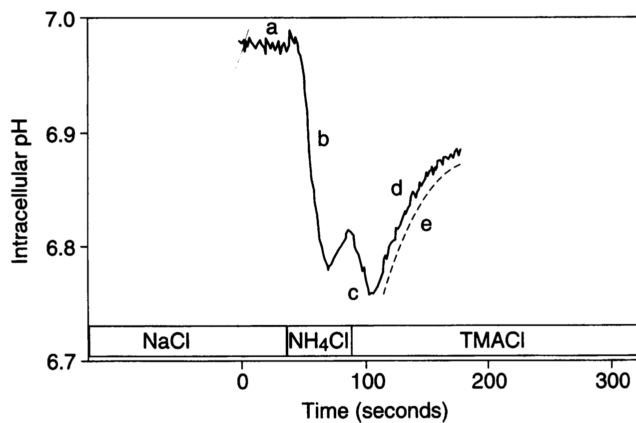

Figure 3. Na^+-independent H^+ extrusion in osteoclasts plated on bone slices. NH^+ addition produces intracellular acidification (*trace b;* 0.550±0.130 pH U/min, $n = 27$), after brief alkalinization (*trace a*), which is sometimes absent. NH_4^+ removal causes further acidification (*trace c*) followed by spontaneous recovery in a Na^+-free medium (*TMACl; trace d;* 0.228±0.011 pH U/min, $n = 25$). 10^{-7} M bafilomycin A_1 fails to significantly inhibit this H^+ extrusion (*trace e*). This is a representative trace of measurements from 25 separate cells.

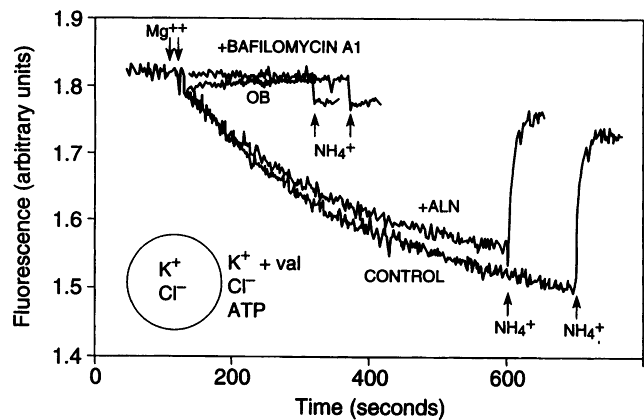

Figure 4. Effects of bafilomycin and alendronate on ATP-dependent vacuolar H^+-pump activity in isolated osteoclastic membrane vesicles. Acridine orange quenching shows Mg^{2+}-dependent H^+-ATPase activity in inside out vesicles that is bafilomycin A_1 sensitive (10^{-8} M) and alendronate (1 mM) insensitive. 75 mg protein was used per experiment. Membrane vesicles isolated from osteoblasts alone, used for co-culture of cells, did not show acidification (*OB*). NH_4Cl (50 mM) was used to dissipate the H^+ gradient. This is a representative figure of six traces from two independent membrane preparations.

lularly generated CO_2/HCO_3^- and the anion exchanger play a direct or indirect role in this process. Surprisingly however, this transport was not significantly inhibited by the macrolide antibiotic bafilomycin A_1 (at up to 10^{-7} M), a membrane permeable inhibitor of the vacuolar $(V)H^+$ pumps (Fig. 3, *trace e*). The same bafilomycin solutions were fully effective in inhibiting the $(V)H^+$ pump of rat kidney proximal tubule cells (data not shown; see reference 36, 36a). Incubation with a higher concentration of bafilomycin A_1 (10^{-5} M) for 20 min or more at 37°C was inhibitory, but at these concentrations this agent is not selective and affects other ATPases, i.e., Na^+/K^+-ATPase, Ca^{2+}-ATPase, etc. (12). Finally, resting pH_i was not increased after removal of Na^+ (tetramethylammonium replacement) neither in osteoclasts attached to bone nor glass (data not shown).

