Proton extrusion into an extracellular resorption compartment is an essential component of bone degradation by osteoclasts. Chronic metabolic acidosis is known to induce negative calcium balance and bone loss by stimulating osteoclastic bone resorption, but the underlying mechanism is not known. The present studies were undertaken to evaluate whether chronic acidosis affects proton extrusion mechanisms in osteoclasts cultured on glass coverslips. Acidosis, mimicked experimentally by maintaining the cells at extracellular pH 6.5, rapidly lowered intracellular pH to 6.8. However, after 2 hours, a proportion of cells demonstrated the capacity to restore intracellular pH near normal levels. To define the mechanism responsible for this recovery, the activity of individual H^+ transport pathways was analyzed. We found that chronic acid treatment for up to 6 h did not significantly affect the cellular buffering power or Na^+/H^+ antiport activity. In contrast, chronic acidosis activated vacuolar H^+ pumps in the osteoclasts. Although only ~5% of the control cells displayed proton pump activity, about 40% of cells kept at extracellular pH 6.5 for 4–6 h were able to recover from the acute acid load by means of bafilomycin A1-sensitive proton extrusion. Conversely, the H^+-selective conductance recently described in the plasma membrane of osteoclasts was clearly inhibited in the cells exposed to chronic acidosis. Following acid treatment, the activation threshold of the H^+ conductance was shifted to more positive potentials, and the current density was significantly reduced. Considered together, these results suggest that induction of plasmalemmal vacuolar type ATPase activity by chronic acidosis, generated either systemically due to metabolic disease or locally at sites of inflammation, is likely to stimulate osteoclastic bone resorption and thus to promote bone loss.

Bone resorption is a multistep process involving migration of osteoclasts and/or osteoclast precursors to the bone surface, attachment to the bone matrix, and subsequent degradation of the underlying bone mineral by local acidification of the osteoclast-bone interface. When resorbing bone, osteoclasts display a specialized attachment zone, called the clear zone, which delimits a sealed compartment characterized by the presence of an extensive ruffled cell membrane. Demineralization of the bone matrix requires acidification of this extracellular compartment. Two lines of evidence suggest that the primary cellular mechanism responsible for this acidification is a vacuolar type H^+-ATPase (V-ATPase) localized to the ruffled border of these cells. First, immunohistochemical studies demonstrated a marked accumulation of V-ATPases on the ruffled membrane of osteoclasts adherent to bone (2, 3). Second, the bone-resorbing capacity of osteoclasts is effectively inhibited by the specific V-ATPase inhibitor bafilomycin A1 (4, 5). Considered together, these observations indicate a central role for the plasmalemmal V-ATPase in osteoclastic bone resorption.

Several systemic factors have been shown to influence bone resorption (for review, see Refs. 6 and 7). Among these, parathyroid hormone and 1,25-dihydroxyvitamin D3 are known to stimulate bone resorption indirectly by inducing osteoclasts to release osteoclast-activating factors into the local milieu (8, 9). It has also been reported, however, that a decrease in environmental pH can directly increase the resorptive activity of osteoclasts in vitro (10–16). The precise mechanism by which experimental or pathophysiological acidosis exerts this effect is incompletely understood. Extracellular acidification appears to increase podosome formation, thereby promoting osteoclast-matrix attachment (17). In addition, incubation of cells in acidic medium has been shown to increase carbonic anhydrase type II gene expression (18). Extra cellular carbonic anhydrase could facilitate the supply of H^+ to the V-ATPase by promoting the hydration of CO_2 generating carbonic acid. Finally, low extracellular pH could favor direct proton leakage into the resorption lacunae or alternatively could increase the availability of intracellular protons ultimately destined for V-ATPase-mediated translocation into the extracellular resorption zone.

