Role of Na-H Exchangers and Vacuolar H+ Pumps in Intracellular pH Regulation in Neonatal Rat Osteoclasts

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ABSTRACT Osteoclasts resorb bone by pumping of H+ into a compartment between the cell and the bone surface. Intracellular pH (pHi) homeostasis requires that this acid extrusion, mediated by a vacuolar-type H+ ATPase, be complemented by other acid-base transporters. We investigated acid-extrusion mechanisms of single, freshly isolated, neonatal rat osteoclasts. Cells adherent to glass coverslips were studied in the nominal absence of CO2/HCO3−, using the pH-sensitive dye BCECF and a digital imaging system. Initial pHi averaged 7.31 and was uniform throughout individual cells. Intrinsic buffering power (βi) decreased curvilinearly from ~25 mM at pHi = 6.4 to ~6.0 mM at pHi = 7.4. In all polygonally shaped osteoclasts, and ~60% of round osteoclasts (~20% of total), pHi recovery from acid loads was mediated exclusively by Na-H exchange. In these pattern-1 cells, pHi recovery was 95% complete within 200 s, and was blocked by removing Na+, or by applying 1 mM amiloride, 50 μM ethylisopropyramidolamide (EIPA), or 50 μM hexamethylenemalamide (HMA). The apparent K1/2 for HMA ([Na+]o = 150 mM) was 49 nM, and the apparent K1/2 for Na+ was 45 mM. Na-H exchange, corrected for amiloride-insensitive fluxes, was half maximal at pHi 6.73, with an apparent Hill coefficient for intracellular H+ of 2.9. Maximal Na-H exchange averaged 741 μM/s. In the remaining ~40% of round osteoclasts (pattern-2 cells), pHi recovery from acid loads was brisk even in the absence of Na+ or presence of amiloride. This Na+-independent pHi recovery was blocked by 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl), a vacuolar-type H+ pump inhibitor. Corrected for NBD-Cl insensitive fluxes, H+ pump fluxes decreased approximately linearly from 96 at pHi 6.8 to 11 μM/s at pHi 7.45. In ~45% of pattern-2 cells, Na+ readdition elicited a further pHi recovery, suggesting that H+ pumps and Na-H exchangers can exist.
simultaneously. We conclude that, under the conditions of our study, most neonatal rat osteoclasts express Na-H exchangers that are probably of the ubiquitous basolateral subtype. Some cells express vacuolar-type H\(^+\) pumps in their plasma membrane, as do active osteoclasts in situ.

INTRODUCTION

Physiological bone resorption is required for the development and growth of the skeleton, for its remodeling throughout life, and for supplying free Ca\(^{2+}\) to the systemic fluids. This process, which involves the degradation of a mineralized extracellular matrix, is performed by the osteoclast, a large, multinucleated cell derived from the hematopoietic bone marrow. To perform its function, the osteoclast attaches firmly to the bone surface, forming a specialized circumferential region, the “sealing zone,” where actin and other cytoskeletal proteins accumulate in the cytoplasm and integrin receptors are present in the plasma membrane. This zone of attachment surrounds a highly folded region of the plasma membrane, termed the “ruffled border,” which directly faces the bone surface. The osteoclast actively acidifies and secretes enzymes into the bone-resorbing compartment, which is surrounded by the sealing zone and lies between the ruffled-border membrane and the bone surface. This leads to the formation of a resorption cavity (Baron, Neff, Louvard, and Courtoy, 1985; Baron, 1989). Carbonic anhydrase is highly expressed in the osteoclast cytosol (Gay, Ito, and Schraer, 1983), and presumably plays a key role in generating H\(^+\) from CO\(_2\). The protons thus formed are translocated across the plasma membrane at the ruffled border, which is very highly enriched in vacuolar-type H\(^+\) pumps (Blair, Teitelbaum, Ghiselli, and Gluck, 1989; Vaananen, Karhukorpi, Sundquist, Wallmark, Roininen, Hentunen, Tuukkanen, and Lakkakorpi, 1990; Chatterjee, Chakraborty, Leit, Neff, Jamsa-Kellokumpu, Fuchs, and Baron, 1992). Hence, although the osteoclast is not part of an epithelium, it is a polarized cell. It has an apical pole, the ruffled border, where secretion and H\(^+\) transport occur, and a basolateral domain, where high numbers of Na-K pumps are present (Baron, Neff, Roy, Boisvert, and Kaplan, 1986).

Regulation of the intracellular pH (pHi) may be expected to be of critical importance for resorbing osteoclasts. If unopposed, the extrusion of large amounts of acid would cause pHi to rise, with detrimental effects for many cellular processes. Therefore, osteoclasts may be expected to possess various acid-base transporters that operate simultaneously with the proton pumps, and thereby keep pHi within a favorable range. Consistent with this view are the observations that blockade of Na-H exchange or Cl-HCO\(_3\) exchange impairs the osteoclast's ability to form resorption pits in bone slices (Hall and Chambers, 1989, 1990; Baron, Bartkiewicz, Chakraborty, Chatterjee, Fabricant, Hernando, Horne, Lomri, Neff, Ravesloot, and Su, 1992). Direct measurements of pHi confirmed the presence of anion exchangers in avian osteoclasts (Teti, Blair, Teitelbaum, Kahn, Koziol, Konsek, Zambonin-Zallone, and Schlesinger, 1989).

Despite their importance for bone resorption, not much is known about the roles played in pHi regulation by Na-H exchangers or other transporters that defend avian or mammalian osteoclasts against acid loads. The main focus of the present study was
to identify the mechanisms by which osteoclasts, freshly isolated from neonatal rats and attached to glass coverslips, defend themselves against acute cytoplasmic acid loads. By conducting the experiments in the nominal absence of CO$_2$/HCO$_3^-$, we minimized the contribution of HCO$_3^-$-dependent acid-base transporters to pHi regulation. Because osteoclasts cannot be isolated from endosteal bone surfaces in sufficient amounts and with sufficient purity to allow population studies, we used the digital imaging of the fluorescent pH-sensitive dye BCECF to monitor pHi in single cells. We found that most if not all osteoclasts express Na-H exchange activity that is highly sensitive to inhibition by hexamethylenemiloridime. This suggests that the exchangers may be of the ubiquitous, basolateral subtype. In addition, a distinct subpopulation of osteoclasts possess an Na$^+$-independent, amiloride-insensitive acid-extrusion mechanism that is blocked by 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl), an inhibitor of vacuolar-type proton pumps. Finally, we found that intrinsic intracellular buffering power is a curvilinear function of pHi, decreasing with increasing pHi values.