To try to reconcile these observations with the reported presence of bafilomycin A_1-sensitive H^+ transport in osteoclast-derived vesicles (7–11) we prepared membrane vesicles from a highly enriched population of the mouse osteoclasts used here (see Methods). Bafilomycin A_1-sensitive H^+ transport was clearly demonstrated in this preparation and under the same conditions no bafilomycin-insensitive mechanism was present (Fig. 4). The observed H^+ pumping was insensitive to 1 mM ALN and under our conditions no bafilomycin-insensitive transport was observed. These data suggest that although a bafilomycin sensitive vacuolar H^+ pump is present in these cells, it does not appear to mediate most of the Na^+-independent acid extrusion, reflected in pH_i changes, in the whole osteoclasts seeded on bone.

The distribution of Na^+-independent H^+ extrusion rates in cells attached to bone versus glass in shown in Fig. 5. H^+ extrusion was detectable in most osteoclasts on bone and was absent or significantly lower in cells on glass. For reasons that are not known, the range of pH_i recovery rates was large (from 0 to 1 pH U/min).

Fig. 6 shows that osteoclasts on bone had a significant NH_4^+ influx pathway that showed a positive correlation with the presence and the rate of Na^+-independent H^+ extrusion ($r = 0.80$), but the nature of this pathway remains to be elucidated. Due to similar hydrated ionic radii (37), NH_4^+ and K^+ could share similar transmembrane pathways; however, the observed influx was not significantly modified by modulators of K^+ fluxes, including K^+ channel blockers TEA (10 mM), 4-aminopyridine (1 mM), gadolinium (20 μM), Na^+/K^+ ATPase inhibitor ouabain (1 mM), and $Na^+,K^+/2Cl^-$ transport inhibitor furosemide (1 mM). It is possible that these inhibitors might not have reached their target if it is located on the ruffled border inside the sealed lacuna.

Calcitonin effects on Na^+-independent H^+ extrusion. Osteoclasts attached to bone for 12 h were treated with salmon calcitonin (10^{-8} M) for 1 h at 37°C (Fig. 7) before loading with

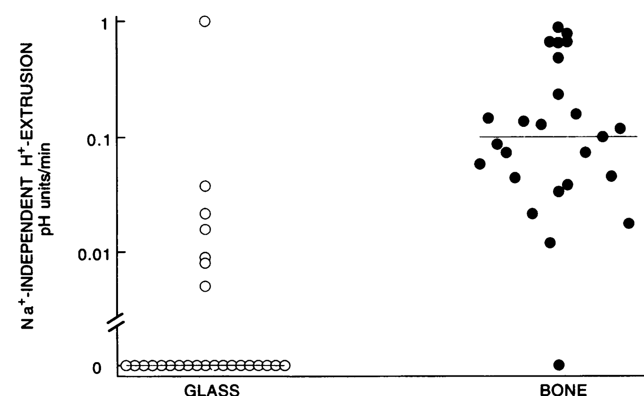

Figure 5. Distribution of Na^+-independent H^+ extrusion rates in osteoclasts attached to bone or glass. The cells were attached to their substrate in the presence of 10% FBS and after 12 h the measurements were performed under serum free conditions as described in Methods. Medians are shown as horizontal lines. Na^+-independent H^+ extrusion on glass: 0.043±0.007 pH U/min, $n = 26$. Na^+-independent H^+ extrusion on bone: 0.233±0.051 pH U/min, $n = 27$. Note the logarithmic scale.