Since plasmalemmal V-ATPases are the ultimate effectors of acidification of the resorption lacunae, one further possible mechanism underlying the stimulatory effect of acidosis on bone resorption might be that low pH directly stimulates V-ATPase activity. In this regard, the number of V-ATPases in the apical membranes of renal epithelial cells has been reported to increase when animals are exposed to chronic acidosis (19–21). In previous studies, we evaluated the pH regulatory

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1 The abbreviations used are: V-ATPase, vacuolar type ATPase; pH, intracellular pH; BCECF, 2,7’-bis(carboxyethyl)-5(6)-carboxyfluorescein; PIPES, piperezene-N,N’-bis(2-ethanesulfonic acid); MES, 2-(N-morpholino)ethanesulfonic acid; TRAP, tartrate-resistant acid phosphatase; pF, picofarad; FCS, fetal calf serum.

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mechanisms in osteoclasts plated on glass coverslips by examining their ability to recover from a cytosolic acid load. pH recovery in these cells was almost completely insensitive to treatment with bafilomycin A1, indicating a paucity of V-ATPase activity in the plasma membrane (22). Based on this observation, we reasoned that cultured osteoclasts represent an excellent model for studying the effect of chronic acidosis on plasmalemmal V-ATPase activity. The present studies demonstrate that chronic exposure to moderately acidic extracellular pH (pH 6.5) augments plasmalemmal V-ATPase activity in osteoclasts cultured on glass coverslips. By contrast, different effects on other proton extrusion mechanisms were observed. Chronic acidosis reduced proton conductance, although it had no effect on Na+/H+ antiporter activity. Considered together, these observations suggest that acid-induced osteoclastic bone resorption may be in part related to a specific increase in V-ATPase-mediated proton extrusion into the extracellular resorption zone.

**EXPERIMENTAL PROCEDURES**

**Materials**—HEPES, PIPES, MES, nigericin, zinc chloride, ammnonium chloride, cycloheximide, actinomycin D, and RPMI 1640 medium (bicarbonate-free) were obtained from Sigma. The acetoxymethyl ester of 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein (BCECF) was purchased from Molecular Probes Inc. (Eugene, OR). Bafilomycin A1 was from Kamiya Biomedical Co. (Thousand Oaks, CA). All other reagents were of analytical grade and obtained from Sigma, Aldrich, Fisher, or BDH.

**Solutions**—The K+ medium used during single cell fluorescence experiments contained 140 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 20 mM HEPES (pH 7.4; 290 ± 5 mosm). The Na+ medium was made by iso-osmotic replacement of KCl with NaCl. For patch clamping, the pipette filling solution (CsAsp, pH 6.5) contained 100 mM MES, 77.5 mM aspartic acid, 122.5 mM CsOH, 1 mM EGTA, and 1 mM MgCl2. In the patch clamp experiments in which Zn2+ was used, EGTA was omitted from the bath solution.

**Cell Isolation, Characterization, and Acid Loading**—Rabbit osteoclasts were isolated as described previously (23). Briefly, 1-day-old New Zealand White rabbits were sacrificed by decapitation, and the long bones were removed and cleaned of muscle. Cells suspensions containing osteoclasts were obtained from the inside of the bony shafts by scraping out the trabecular bone, suspending these particles in culture medium, and releasing the cells by pipetting. Medium 199 (Life Technologies, Inc.) supplemented with 15% fetal bovine serum and antibiotics was used as the plating medium. Osteoclasts were identified visually for use during single cell fluorescence and patch-clamping studies. Identifying features were the presence of trilaminar lamellae and the presence of large multinucleated cells (24). Osteoclasts containing at least three clearly discernible nuclei were studied. At the conclusion of the experiment, this visual identification was confirmed by staining for tartrate-resistant acid phosphatase (TRAP), an established marker of osteoclasts (24). Briefly, cells were fixed with 10% neutral buffered formalin for 10 min and washed with phosphate-buffered saline. The cells were then treated with a cold 0.5% cyanate chloride solution (98.5% methanol, 1% N-methylmorpholine, and 0.5% cyanate chloride) for 6 h, rinsed with 0.2 mM sodium acetate buffer for 10 min, and subsequently stained for TRAP using Naphot AS-MX phosphate as the substrate. The cells were visualized with a Nikon microscope at ×40 magnification and photographed. The grid, which had been previously etched onto the coverslip, allowed identification of those cells used during the experiment as staining positive for TRAP.