Portions of this work have been presented elsewhere in abstract form (Eisen, Su, Baron, and Boron, 1991; Ravesloot, Boron, and Baron, 1992).

MATERIALS AND METHODS

Cell Isolation

Osteoclasts were isolated from the long bones of 1–5-d old neonatal Wistar rats essentially as described by Boyde, Ali, and Jones, (1984) and Chambers, Reveli, Fuller, and Athanasou, (1984). Tibiae, femora and humeri were removed and placed in culture medium, which consisted of the alpha modification of minimal essential medium (α-MEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal-calf serum (Sigma Chemical Co.) and antibiotics (penicillin and streptomycin, Sigma Chemical Co.). After shredding the bones, we dispersed the cells by passing the bone fragments gently through a Pasteur pipette. The resulting cell suspension, usually 4 ml, was plated on glass coverslips. Before the cells were plated on them, the coverslips were exposed to serum for 40 rain or, in a few experiments, coated with typed collagen (Sigma Chemical Co.) to improve adherence of the cells. Cells were allowed to sediment for 40–60 min in a 5% CO$_2$ incubator at 37°C. Finally, the coverslips were washed with α-MEM to remove nonadherent cells, and incubated in culture medium until use (with a maximum delay of 10 h).

Solutions

Table I lists the composition of the solutions used. In some experiments, flame photometry was used to confirm the Na$^+$ concentration of the solutions. Using a vapor-pressure osmometer (Wescor Inc., Logan, UT), we verified that the osmolality of the solutions was between 290 and 310 mOsm/kg H$_2$O. 2’,7’-bis-2-carboxyethyl)-5(and-6)carboxyfluorescein/tetraacetoxymethyl ester (BCECF-AM, Molecular Probes, Inc., Eugene, OR) was prepared as a 10-mM stock solution in dimethylsulfoxide (DMSO, Sigma Chemical Co.). Nigericin (Sigma Chemical Co.) was prepared as a 10-mM stock solution in ethanol. The amiloride analogues ethylisopropylamiloride (EIPA), and hexamethylenemiloridime (HMA) were purchased from E. J. Cragoe, Jr. (Nacogdoches, TX) and were prepared as a 50-mM stock solution in DMSO. 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl, Sigma Chemical Co.) was prepared as a 100-mM stock in DMSO before each experiment.
Fluorescence Experiments

Cells were loaded with the acetoxymethyl ester of the pH sensitive dye BCECF (BCECF-AM). To this end the coverslips were placed in a HEPES-buffered solution (solution 1, Table I) containing 10–15 μM of BCECF-AM at 37°C for 20–40 min in an air-gassed incubator. The coverslip was then placed and secured in a chamber on the stage of a Zeiss IM35 inverted microscope equipped with differential interference contrast (DIC) microscopy optics and apparatus for epillumination. The chamber was continuously superfused with prewarmed solutions to give a bath temperature of 37°C. This temperature was monitored continuously by a thermistor. The chamber had two inlets connected to the tubing of two separate solution delivery systems. One system was used exclusively for the delivery of the nigericin containing high-K⁺ solutions (solution 3), the second one for all other solutions. Using DIC optics, osteoclasts were easily identified among the many mononuclear bone marrow cells by their shape, size and many nuclei. Routinely, two DIC images of the osteoclast were stored, one before and one after the experiment. The entire microscopic field, including the osteoclast selected for fluorescence experiments, was alternately excited with light of wavelengths of 490 and 440 nm and the light emitted by the dye of wavelength of 530 nm was imaged for both exciting wavelengths (1490 and 1440). The light source in the fluorescence experiments was a 100-W Tungsten halogen lamp. The arrangement of dichroic mirrors, filters and computer-controlled shutters, used to generate the 490- and 440-nm light, is described elsewhere (Boyarsky, Ganz, Sterzel, and Boron, 1988). To limit both photobleaching of the dye and

| Solute | Solution 1 | Solution 2 | Solution 3 | Solution 4 | Solution 5 | Solution 6 |
|--------|------------|------------|------------|------------|------------|------------|
| Na⁺    | 142.8      | 122.8      | 0          | 15.5       | 0          | 0          |
| NH₄⁺   | 0          | 20.0       | 0          | 0          | 0          | 40.0       |
| NMDG⁺  | 0          | 0          | 32.8       | 129.5      | 145        | 102.8      |
| K⁺     | 5.0        | 5.0        | 100.0      | 5.0        | 5.0        | 5.0        |
| Ca²⁺   | 2.0        | 2.0        | 2.0        | 2.0        | 2.0        | 2.0        |
| Mg²⁺   | 2.4        | 2.4        | 2.4        | 2.4        | 2.4        | 2.4        |
| mEq/l (+) | 152.2     | 152.2      | 137.2      | 154.4      | 154.4      | 152.2      |
| Cl⁻    | 132.0      | 132.0      | 123.9      | 134.2      | 134.2      | 132.0      |
| H₂PO₄⁻ | 0.4        | 0.4        | 0.8        | 0.4        | 0.4        | 0.4        |
| HPO₄²⁻ | 3.2        | 3.2        | 2.4        | 3.2        | 3.2        | 3.2        |
| HEPES⁻ | 14.2       | 14.2       | 7.7        | 14.2       | 14.2       | 14.2       |
| SO₄²⁻  | 2.4        | 2.4        | 2.4        | 2.4        | 2.4        | 2.4        |
| mEq/l (-) | 152.2     | 152.2      | 137.2      | 154.4      | 154.4      | 152.2      |
| Glucose| 10.5       | 10.5       | 10.5       | 10.5       | 10.5       | 10.5       |
| HEPES⁰ | 17.8       | 17.8       | 24.3       | 17.8       | 17.8       | 17.8       |
| Mannitol| 0          | 0          | 25.0       | 0          | 0          | 0          |
| mM (neutral) | 28.3 | 28.3 | 59.8 | 28.3 | 28.3 | 28.3 |
| pH     | 7.4        | 7.4        | 7.0        | 7.4        | 7.4        | 7.4        |