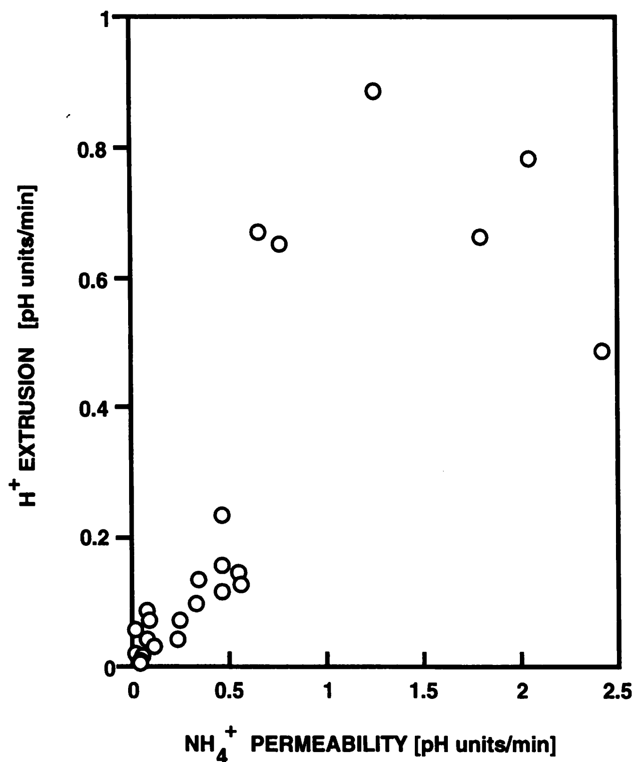

Figure 6. Correlation of NH_4^+ influx and Na^+-independent H^+ extrusion in osteoclasts attached to bone. The exact pathway for NH_4^+ influx has not yet been established (see text) but its presence correlates with that of Na^+-independent H^+ extrusion ($r = 0.80$).

BCECF. The calcitonin-treated osteoclasts did not extrude acid under Na^+-free conditions, their pH_i regulation being similar to that of osteoclasts attached to glass. With 10^{-12} M calcitonin (not shown) the inhibition was present, but not complete as with 10^{-8} M (acid extrusion rate medians: 0.001 pH U/min for 10^{-8} M and 0.03 pH U/min 10^{-12} M, $n = 4$).

Effects of ALN on Na^+-independent H^+ extrusion. When cells were attached to bone slices preincubated in ALN, containing 0.22 nmol ALN/mm² bone, no Na^+-independent H^+ extrusion was observed (Fig. 8). A time course for this inhibitory effect was conducted at 0.22 nmol ALN/mm² bone. The earliest effect was observed at 2.5 h after cell seeding, the time required for cell attachment, BCECF loading and the assembly of the measuring chamber. The ALN effects were dose dependent with a 50% inhibitory dose being around 0.022 pmol/mm² bone (Fig. 9). Etidronate (1-hydroxyethylidene-1,1--bisphosphonate), another bisphosphonate, had similar effects at 2.2 nmol/mm² bone.

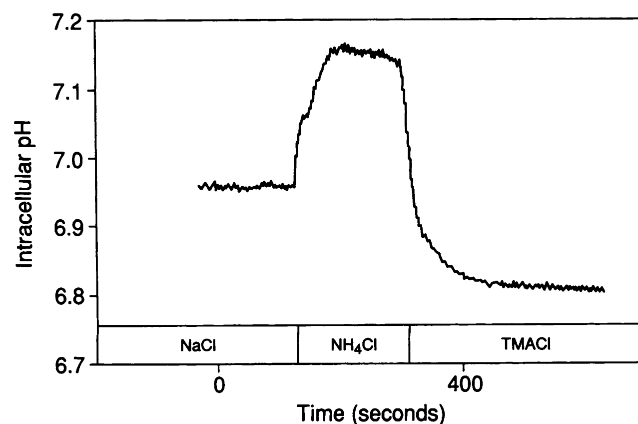

Figure 7. Calcitonin inhibits Na^+-independent H^+ extrusion in osteoclasts on bone. Osteoclasts attached to bone were incubated with salmon calcitonin (10^{-8} M) for 1 h at 37°C followed by loading with BCECF. After intracellular acidification by NH_4^+ pulse, Na^+-independent pH_i recovery was measured and found absent ($n = 4$).