**Culture of Osteoclasts on Bone Slices and Quantitation of Resorption Lacunae**—An aliquot of the cell suspension (100 μl, containing 50–100 multinucleated osteoclasts) was plated onto sterile, devitalized bovine bone slices (diameter, 6 mm) placed in the small wells of the cover plates of 96-well microculture plates. After incubation at 37°C for 90 min the nonattached cells were gently washed off, and the bone slices were transferred to 35-mm culture dishes of medium (α-minimum Eagle’s medium without bicarbonate, containing 25 mM PIPES, 15% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml gentamicin, adjusted to the required pH) and incubated at 37°C under air for up to 48 h. Where indicated, the pH was adjusted by addition of 0.1 N HCl. Before adding the cells, freshly prepared media were equilibrated for 20 h under humidified air at 37°C to stabilize the pH. During this initial period pH tended to increase, likely due to loss of the bicarbonate in serum. Therefore, the initial solutions were titrated to pH 7.2, 6.7, and 6.2 for eventual equilibration at pH 7.5, 7.0, and 6.5, respectively.

At the end of the incubation, TRAP-positive mononuclear and multinucleated cells were counted using light microscopy. TRAP-positive cells with two or more nuclei were classified as multinucleated. After counting the TRAP-positive cells, bone slices were sonicated in phosphate-buffered saline for 5 min to remove the cells and fixed again in 4% paraformaldehyde for 6 h at 4°C. Resorption pits were then stained using an anti-collagen type I polyclonal antibody (15). The number of resorption pits and their total area were quantitated using a Zeiss Photo microscope II and a Zeiss Zidas system.

**Single Cell Measurements of pH**—For microfluorimetric studies, osteoclasts were analyzed essentially as described earlier for macrophages (25). Osteoclasts were plated for 24 h on acid-washed glass coverslips and placed into a Leiden CoverSlip dish (Medical Systems Corp., Greenvaille, NY) and maintained at 37°C. Cells were then loaded with BCECF by incubating them with a 1 μM concentration of the parent acetoxymethyl ester for 15 min at 37°C. The cells were next washed with RPMI 1640 medium and incubated in the indicated bathing medium. Single cell fluorescence was monitored using a Nikon TMD-Diaphot microscope attached to an M Series dual wavelength imaging system (Photon Technology International) (26). Illumination was shuttered on and off for 2 and 20 s, respectively, and the photometric data were recorded at a rate of 5 points/s. Mean values for each 2-s illumination period were plotted against time.

To load the osteoclasts with acid, adherent cells were incubated in HEPES/RPMI 1640 medium containing 40 μM NH4Cl for 12 min at 37°C and then rapidly transferred to a NH4+-free Na+ or K+-containing medium. Where indicated, this technique was used sequentially to examine intracellular pH (pH) recovery under different conditions in a single cell. Calibration of the fluorescence ratio versus pH was performed using the K+/-H+ ionophore nigericin. Cells were equilibrated in K+ medium (140 mM) of varying pH in the presence of 5 μM nigericin, and calibration curves were constructed by plotting the extracellular pH (27) against the corresponding fluorescence ratio. The resulting curve was sigmoidal, with an inflection point at pH 7.0, as expected from the reported pK of BCECF. A recent report by Boyarski et al. (28) found that the conventional K+ and nigericin calibration procedure may overestimate pH, systematically, likely as a result of underestimating the intracellular K+ activity and/or due to changes in internal K+ during the course of the calibration procedure. If occurring in our experiments, this systematic error would be ≤0.2 pH units around neutral pH and even smaller at acidic pH (28). More importantly, none of the conclusions reached in this article would be affected by this imperfection of the calibration procedure.