*Solutions 1 and 2 were titrated with NaOH. For solutions 3–6, the free base NMDG was titrated with HCl. All titrations were done at 37°C. The concentrations of the HEPES-free acid (HEPES⁰) and its conjugate weak base (HEPES⁻) were computed assuming a pK of 7.5. The concentrations of inorganic phosphate species were computed assuming a pK of 6.8.
photodynamic damage of the cells, illumination was limited to 450 ms for the 490-nm light, followed immediately by 450 ms for the 440-nm light. This pair of excitations was repeated at intervals varying from 5–20 s; between the excitations, the cells were in the dark. The emitted light was collected through a 63 × 1.25 NA oil-immersion objective. The light passes through a 510-nm long-pass dichroic mirror and a 550-nm long-pass filter located in the filter cube underneath the turret of the microscope. The light was then amplified by an image intensifier (KS-1381 intensifier, Video Scope International, Ltd, Washington, DC), before it was captured with a charged-coupled device camera (CCD 72, Dage M.T.I., Michigan City, IN). Each cycle of excitations had the following protocol: the shutters gating the 490-nm light were opened. After a delay of 100 ms, to allow for stabilization of the signal, we collected, digitized and averaged eight successive TV frames using an image processor (Itex 151, Imaging Technology Inc, Woburn, MA). After storage of the averaged image, the 490-nm shutters were closed, the 440-nm shutters were opened after a 166-ms delay and an averaged 440-nm image was obtained in the same way. No background subtraction was performed (see below). The averaged images were transferred in real time to a Compaq 386/20 personal computer with 300-MB storage capacity. At the conclusion of each experiment, the data were transferred to a 2.1-Gb digital tape (Emerald Systems, San Diego, CA) for later analysis. The DOS-based computer software used to control the data acquisition, as well as the Windows-based software used to perform the off-line analysis, was developed in our laboratory. For the analysis, we used a mouse to outline areas of interest, which could be the entire cell or portions thereof. Typically, we grouped together and analyzed the pixels that corresponded to the nuclei as well as the cytoplasm between or surrounded by the nuclei. The sum of the $I_{490}$ values of the pixels in the area of interest was divided by the sum of the corresponding $I_{440}$ values. The resulting ratio is strongly dependent on pH, but relatively insensitive to other factors, such as dye concentration.

**Calculation of Intracellular pH**

Conversion of the $I_{490}/I_{440}$ ratios to pH values was done according the high-K+/nigericin technique of Thomas, Buschbaum, Zimniak, and Racker (1979), as modified by Boyarsky et al. (1988). At the end of each experiment (see Fig. 6 C), the osteoclast was exposed to a high-K+ solution (solution 3), titrated to pH 7.00, to which 10 μM nigericin was added. The potential limitations of this approach are discussed elsewhere (Chaillet and Boron, 1985). The $I_{490}/I_{440}$ ratios of the entire experiment were normalized by dividing them by the $I_{490}/I_{440}$ ratio corresponding to pH 7.00. pH, was then calculated using the following equation (Boyarsky et al., 1988):

$$\frac{I_{490}/I_{440}}{(I_{490}/I_{440})_{pH=7}} = 1 + b \left[ \frac{1}{1 + 10^{(pK-pH)}} - 1 + 10^{(pK-7)} \right].$$

The values of $pK$ and $b$ were determined from experiments in which osteoclasts were exposed to a series of 2–11 10 μM nigericin-containing solutions at different pH, always including pH 7.00. An example of such an experiment is shown in Fig. 1 A. Fig. 1 B shows the values of $(I_{490}/I_{440})/(I_{490}/I_{440})_{pH=7}$ as a function of pH, obtained from a total of 75 osteoclasts. The data were fitted by the above equation, which forces the best-fit curve to pass through unity at pH = 7.00, using a nonlinear least-squares method. The best values were $pK = 7.34 \pm 0.02$ (SD) and $b = 1.80 \pm 0.02$ (SD). The background fluorescence always was < 1% of the dye signal. The average background fluorescence intensity as determined in 13 cells was 0.321 ± 0.039% (n = 40) of the $I_{490}$ signal and 0.318 ± 0.043% (n = 40) of the $I_{440}$ signal as recorded at the end of the nigericin calibration procedure. For these reasons the background fluorescence was not subtracted from the $I_{490}$ and $I_{440}$ values.
Determination of the Intrinsic Buffering, $\beta_i$

Acid-loaded osteoclasts were exposed to a series of nominally Na+-free solutions that contained 20, 10, 5, 2.5, 1, 0.5, and 0 mM total ammonium (see Fig. 7A), following the approach of Boyarsky et al. (1988). $\text{NH}_2/\text{NH}_4^+$-containing solutions were prepared by mixing solution 5 and solution 6 (Table I). With each stepwise decrease in $[\text{NH}_2/\text{NH}_4^+]_o$, the amount of protons delivered to the cytoplasm ($\Delta[\text{acid}]_c$) was considered equal to the resultant change in $[\text{NH}_4^+]_i$. If it is assumed that the $[\text{NH}_2]_c$ equals $[\text{NH}_2]_o$ and that the $\text{pK}_a$ governing the $\text{NH}_2/\text{NH}_4^+$ equilibrium (8.90 at 37°C) is the same in the cytoplasm as in the extracellular fluid, $[\text{NH}_4^+]_i$ can be calculated from the observed $\text{pHi}$. $\Delta\text{pHi}$ was taken as the change in $\text{pHi}$ produced by the stepwise decrease in $[\text{NH}_2/\text{NH}_4^+]_o$. $\beta_i$ was then calculated as $-\Delta[\text{acid}]_c/\Delta\text{pHi}$ (Roos and Boron, 1981). $\beta_i$ was assigned to the mean of the two $\text{pHi}$ values used for its calculation.