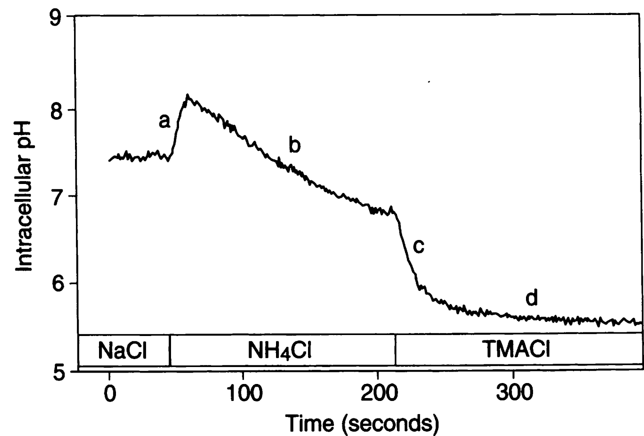

Figure 8. Presence of ALN on bone slices abolishes Na^+-independent H^+ extrusion. Osteoclasts attached for 12 h to a bone slice with prebound ALN 0.22 nmol/mm² show no Na^+-independent H^+ extrusion (0.004±0.000 pH U/min, $n = 21$). A control trace on bone without ALN is depicted in Fig. 3. This is a representative trace from experiments on 23 separate cells. The abbreviations a,b,c,d are explained in Fig. 1.

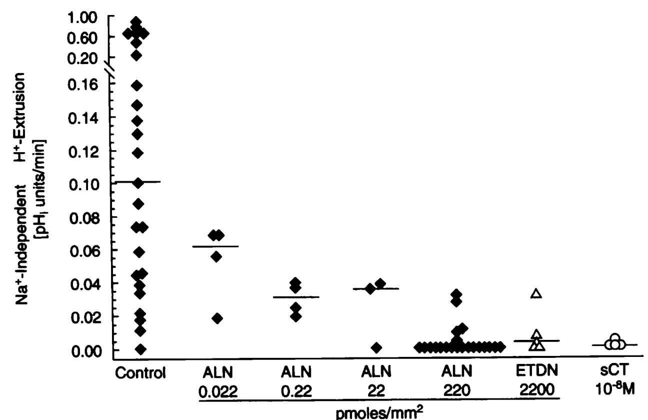

Figure 9. Alendronate, etidronate, and calcitonin effects on Na^+-independent H^+ extrusion rates on bone. Summary of individual experiments on bone is shown. Salmon calcitonin (10^{-8} M), similarly to alendronate causes inhibition of Na^+-independent H^+ extrusion on bone after 60-min incubation at 37°C followed by BCECF loading. Alendronate inhibition increases with the amount of ALN bound to bone, with 50% inhibitory effects observed ~ 0.022 pmol/mm² bone after 12 h attachment. Another bisphosphonate, etidronate displays similar effects to alendronate. The horizontal lines represent median values. Controls on bone are from Fig. 2 for reference.

Discussion

This study describes a potent Na^+-independent acid extrusion mechanism in osteoclasts, which is induced by osteoclast attachment to bone and is inactive in osteoclasts treated with salmon calcitonin or seeded on bone preincubated in bisphosphonate. Using restricted monochromatic excitation light and restricted emission we were able to visualize osteoclasts loaded with BCECF with negligible detection of bone autofluorescence. To single out the osteoclasts, the biggest cell in the field was chosen for these experiments. These cells had been seeded on bone 12 h earlier, and under many there were visible resorption pits. TRAP staining was performed on a sample of cells for confirmation of osteoclastic phenotype, especially in cells treated with ALN, which had no resorption pits. Only viable cells were tested, as estimated by their attachment, by esterase activity and by membrane integrity, evidenced by BCECF retention.