**Patch Clamping**—The whole cell configuration of the patch clamp technique was used to record ionic currents in osteoclasts (23, 26, 29). Patch electrodes had resistances ranging from 5 to 7 megohms, and junction potentials were neutralized using the appropriate circuitry of the Axopatch-1D amplifier (Axon Instruments, CA). Successful pipette-to-cell attachments resulted in seal resistances varying from 10 to 50 gigaohms. Access resistance was monitored after break-in to establish stability of the patch. Under the conditions used, input resistance in the whole cell mode was found to range from ~5 to 50 gigaohms at normal pH. Bath perfusion with the appropriate solutions was initiated only after successful establishment of a patch. The capacitance of the cells was found to range from 35 to 80 pF. Prior to data collection, the capacitance was compensated using the circuitry built into the Axopatch-1D amplifier. Currents in response to voltage steps were filtered at 100 Hz with a four-pole Bessel filter and digitized on line at 3-msec intervals using pClamp Clampex software (Axon Instruments). Data analysis was carried out using pClamp Clampfit and Clampan (Axon Instruments) and Sigma Plot (Jandel Scientific) software. To facilitate comparison between experiments, current traces were normalized using the capacitance determined for the corresponding cell. Leak current was determined by stepping the voltage from the holding potential of −60 to −90 mV in two 15-mV steps. When present, a significant linear leakage was subtracted from the current traces prior to analysis. All patch-clamping experiments were carried out at room temperature (22°C).

**Other Methods**—Bone resorption and osteoclast morphology were assessed as described (15, 18). Data are presented as representative traces of at least three similar experiments with cells from different animals or as means ± S.E. of the number of experiments indicated. Significance was assessed using Student’s t test for independent samples.
Acid Induction of H\(^{+}\) Pump in Osteoclasts

### RESULTS

**Effect of Extracellular Acidification on Bone Resorption and Osteoclast Morphology**—As mentioned in the Introduction, a decrease in extracellular pH (pHo) can increase the resorptive activity of osteoclasts in vitro (10–14, 16). The mechanism underlying this effect is not known, but changes in pHi are likely involved. The purpose of the present study was to analyze pHi homeostasis in osteoclasts incubated in acidic media. We first ensured that, under our experimental conditions, the reported stimulation of bone resorption would indeed occur. The results of these experiments are summarized in Table I. When measured at 24 h, cells incubated at pH 7.0 and 6.5 resorbed bone approximately six times more efficiently than did cells at pH 7.5. By 48 h, resorption was still 4–5-fold greater (not shown).

Morphological alterations were associated with the functional changes detailed above (Table I). As described earlier (22), rabbit osteoclasts cultured on plastic or glass at physiological pH display a variety of phenotypes; a majority of the cells show a “flat” morphology, with well-developed lamellipodia. Migrating osteoclasts displaying the “spread” phenotype can also be observed. Finally, “rounded and compact” cells constitute 8–9% of the total osteoclast population under control conditions (Table I; see Refs. 15 and 18 for technical details). Following incubation for 24 h in acidic medium (pH 7.0), the fraction of compact cells increased to 24.5% at the expense of flat and spread cells, which were observed less frequently. At pH 6.5, 30–40% of the cells assumed the compact morphology after 4 h (not shown).

**Effect of Extracellular Acidification on Cytoplasmic pH Regulation**—Earlier studies indicated that proton extrusion by osteoclasts cultured on glass coverslips was predominantly mediated by the Na\(^+/\)H\(^{+}\) exchanger as well as a proton conductive pathway. By contrast, the plasma membrane V-ATPase played a relatively minor role (22). To study the effect of extracellular acidosis on proton extrusion mechanisms, osteoclasts plated on glass coverslips were cultured in HEPES-buffered and nominally bicarbonate-free medium at pH 7.5, 7.0, or 6.5. Cytoplasmic pH was then determined individually at various time points in ~15–20 BCECF-loaded cells observed on each coverslip. The effect of reduced extracellular pH on pHi measured after 5 h, is summarized in Table I (last column). The cytoplasmic pH was similar at pHi 7.5 and 7.0 and only slightly more acidic in cells incubated at pH 6.5.

Transient pHi changes and secondary adjustments may have occurred by 5 h. To evaluate this possibility, a more detailed analysis of the time course of pHi changes was performed (Fig. 1). In these and subsequent experiments only pH 6.5 and 7.5 were compared, to accentuate any differential behavior, thereby facilitating its detection. When the extracellular pH was lowered to 6.5, pHi gradually decreased to a nadir pH of ~6.8 2 h after the initiation of incubation. Subsequent to this time point, a partial recovery was observed when the population was considered as a whole (Fig. 1A). However, the response was heterogeneous (Fig. 1B). In a significant proportion of cells, a sizable recovery phase was observed. By 4 h, the cytoplasmic pH of these cells had recovered to near physiological range and stayed at this level for a further 6 h. Other cells remained acidic for the duration of the experiment.