**Figure 1.** Multipoint intracellular calibration of the pH-sensitive dye BCECF present in the cytoplasm of neonatal rat osteoclasts. (A) Typical example of the changes of the $I_{490}/I_{440}$ ratio observed in an osteoclast exposed to a series of high-K+10-μM nigericin solutions (solution 3) titrated to the pH values indicated. Normalized $I_{490}/I_{440}$ ratios are obtained by dividing the raw $I_{490}/I_{440}$ ratio by the raw ratio corresponding to a pH $= 7.00$. Note that the $I_{490}/I_{440}$ ratios corresponding to pH $= 7.00$ at the beginning and the end of this 24-min experiment are virtually identical. (B) Plot of the mean value of the normalized ratios versus pH. The figure summarizes data obtained from 75 experiments similar to that shown in A; each symbol represents at least three observations. The curve through the points is the result of a nonlinear least-squares fit. From the fitted curve, the pH$_i$ values corresponding to normalized ratios of all subsequent experiments were determined. Therefore, all experiments were concluded by exposing the osteoclasts to high-K+/nigericin solution titrated at pH $= 7.00$ (see also Fig. 6 C). Vertical bars represent the SEM, and are omitted if the SEM does not exceed the symbol radius by at least 25%. The filled symbol marks the position corresponding to a normalized ratio of 1.0 at pH $= 7.00$.

**Data Analysis**

First to fourth order polynomial functions were fitted to the pH$_i$ transients using least-squares fitting methods. The rate of pH$_i$ change per unit time (dpH$_i$/dt) was calculated by differentiating the polynomial function at any selected pH$_i$. The software package used for this purpose was developed in our laboratory. Statistical analysis as well as some of the nonlinear least-squares fits (Hill equation, buffering power vs pH of monoprotic weak acid) were performed with the software package "Systat" (Systat Inc., Evanston, IL). Two sets of data were considered significantly different, if the $P$ value of the (paired) $t$ test was < 0.05. Results are given as mean ± SEM, with the number of cells ($n$) from which it was calculated, unless stated otherwise.
RESULTS

Cell Morphology

Under our conditions for isolating and culturing osteoclasts, we recognized two major types of cell morphology. The cells of the first group, which represented the majority, were well spread, and displayed irregularly shaped cytoplasmic extensions that projected from an elongated and/or polygonal cell body. A digital fluorescence image of such a cell, excited at 440 nm, is shown in Fig. 2 A. Sometimes the extension (apron) was devoid of subcellular structures that could be resolved with DIC microscopy. The nuclei of these cells tended to be arranged linearly, and the cytoplasm had a granular appearance. These cells often rearranged the shape of their extensions over the course of an experiment (15–40 min). The cells of the second group had a round shape, no cytoplasmic extensions, and the nuclei were clustered in the center (see Fig. 2 B). These cells, too, were well spread. In general, the aprons of these cells surrounded the cytoplasm completely. We used both types of osteoclasts in our experiments.

Dye Behavior

In examining individual $I_{490}$ and $I_{440}$ images of BCECF-loaded osteoclasts, we generally observed three regions with different, though uniform, fluorescence intensities. The highest fluorescence intensity was found over the nuclei. The fluorescence intensity was usually lower in the perinuclear region, which corresponded to the granular, organelle-containing part of the cytoplasm. Finally, the fluorescence intensity was lowest, and in fact often negligible, in the apron. The regional differences in intensities, however, did not reflect regional pH$_i$ differences, inasmuch as pseudo-images, constructed from the predominantly pH$_i$-sensitive $I_{490}/I_{440}$ ratios, showed no important regional variation. Similarly, pH$_i$ values averaged over the nuclei were virtually identical to those measured over the perinuclear region in all cells tested.

Distribution of Initial pH$_i$ Values

We found that the distribution of initial pH$_i$ values for osteoclasts studied in the nominal absence of CO$_2$/HCO$_3^-$ was approximately Gaussian (not shown), with a slight skewness toward alkaline pH$_i$ values. The median value, 7.27, differs only slightly from the mean initial pH$_i$ of 7.31 ± 0.01 (n = 245). A relatively small number of osteoclasts (26 or 11% of the total) had initial pH$_i$ values > 7.6, contributing to the skewness. Monitoring pH$_i$ for 15–35 min revealed a slow decline with time; the rate of the decline ranged between 0 and $-1.0 \times 10^{-4}$ pH/s, and averaged $-0.71 \pm 0.29 \times 10^{-4}$ (n = 7).

Osteoclasts Recover from Acute Acid Loads

To test for the presence of acid-extruding transporters, we acid loaded osteoclasts in the nominal absence of CO$_2$/HCO$_3^-$ by means of an NH$_4^+$ prepulse. To this end, we exposed osteoclasts to a HEPES-buffered solution in which 20 mM NaCl was replaced by 20 mM NH$_4$Cl. At a pH of 7.4, this solution contains ~0.6 mM NH$_3$. As shown in Fig. 3, the application of NH$_3$/NH$_4^+$ causes a rapid increase in pH$_i$, due to
FIGURE 2. Digital fluorescence images of freshly isolated osteoclasts. (A) A polygonally shaped cell. (B) A round cell. Intracellular BCECF was excited at 440 nm.
the influx of NH₃ and its subsequent association with H⁺ to form NH₄⁺. The slow pHᵢ decrease during the subsequent plateau phase is the result of acidifying processes, such as the entry of NH₄⁺, a fraction of which will dissociate into NH₃ and H⁺. After the extracellular NH₃/NH₄⁺ is removed, the resulting efflux of NH₃ causes dissociation of all intracellular NH₄⁺. At the end of this acidification phase, the cell is virtually free of NH₃/NH₄⁺, and the protons thus generated cause pHᵢ to fall far below the initial value. The amount of acid loaded into the cell, which is a function of the duration of the plateau-phase NH₃/NH₄⁺ exposure, determines the degree of the cytoplasmic acidification. In a total of 51 experiments with an 85-s plateau-phase, pHᵢ fell during the acidification phase by an average of 0.43 ± 0.02 below the initial pHᵢ.