We observed that these osteoclastic cells exhibit at least two patterns of pH_i regulation. On glass, like most other cell types, they do not extrude acid in an Na^+ and HCO_3^--independent manner. However, upon attachment to bone, an Na^+-independent mechanism of acid extrusion is being activated. This H^+ extrusion is similar to that of cells that have proton pumping capability (36, 38, 39). The mechanism for acquiring the H^+-pumping ability on bone may result from osteoclast polarization, with the formation of a specialized new membrane in which the ion pathways have been inserted. Several membrane transport processes, including H^+ pumps, have been reported to be regulated by insertion/removal into the plasma membrane (40). Moreover, membrane capacitance measured by patch clamping indicates that osteoclasts have an increased plasma membrane surface on bone, consistent with the presence of a ruffled border (41).

The coactivation of a NH_4^+-permeable pathway which correlates with the appearance of the acid extruder, might reflect simultaneous activation or insertion into the ruffled membrane. This pathway probably transports a different ion in vivo. It has been suggested that NH_4^+ is translocated via K^+ pathways, but in this study it was insensitive to several K^+ pathway blockers. This could also be due to the inaccessibility of the inhibitors to their target. The NH_4^+ uptake could represent a charge compensatory pathway, but as yet we have no evidence for a causal relation between NH_4^+ uptake and the H^+ extrusion observed here.

Surprisingly, the bulk of the Na^+-independent H^+ extrusion observed in our whole cell assays was bafilomycin A_1 insensitive (Fig. 3). A bafilomycin highly sensitive H^+-ATPase clearly exists in osteoclasts (7, 9–11) and we demonstrated its presence in vesicles prepared from the osteoclasts used in this study. However, the findings reported here suggest the existence of an alternative or additional bafilomycin-insensitive H^+ extruder in whole osteoclasts that was not detectable under our conditions in isolated membrane vesicles. The reported inhibition of bone resorption by bafilomycin (42) could occur via other bafilomycin effects; for example, on vesicular traffic (43–45). Alternative H^+ pathways have also been observed in several phagocytic cells (46). Moreover, in osteoclasts, numerous bafilomycin-insensitive acidic vacuoles (47) and some alternative forms of vacuolar pumps have been reported (16, 17). The existence of bafilomycin-insensitive H^+ extrusion pathway in osteoclasts was previously reported by us (36a) and others (48).

The Na^+-independent H^+ extrusion observed here was inhibited by calcitonin (Figs. 7 and 9) which suggests that it may play a role in the bone resorption process. Moreover, bone pretreatment with a potent bone resorption inhibitor, the aminobisphosphonate ALN, produced a similar effect and reverted osteoclast behavior to that seen on glass, analogous to that of most other cell types without Na^+- independent H^+-extruders (33). This observation further supports a relationship between H^+ extrusion and bone resorption. It also provides a clear demonstration of an alendronate effect on a discrete physiological process in an isolated osteoclast. Our findings do not exclude indirect effects of bisphosphonates via osteoblasts (28), but focus on the events occurring at the osteoclast level. The molecular mechanism for the H^+ extrusion observed here, which may reflect osteoclast activation, has not been elucidated. Alendronate and calcitonin produce in osteoclasts (3–5) similar morphological changes to those caused by *src* knockout in mice where the lack of ruffled membrane is associated with compromised bone resorption (6). The ruffled membrane is probably formed by the insertion of vesicles which could contain the H^+extrusion apparatus detected here. Due to the similarity in the structure of several bisphosphonates to nucleoside phosphates, their putative target could include kinases (6), such as phosphatidyl inositol-3-kinase (49), phosphatases, small GTPases (44) such as *rho* (50), or some other molecule that may play a role in the ruffled border formation and the acid extrusion described in this paper.

In conclusion, we demonstrate that upon osteoclastic attachment to bone, osteoclasts display a strong Na^+-independent acid extrusion mechanism. This process is inhibited by calcitonin, and by pretreatment of bone with alendronate. In addition, these observations provide an experimental system for studying osteoclast activation and for identification of the molecular target(s) of bisphosphonates or other osteoclast inhibitors.

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