**Effect of Chronic Acid Treatment on Na\(^+/\)H\(^{+}\) Antiporter Activity**—In many cell systems, chronic acidosis has been shown to increase Na\(^+/\)H\(^{+}\) antiporter activity (30–32). To examine the effect of chronic acidosis on Na\(^+/\)H\(^{+}\) exchange in osteoclasts, cells were exposed to media of pH 7.5 or 6.5 for 4 h and then studied for their ability to recover from an acute acid load induced by the ammonium “prepulse” technique (Fig. 2). Conditions were chosen to favor detection of Na\(^+/\)H\(^{+}\) antiporter activity while minimizing the contribution of other pH regulatory systems by using a medium nominally devoid of bicarbonate to obviate anion exchange and by adding bafilomycin A1 to inhibit

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### Table I

| pHo  | Resorption\(^a\) | Compact cells\(^b\) | pHi\(^c\) |
|------|-----------------|-------------------|---------|
| 6.5  | 1.2 ± 0.22      | ND                | 6.99 ± 0.05 |
| 7.0  | 1.2 ± 0.22      | 24.5 ± 6         | 7.17 ± 0.12 |
| 7.5  | 0.2 ± 0.04      | 8 ± 2             | 7.18 ± 0.02 |

\(^a\) Area of lacunae per TRAP-positive cell. Data (mean ± SE) are from five bone slices, each with 50–100 cells.

\(^b\) Percentage of TRAP-positive cells/dish. Data are means ± SE from 10 dishes. ND, not determined.

\(^c\) Cells were incubated in RPMI 1640 medium containing 2% fetal calf serum at the indicated pHo. Cytoplasmic pH was monitored in individual cells using BCECF, as described under “Experimental Procedures.” Data are means ± SE of 20 cells from four separate experiments.

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**FIG. 1.** pHi of osteoclasts incubated in physiological or acidic medium. A, osteoclasts were incubated in bicarbonate-free RPMI 1640 culture medium containing 2% FCS at either pH 7.5 (●) or 6.5 in the absence (○) or presence of 200 nM bafilomycin A1 (Baf. A1, □) at 37 °C in an air incubator for the periods specified. Cytoplasmic pH was monitored in individual cells using BCECF. Data are means ± S.E. (bars) of 14–20 cells from four separate experiments. B, scatter diagram showing the pH values of the individual cells recorded in A, measured at 5 h. The horizontal lines indicate the means.
the V-ATPase (33). The cells were acid-loaded by withdrawal of ammonium, and the subsequent recovery phase was studied in Na+-containing medium. By maintaining the cells in Na+-medium the membrane potential remained at normal resting values, thereby minimizing the contribution of the voltage-sensitive H+ conductance (22). As shown in Fig. 2A, the rate of antiport-mediated pH recovery did not differ between cells preincubated for 4 h at pH 6.5 compared with cells maintained at pH 7.5. Amiloride largely inhibited this response in cells preincubated at pH 7.5 (Figs. 2A and 4), confirming that it was mediated by the Na+/H+ antiporter. Furthermore, the extent of antiport-mediated pH recovery was comparable in control cells and cells exposed to chronic acid treatment (Fig. 2B; pH 7.5 ± 0.05 for controls; n = 16; versus 7.23 ± 0.04 for acid-treated cells; n = 19). We measured independently the buffering capacity of cells preincubated at pH 7.5 and 6.5 over a wide range of intracellular pH values, using weak electrolyte pulses (34). Buffering power increased as pH decreased, as reported for other cells (34); at pH 7.0 buffering power averaged 34 mEq/mg, whereas at pH 6.5 it reached 68.5 mEq/mg. The appropriate values were used to calculate the net H+ (equivalent) efflux induced by Na+ as a function of pH.2 For comparable pH levels, similar buffering capacities were calculated for cells incubated at pH 7.5 or 6.5. As shown in Fig. 2C, the pH dependence of the antiporter was not noticeably affected by preincubation in acid medium. Thus, neither the turnover number nor the set point of the antiporter was significantly altered by prolonged acidification.