In other cells, the intracellular acid load produced by an NH₃/NH₄⁺ prepulse stimulates acid-base transporters in the plasma membrane to extrude acid, thereby returning pHᵢ toward normal (see Boron, 1983). This also was the case for all the 51 osteoclasts we studied. As shown in Fig. 3, we found that the rate of pHᵢ recovery (dpHᵢ/dt) was pHᵢ dependent. It was maximal at, or close to, the lowest pHᵢ value, and decreased with increasing pHᵢ. In 15 osteoclasts, in which the cytoplasm was acidified to values between 6.70 and 6.85 (mean: 6.79 ± 0.01), the mean dpHᵢ/dt was 36 ± 4 × 10⁻⁴ pH/s at a pHᵢ of 6.85. This mean dpHᵢ/dt will be referred to as the mean pHᵢ recovery rate under "control conditions." The time necessary for pHᵢ to reach the midpoint (6.96 ± 0.02) between the lowest and final pHᵢ values was 65 ± 8 s. At this midpoint, dpHᵢ/dt had decreased to 24 ± 3 × 10⁻⁴ pH/s. The time necessary for pHᵢ to reach 95% of its final value was 190 ± 20 s. The mean final pHᵢ for all 51 osteoclasts examined was 7.19 ± 0.02, which is 0.085 lower than the average initial pHᵢ observed in this group of osteoclasts (P < 10⁻⁷).

In a few experiments, the osteoclast was submitted to a second 85-s NH₃/NH₄⁺ prepulse after it had recovered from the first. As illustrated in Fig. 3, the recovery of pHᵢ from the second acute acid load was similar to that observed for the first. On average (see Table II), neither the minimum pHᵢ value after washout of the NH₃/ NH₄⁺ prepulse, the magnitude of this acidification, the maximal dpHᵢ/dt value during the pHᵢ recovery, the dpHᵢ/dt value determined at a pHᵢ of 6.90, nor the final pHᵢ value reached after the pHᵢ recovery were significantly different between the first and second pHᵢ recoveries.

Recovery from Acid Loads, under Conditions of Reduced Extracellular [Na⁺], Follows One of Two Patterns

In the nominal absence of CO₂/HCO₃⁻, the recovery of pHᵢ from an acid load in most mammalian cells is mediated by Na-H exchange. To test whether osteoclasts express
Table II

Comparison of Paired NH₃/NH₂⁺ Pulses under Control Conditions*

| Parameter                      | First pulse | Second pulse | n  | Significance |
|--------------------------------|-------------|--------------|----|--------------|
| Minimum pHi                   | 6.78 ± 0.04 | 6.71 ± 0.05  | 9  | NS           |
| Initial vs minimum pHi        | -0.38 ± 0.03| -0.38 ± 0.04 | 9  | NS           |
| Max dpHi/dt (10⁻⁴ pH/s)       | 26 ± 6.4    | 28 ± 3.6     | 9  | NS           |
| pHbelow max dpHi/dt           | 6.82 ± 0.04 | 6.74 ± 0.07  | 9  | NS           |
| dpHi/dt at pH 6.90 (10⁻⁴ pH/s)| 17 ± 5      | 13 ± 5       | 7  | NS           |
| Final pHi                      | 7.10 ± 0.06 | 7.11 ± 0.07  | 8  | NS           |

*The experiments followed the protocol of Fig. 3. Minimum pH is the lowest pH achieved after washout of NH₃/NH₂⁺. Initial vs minimum pH is the difference between the pH value prevailing before application of the NH₃/NH₂⁺ and the minimum pH. Max dpHi/dt is the maximal rate of pH recovery, measured at pH below max dpHi/dt; this pH is slightly higher than the minimum pH. dpHi/dt at pH 6.9 is the pH recovery rate measured at pH 6.9. Final pH is value prevailing after recovery of pH from the acid load was complete. n is the number of observations. Significance was judged on the basis of paired t tests, comparing values for the first and second pulses.

Na-H exchangers, we used three experimental protocols to examine the effects of lowering of the extracellular Na⁺ concentration ([Na⁺]₀) on the rate of pH recovery from acid loads. In the first, shown in Fig. 4 A, we twice acid loaded the osteoclast by means of a 85-s NH₃/NH₂⁺-prepulse, as described in the preceding paragraph. The pH recovery from the first acid load, under control conditions, had a maximal rate of

![FIGURE 4. Recovery from acid loads in pattern-1 cells is Na⁺ dependent. (A) Protocol 1. The osteoclast was twice exposed for 85 s to 20 mM NH₃/NH₂⁺ (solution 2). After the first acid load, the cell was exposed to the standard HEPES-buffered saline (solution 1) and allowed to recover from the acid load. After the second, the osteoclast was exposed to a solution containing only 15.5 mM Na⁺ (solution 4); this decreased the rate of pH recovery by 72%. Restoring [Na⁺], to 150 mM (solution 1) caused dpHi/dt to increase to control values. (B) Protocol 2. The osteoclast was acid loaded by an 85-s exposure to 20 mM NH₃/NH₂⁺ (solution 2). Subsequently, the cell was exposed to solutions containing 15.5 mM Na⁺ (solution 4) and 150 mM Na⁺ (solution 1). In this cell, the rate of pH recovery, relative to that in 150 mM Na⁺, was reduced 82% in the presence of 15.5 mM Na⁺. (C) Protocol 3. The osteoclast was exposed for 85 s to 20 mM NH₃/NH₂⁺ (solution 2). Subsequently, the external solutions were switched to ones containing nominally no Na⁺ (solution 5), 15.5 mM Na⁺ (solution 4) and 150 mM Na⁺ (solution 1). No recovery from the acid load was observed in the Na⁺-free solution, whereas in the presence of 15.5 mM Na⁺ the rate of recovery was 84% lower than that observed in the presence of 150 mM Na⁺. ]
47 ± 6.3 × 10⁻⁴ pH/s at an average pHᵢ of 6.83 ± 0.04 (n = 13). The pHᵢ recovery from the second acid load, under conditions in which [Na⁺]₀ had been reduced to 15.5 mM, followed one of two patterns. In five of the 13 cells, which we define as pattern-2 cells, pHᵢ recovered rapidly in the presence of 15.5 mM Na⁺, with an average \( \frac{dpHᵢ}{dt} \) of \( 35 ± 4.6 \times 10⁻⁴ \) pH/s determined at a mean pHᵢ of 7.08 ± 0.06. This was only modestly slower than the matched pHᵢ recovery in these same cells under control [Na⁺]₀ conditions, \( 54 ± 12 \times 10⁻⁴ \) pH/s (mean pHᵢ = 6.83 ± 0.06; n = 5). These pattern-2 cells, which appear to have substantial Na⁺-independent acid-extrusion activity, are discussed at the end of Results. In the other eight cells, which appeared to lack such an acid extruder, the pHᵢ recovery was very slow when [Na⁺]₀ was 15.5 mM, as typified by the experiment shown in Fig. 4A. Subsequently raising [Na⁺]₀ to 150 mM caused pHᵢ to recover rapidly, consistent with the presence of a Na-H exchanger. As summarized in Table III (protocol 1), the mean maximal

| [Na⁺]₀ | Protocol 1 (n = 8) | Protocol 2 (n = 10) | Protocol 3 (n = 21) |
|--------|-------------------|-------------------|-------------------|
| mM     | \( 10⁻⁴ \) pH/s  | \( 10⁻⁴ \) pH/s  | \( 10⁻⁴ \) pH/s  |
| 0      | —                 | —                 | —                 |
| 15.5   | 15 ± 1            | 6.71 ± 0.03       | 6.3 ± 1.1         |
| 150    | 40 ± 5            | 6.88 ± 0.03       | 46 ± 9            |

*In Protocol 1 (Fig. 4A), the osteoclast was twice acid loaded via NH₃/NH₂⁺ prepulses. After the first acid load, the NH₃/NH₂⁺ was washed out into 150 mM Na⁺ (not summarized here). After the second, the NH₃/NH₂⁺ was washed out into a solution containing 15.5 mM Na⁺, which was then switched to one containing 150 mM Na⁺. In Protocol 2 (Fig. 4B), the osteoclast was acid loaded only once. The NH₃/NH₂⁺ was washed out into a solution containing 15.5 mM Na⁺; this was then switched to one containing 150 mM Na⁺. In Protocol 3 (Fig. 4C), the osteoclast was acid loaded only once. The NH₃/NH₂⁺ was washed out into a Na⁺-free solution, which was switched to ones containing 15.5 mM and then 150 mM Na⁺.

dpHᵢ/dt in eight experiments on pattern-1 cells was \( 13 \times 10⁻⁴ \) pH/s (pHᵢ = 6.71) with cells in 15.5 mM Na⁺, and \( 40 \times 10⁻⁴ \) pH/s when [Na⁺]₀ was subsequently increased to 150 mM. The matched dpHᵢ/dt in these same pattern-1 cells under control conditions (first NH₂⁺ pulse) averaged \( 42 ± 6.9 \times 10⁻⁴ \) pH/s (mean pHᵢ = 6.83 ± 0.05; n = 8). Thus, regardless of which of the two 150-mM Na⁺ values is used, reducing [Na⁺]₀ to 15.5 mM caused the pHᵢ recovery rate to decrease by ~70% in these cells.

We made similar observations using two additional experimental protocols (protocols 2 and 3, summarized in Table III). Protocol 2 (Fig. 4B) consisted of a single 85-s NH₃/NH₂⁺ prepulse, followed by an exposure to solutions having [Na⁺]₀ values of 15.5 and 150 mM (i.e., identical to the second half of Fig. 4A). Again, in 16 osteoclasts we noted two patterns of pHᵢ recovery. In six pattern-2 cells, pHᵢ recovered rapidly, with an average dpHᵢ/dt of \( 23 ± 4.0 \times 10⁻⁴ \) pH/s (mean
pH$_i$ = 7.03 ± 0.07) in the presence of 15.5 mM Na$^+$. The mean rate of pH$_i$ recovery in ten pattern-1 cells (those with slow recovery in 15.5 mM Na$^+$) increased from $6.3 \times 10^{-4}$ pH/s (pH$_i$ = 6.86) to $46 \times 10^{-4}$ pH/s upon raising [Na$^+$]$_o$ from 15.5 to 150 mM (Table III). Protocol 3 (Fig. 4 C) was similar to protocol 2, except that the NH$_3$/NH$_4^+$ prepulse was followed by exposures to solutions having [Na$^+$]$_o$ values of 0, 15.5 and 150 mM. As in the previous two protocols, we noted two patterns of pH$_i$ recovery. In the minority, pattern-2 cells, pH$_i$ recovered rapidly (dpH$_i$/dt > $18 \times 10^{-4}$ pH/s) in the absence of Na$^+$. In the pattern-1 cells, the pH$_i$ recovery was virtually absent in Na$^+$-free solutions, as shown in Fig. 4 C. For these cells, dpH$_i$/dt ranged from $-7 \times 10^{-4}$ to $+4 \times 10^{-4}$ pH/s, having a mean value of $-1.3 \times 10^{-4}$. When [Na$^+$]$_o$ was subsequently increased from 0 to 15.5 mM in the experiment shown in Fig. 4 C, pH$_i$ recovered at a rate higher than observed for the other two protocols (i.e., when the cells had not been exposed to a Na$^+$-free solution). The mean maximal dpH$_i$/dt (measured at a mean pH$_i$ of 6.66) for these pattern-1 cells in the presence of 15.5 mM Na$^+$ was $31 \times 10^{-4}$ pH/s (Table III). Still in the 15.5 mM Na$^+$ solution, but just before [Na$^+$]$_o$ was increased to 150 mM, dpH$_i$/dt had decreased to $5.1 \pm 1.3 \times 10^{-4}$ pH/s (n = 21). Subsequently increasing [Na$^+$]$_o$ from 15.5 to 150 mM caused dpH$_i$/dt to increase to an average rate of $56 \times 10^{-4}$ pH/s. Thus, at least in the pattern-1 cells, the recovery of pH$_i$ from an acid load is markedly Na$^+$ dependent, consistent with the presence of a Na-H exchanger. 