Effect of Chronic Acid Treatment on V-ATPase Activity—
Since increased Na+/H+ exchange activity was not found to contribute to the restoration of cytosolic pH during chronic acidification, we examined whether enhanced V-ATPase activity might be responsible. When cells were exposed to pH 6.5 in the presence of 200 mM bafilomycin A1, a more rapid and profound acidification of the cytosol occurred, compared with cells in acidic medium without the inhibitor (Fig. 1). Moreover, bafilomycin A1 precluded the ability of cells cultured at pH 6.5 to restore their cytoplasmic pH following the initial acidification phase. This finding suggests that the recovery of cytoplasmic pH seen in a proportion of cells is mediated by increased V-ATPase activity.

To examine this possibility in further detail, V-ATPase activity in control or acid-treated cells was assessed by measuring pH recovery from an acid load in cells suspended in K+-rich, Na+-free medium containing 100 μM Zn2+. Na+ was omitted to eliminate antiport activity, whereas Zn2+ was used to block the voltage-sensitive proton conductive pathway (35). Unlike cells incubated at pH 7.5, which remained acidic for extended periods following the ammonium prepulse, cells cultured at pH 6.5 for 4–6 h were frequently able to recover from the acute acid load (Fig. 3A). This response was not homogeneous; only a fraction of the cells displayed an alkalization. This finding is reminiscent of the functional and morphological heterogeneity reported earlier for cultured osteoclasts (36–39). To evaluate the percentage of cells responding to the chronic acid treatment, we studied the ability of larger numbers of cells to recover from an acid load in Na+-free, K+-medium containing Zn2+, conditions favoring V-ATPase-mediated recovery (Fig.
In previous studies, we showed that proton efflux mediated by a percentage of responsive cells increased to 37°C in the presence of 200 nM bafilomycin A1 at pH 7.5. The cell's mean pH reached 6.09 ± 0.06. Following preincubation at pH 6.5, the percentage of responsive cells increased to −40%; in 30 determinations the mean pH reached by these cells after 10 min was 6.55 ± 0.09. At pH 7.0 the fraction of responsive cells averaged −15% (not shown).

To confirm that this recovery was mediated by V-ATPase activity, the osteoclasts were exposed to bafilomycin A1 (200 nM) immediately prior to the acid load. Cells exposed to pH 6.5 for 4 h failed to recover from the acute acidification when bafilomycin A1 was present (Fig. 3C). Furthermore, when cells chronically exposed to low pH were acutely acid-loaded for a second time after recovery from an initial acid load, the presence of bafilomycin A1 completely prevented subsequent recovery (data not shown). None of the cells tested were able to recover from the ammonium prepulse in the presence of bafilomycin A1 (Fig. 3D). Considered together, these findings suggest that incubation of osteoclasts at reduced pH levels induces the functional expression of plasmalemmal V-ATPases in osteoclasts.

**Effect of Chronic Acid Treatment on the H⁺ Conducance**—In previous studies, we showed that proton efflux mediated by a proton-conductive pathway was an important pH regulatory mechanism in cultured osteoclasts (22). Vast amounts of H⁺ (equivalents) can be transported through the conductance, which can be measured either as pH changes or electrophysiologically. As illustrated in Fig. 4, the conductive pathway is active in cells chronically incubated in acidic medium, as indicated by the rapid pH recovery following an acid load in K⁺ medium with bafilomycin A1, added to prevent pumping. That recovery is mediated by the conductance is suggested by the blocking effect of micromolar Zn²⁺, a hallmark of this pathway (22, 35). Under conditions designed to reveal the activity of the conductance, all the cells tested recovered in the absence of Zn²⁺ (final pH_i, 7.06 ± 0.03; n = 19), yet none did in the presence of the cation (final pH_i, 5.99 ± 0.03; n = 18) (Fig. 4B). The recovery was also absent when the membrane potential was normal (negative inside), i.e., in cells suspended in Na⁺ medium with amiloride, added to prevent Na⁺/H⁺ exchange (Fig. 4A, triangles). Depolarization is required for activation of the conductance, which shows sharp outward rectification (see below and also Ref. 22).

The presence of an active conductance was confirmed electrophysiologically, patch-clamping the cells in the whole-cell configuration (Fig. 5). The ionic conditions were selected to minimize interference by other channels (see Refs. 22 and 25 for details). In keeping with an earlier report (22), control cells displayed a slowly activated current with marked outward rectification (Fig. 5A). The reversal potential of the tail currents approximated the H⁺ reversal potential (calculated from the transmembrane ΔpH; not shown), confirming the identity of the charge-carrying species. The conductance was also present in cells preincubated chronically in acidic media (Fig. 5B).

The outward rectification and selectivity of the current remained unchanged, as determined by tail current analysis. Interestingly, however, other features of the conductance were altered. First, as shown in Fig. 5C, the current density, measured at +75 mV and normalized per capacitance, was lower after chronic acidosis (controls, 8.8 ± 0.79 pA/pF; n = 13; versus acid-treated, 5.2 ± 0.73 pA/pF; n = 17). It is noteworthy that all the measurements were carried out under identical conditions, i.e., the same pH_i and pipette perfusion medium were used during the assay, independently of the preincubation conditions. Second, the activation threshold of the current was shifted to more positive potentials following prolonged acidification (Fig. 5D). In control cells, current activation was first
detectable at $-30 \text{ mV}$, and the majority became activated at or below $-15 \text{ mV}$. By contrast, most acid-loaded cells were activated only at or above $0 \text{ mV}$. It is important to note that the current was comparably inhibited by $\text{Zn}^{2+}$ in both control and acid-pretreated cells, implying that the $H^+$-selective current was being measured in all instances. Therefore, the magnitude of the proton current and its voltage sensitivity were reduced in cells exposed to low pH for a prolonged duration.

**DISCUSSION**

Chronic metabolic acidosis is associated with an increased plasma calcium concentration (40), due to increased release of skeletal calcium. Accordingly, there is considerable experimental evidence indicating that the resorptive activity of osteoclasts cultured on bone or present in organ cultures of bone increases when incubated in acidic media (10, 12–16). Nevertheless, the exact mechanism whereby chronic acidosis promotes resorption remains controversial. Direct activation of osteoclasts has been proposed (10), but indirect effects on osteoclasts (11) or on the bone matrix itself have also been invoked (41, 42).

In this report, we show that the cytosolic pH of osteoclasts changes at least transiently during acidic incubation and, more importantly, that chronic acidosis exerts significant regulatory effects on at least two $H^+$ transport systems. In our experiments, osteoclasts subjected to low pH$_o$ underwent a sizable cytoplasmic acidification, which was maximal around 2 h and partially recovered thereafter (Fig. 1). The maximal ApH recorded ($-0.4 \text{ units}$) is greater than that reported by Carano et al. (14), $\pm 0.1 \text{ pH unit}$, but the sampling time used by these investigators was not specified. It is possible that their determinations were made before or after the nadir of pH$_o$ was reached. The relevance of cytosolic acidification is stressed by the observation that, at constant pH$_o$, reduction of pH$_i$ by addition of butyrate promoted the expression of cell matrix attachment structures (podosome formation) (17).

In renal tubule cells, the activity of the Na$^+$/H$^+$ antiporter has been found to increase following chronic acidosis (30, 31). Similar results have been reported in lymphocytes (32), which are likely to express the same isoform as osteoclasts, namely the nonepithelial housekeeping isofrom NHE-1 (Na/H exchanger-1). It was therefore of interest to determine whether a comparable stimulation occurred in osteoclasts. We found that, although the antiporter could be shown to play an important role in pH$_o$ maintenance, there were no alterations in its activity following chronic acidosis.