Recovery from Acid Loads Is Inhibited by Amiloride and 5-Amino-substituted Amiloride Analogues in Pattern-1 Cells

The Na-H exchangers of osteoclasts are active at resting pH$_i$. Most Na-H exchangers are characterized by their sensitivity to inhibition by amiloride and 5-amino-substituted amiloride derivatives (Kleyman and Cragoe, 1988). We therefore evaluated the effects of amiloride, ethylisopropylamiloride (EIPA) and hexamethyleneamiloride (HMA), employing three experimental protocols. The first is shown in Fig. 5 A. To test whether Na-H exchangers are active in the normal steady state, we examined the effect of applying 1 mM amiloride under resting conditions. This maneuver led to a reversible acidification that averaged 0.10 ± 0.01 pH units over a period of 116 ± 12 s (n = 7). The trajectory of the acidification was nearly linear, having a mean dpH$_i$/dt of $-13 \pm 2.0 \times 10^{-4}$ pH/s, determined at an average pH$_i$ = 7.24 ± 0.04. These results indicate that, under resting conditions, an amiloride-sensitive acid-extruder, presumably a Na-H exchanger, exactly balances background acid-loading processes. 

The Na-H exchangers of osteoclasts are blocked by amiloride. Next, the cell shown in Fig. 5 A was acid loaded using a 85-s NH$_3}$/NH$_4^+$ prepulse, and pH$_i$ was allowed to recover under control conditions. At a mean pH$_i$ of 6.91 ± 0.03, the maximal pH$_i$ recovery rate averaged $37 \pm 5 \times 10^{-4}$ pH/s (n = 13) for this group of cells. After the pH$_i$ recovery was complete, we applied a second 85-s NH$_3}$/NH$_4^+$ prepulse, but this time introduced 1 mM amiloride during the NH$_3}$/NH$_4^+$ washout. Again, we noted two patterns of pH$_i$ recovery. In four pattern-2 cells, pH$_i$ recovered rapidly, having an average dpH$_i$/dt of $30 \pm 11 \times 10^{-4}$ pH/s (mean pH$_i$ = 6.76 ± 0.06) in the presence of amiloride. This was only modestly slower than the matched pH$_i$ recovery in these same cells under control conditions after the first NH$_4^+$ pulse, $55 \pm 6 \times 10^{-4}$ pH/s (mean pH$_i$ = 6.95 ± 0.02). In the remaining, pattern-1 cells (such as that illustrated
in Fig. 5A), amiloride substantially reduced the rate of pH recovery, consistent with
the presence of a Na-H exchanger. As summarized in Table IV (protocol 1), the
maximal dpH/dt averaged \(5.7 \times 10^{-4}\) pH/s, considerably less than the matched pH
recovery in these same cells under control conditions (first NH\(_3\) pulse), \(29 \times 10^{-4}\)
pH/s. Thus, 1 mM amiloride caused an 80% reduction in maximal recovery rates. Inive cells (see Fig. 5A), the inhibition was at least partially reversible, removal of
amiloride causing dpH/dt to increase to an average maximum value of \(18 \pm 5 \times 10^{-4}\) pH/s.

The pH recovery is Na\(^+\) dependent and amiloride sensitive in the same cells (pattern-1).
Our observation that osteoclasts display two patterns of pH recovery raises the
question of whether the pH recovery is both Na\(^+\) dependent and amiloride sensitive
in the same cells. Therefore, we employed a second experimental protocol to answer
this question. As shown in Fig. 5B, cells were twice acid loaded by means of NH\(_3\)/

\[\text{FIGURE 5. Recovery from acid loads in pattern-1 cells is amiloride sensitive. (A) A brief, 90-s application of 1 mM amiloride (Amil.), dissolved in solution 1, led to a reversible pH decrease of 0.11 under resting conditions. The osteoclast was subsequently acid loaded twice by means of 85-s NH\(_3\)/NH\(_4\) prepulses (solution 2). In the first case, the cell recovered in solution 1. In the second, in which 1 mM amiloride was dissolved in solution 1, the pH recovery rate was inhibited > 95%. Removal of the amiloride partially reversed this inhibition. (B) The osteoclast was twice acid loaded by means of 85-s NH\(_3\)/NH\(_4\) prepulses (solution 2). After the first, reducing [Na\(^+\)]\(_o\) to 15.5 mM (solution 4) caused pH to recover at a rate that was 87% reduced when compared to the rate of pH recovery observed when [Na\(^+\)]\(_o\) was subsequently restored to 150 mM (solution 1). Recovery from the second acid load was largely blocked by 1 mM amiloride (dissolved in solution 1).} \]

NH\(_4\)\(^+\) prepulses. During the first washout of NH\(_3\)/NH\(_4\)\(^+\), [Na\(^+\)]\(_o\) was reduced to 15.5 mM, whereas during the second washout, 1 mM amiloride was introduced in the presence of 150 mM Na\(^+\). Consistent with our previous observations, we noted again two patterns of pH recovery. In three of 16 cells (i.e., pattern-2 cells), pH rapidly recovered from both acid loads, despite the lowered [Na\(^+\)]\(_o\) or the presence of the amiloride (see discussion of Fig. 11B, below). In the experiment on the pattern-1 cell illustrated in Fig. 5B, however, recovery from the acute acid load was impeded at an [Na\(^+\)]\(_o\) of 15.5 mM, but proceeded at higher rates once [Na\(^+\)]\(_o\) was restored to 150 mM. Similarly, the pH recovery was greatly inhibited by amiloride after the second acid load. In 12 of 13 osteoclasts in which the pH recovery from the first acid load was substantially inhibited by 15.5 mM Na\(^+\), amiloride profoundly reduced dpH/dt during the recovery from the second acid load. The mean dpH/dt for these 12 cells
in the presence of amiloride after the second NH$_4^+$ pulse was $9.6 \times 10^{-4}$ pH/s (see Table IV, protocol 2), not significantly different from the mean dpHi/dt for these same cells in the presence of 15.5 mM Na$^+$ after the first NH$_4^+$ pulse, $8.8 \pm 1.1 \times 10^{-4}$ (mean pH$_i$ = 6.82 $\pm$ 0.04). However, both recovery rates are more than 70% lower than the matched control dpHi/dt of $35 \times 10^{-4}$ pH/s, observed when [Na$^+$]$_o$ was increased from 15.5 to 150 mM after the first NH$_4^+$ pulse.