In contrast, V-ATPase-mediated $H^+$ pumping was markedly increased by incubating the cells in acidic buffers. Bafilomycin $A_2$-sensitive pumping, which is negligible in cells cultured at normal pH, became apparent in $\sim 40\%$ of the cells following prolonged acidosis. The source of this heterogeneity is presently not clear. Other functional responses, as well as the morphology of the cells, are known to be quite heterogeneous in cultured osteoclasts and may reflect variable stages of differentiation at the time of isolation. On the other hand, it is noteworthy that the morphology and functional responsiveness of the cells may not be stationary, and that short term observations may reveal different stages of a temporal cycle. Indeed, cells plated on bone are known to cycle between flat (inactive), motile, and compact (resorptive) stages (43). It is therefore conceivable that only one form of the cells (e.g. the compact phenotype) is capable of pumping, and that external acidification alters the time of residence in individual stages of this cycle. Consistent with this notion, the increased pumping activity was associated with a greater fraction of compact cells (Table I).

Although the precise mechanism leading to enhanced pumping remains to be defined, several possibilities can be contemplated. First, V-ATPases could be synthesized de novo. This appears unlikely, in that neither cycloheximide nor actinomycin D prevented the stimulatory effect of chronic acidosis (not shown). Alternatively, latent plasmalemmal pumps could have been activated. The activity of V-ATPases is known to be regulated via oxidation of critical sulfhydryl residues (44, 45) and by association with adaptins (46). It is at present not known whether acidification uses these pathways or can independently control pump activity. Finally, it is possible that endomembrane pumps are translocated and inserted exocytically into the plasmalemma. An analogous process is believed to mediate the initiation of pumping in bladder and renal epithelia (19, 21). Two observations would seem to favor this alternative. First, in a related cell type, namely macrophages, cytosolic acidification promotes the subcellular redistribution of lysosomal organelles, which are known to bear V-ATPases (47). Second, although systematic measurements were not performed, our preliminary observations suggest that the capacitance of cells subjected to acidic treatment is on average higher than that of control cells. This is suggestive of an increased surface membrane area, possibly as a result of endomembrane fusion. These speculations must be tempered by the considerable heterogeneity in the size of osteoclasts, which makes statistically meaningful comparisons rather difficult.

Prolonged acidification also resulted in an inhibition of the proton conductance. Because fluxes through this pathway are passive, driven solely by the proton motive force, external acidification would tend to reduce $H^+$ extrusion and might even promote $H^+$ influx (the stringency of the rectification properties of the conductance is a subject of controversy; cf. Refs. 48 and 49). It would therefore be advantageous for the cells to reduce such potentially deleterious acid accumulation. This may explain the susceptibility of the conductive pathway to inhibition by external acid. It must be stressed that the reduction in the $H^+$ current was not due to differing driving forces, since the assay conditions were identical for control and acid-treated cells. This implies that chronic acidosis produced a slowly reversible or possibly irreversible alteration in the properties of the conductive pathway. It is noteworthy that the conductance is active only when the membrane is depolarized and/or when the cytosol becomes acidic (22). The membrane potential and pH$_i$ have not been systematically monitored during the functional cycle of osteoclasts, but conditions that activate the conductance may occur, for instance, when the NADPH oxidase is operating (see Ref. 49 for details) or when cells are otherwise metabolizing very actively.

In summary, our studies revealed a dual modification in the functional properties of acid-treated osteoclasts, accompanied by ostensible changes in cell morphology. The reduction in $H^+$ conductance could represent a defensive mechanism, designed to preclude acid accumulation in the cytosol. Concomitantly, the ability of the cells to extrude $H^+$ via the V-ATPase was found to increase. Such an increase may account, at least in part, for the reported enhancement in bone resorption during metabolic acidosis. As a working hypothesis, we propose that increased pumping results from insertion into the plasmalemma of V-ATPases otherwise resident in endomembrane vesicles. Insertion and retrieval of such vesicles may be a cyclical

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3 Arnett et al. (16) also noted that, at any given pH$_o$, the rate of resorption was greater in the presence than in the absence of bicarbonate. Thus, despite the fact that our experiments were performed in the nominal absence of bicarbonate, to simplify the experimental design and the interpretation, the importance of this anion should not be overlooked.
process, and the relative duration of its phases may be controlled by the extracellular and/or intracellular pH.

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