**High levels of EIPA and HMA cause a paradoxical alkalinization, and yet block Na-H exchange in osteoclasts.** In a third protocol, we applied 50 $\mu$M EIPA or HMA while submitting the osteoclast to the solution changes shown in Fig. 6. Fig. 6 A shows a control experiment (same protocol as in Fig. 4 C), in which the osteoclast was acid loaded by prepulsing with NH$_3$/NH$_2$H$^+$, and the subsequent pH$_i$ recovery was monitored in the presence of 0, 15.5, and 150 mM Na$^+$. Fig. 6, B and C, show experiments otherwise identical to that in Fig. 6 A, except that we introduced either 50 $\mu$M EIPA or HMA while the extracellular solution still was Na$^+$ free. We were surprised to find that EIPA, but particularly HMA, alkalinized the cell even in the absence of Na$^+$ (arrow at A in Figs. 6, B and C). For EIPA, the mean rate of alkalinization was $+10 \pm 2.3 \times 10^{-4}$ pH/s ($n = 4$), and for HMA, $+22 \pm 1.8 \times 10^{-4}$ pH/s ($n = 5$). The application of 0.1% (vol/vol) DMSO, the vehicle in which both EIPA and HMA were dissolved, did not significantly affect the rate of pH$_i$ change ($n = 5$). In general, the EIPA- or HMA-induced alkalinizations were self limited and reversible, as shown for HMA in Fig. 6 C. Even though these compounds, employed at concentrations of 50 $\mu$M, paradoxically increased pH$_i$, they nevertheless inhibited Na-H exchange. Thus, we observed no appreciable increase in the dpHi/dt upon raising [Na$^+$]$_o$ from 0 to 15.5 mM (arrow at B in Fig. 6, B and C) or from 15.5 to 150 mM (arrow c). The mean data for these experiments are summarized in Table V. We did not examine the effects of EIPA or HMA on pH$_i$ in the pattern-2 cells.
We however observed that the pH_i increase elicited by applying EIPA or HMA was associated with a substantial decrease in the I_{440} signal (segment ad, Fig. 6 D), whereas the pH_i decrease elicited by withdrawing EIPA or HMA was associated with an increase in the I_{440} signal (segment de, Fig. 6 D). To investigate the possibility that these effects were caused by an unknown interaction of the amiloride analogues with 

\[
\begin{align*}
&\text{NH}_4^+ \\
&\text{Na}^+ \\
&\text{NH}_4^+ \\
\end{align*}
\]

FIGURE 6. Na-H exchange in pattern-1 rat osteoclasts is highly sensitive to inhibition by 5-amino-substituted amiloride analogues. (A) Control experiment showing the Na\(^+\) dependence of the pH_i recovery from an acid load. After acid loading via an NH\(_4\)/NH\(_4\)^+ prepulse (solution 2), pH_i did not recover in the nominal absence of external Na\(^+\) (solution 5). Raising [Na\(^+\)]\(_o\) from 0 mM to 15.5 mM (solution 4) caused pH_i to recover at a rate that was 87% of the rate observed after restoring [Na\(^+\)]\(_o\) to 150 mM (solution 1). (B) Na-H exchange is blocked by ethylisopropylamiloride (EIPA). Applying 50 \(\mu\)M EIPA in the absence of Na\(^+\) causes pH_i to increase (arrow a). The trajectory of the pH_i increase was not accelerated by raising [Na\(^+\)]\(_o\) to either 15.5 mM (solution 4, arrow b) or 150 mM (solution 1, arrow c). After removal of EIPA (arrow d), pH_i decreased and then started to increase slowly (segment de). (C) Na-H exchange is blocked by hexamethyleneamiloride (HMA). The first part of the protocol is identical to that of B, except that the cell was exposed to 50 \(\mu\)M HMA, rather than EIPA. Removal of HMA (arrow d), led to a marked cytoplasmic acidification (segment de). All experiments were concluded by exposing the cell to a high-K\(^+\)/10-\(\mu\)M nigericin solution (solution 3) at pH 7.00, thereby clamping pH_i to this value. In this experiment, a second pulse of 50 \(\mu\)M HMA (segment fg) was applied in the presence of the high-K\(^+\)/nigericin. Under these conditions, HMA failed to increase pH_i. (D) Time courses of the I_{440} (labeled arrows) and I_{490} (unlabeled arrows) signals from the same experiment shown in C. The first application of HMA caused a substantial fall in I_{440}, but less so in I_{490} (segment ad). Upon removal of the drug (arrow d), both I_{440} and I_{490} recovered (segment de). Exposing the osteoclast to high-K\(^+\)/10-\(\mu\)M nigericin equalized I_{440} and I_{490}. Applying 50 \(\mu\)M HMA under these conditions (arrow f) caused reversible but identical decreases in both I_{440} and I_{490} (segment fg).

BCECF, or by interference with the measurement of BCECF fluorescence, we applied HMA while clamping pH_i to 7.00 by exposing the cell to a high-K\(^+\)/nigericin solution. As illustrated by the last portion of Fig. 6 C, 50 \(\mu\)M HMA failed to change pH_i. Under these conditions, HMA caused both the I_{440} and I_{490} signals to decrease
slightly and to the same extent (segment fg, Fig. 6 D). Similar results were obtained in two other experiments.

Removal of the amiloride analogues, in the continued presence of 150 mM Na⁺, caused either a transient slowing of the pHᵢ increase (not shown), a modest acidification (segment de for EIPA in Fig. 6 B) or a pronounced acidification (de for HMA in Fig. 6 C). In the case of EIPA withdrawal, dpHᵢ/dt temporarily slowed to an average of $1.5 \pm 2.6 \times 10^{-4}$ pH/s, but increased again within 5 min to $7-14 \times 10^{-4}$ pH/s. In the case of HMA withdrawal, the cytoplasm acidified within 3 min an average of $0.12 \pm 0.01$ pH units, after which the cells started to recover slowly ($<6 \times 10^{-4}$ pH/s).

Virtually identical results were obtained in experiments after a protocol similar to that shown in Fig. 4 A, except that 50 μM EIPA (n = 12) or 50 μM HMA (n = 8) were added to the 15.5 mM and 150 mM Na⁺-containing solutions that were applied to the cell after the second NH₃/NH₄⁺ prepulse. In all 20 experiments, the amiloride analogues prevented the pHᵢ recovery upon raising [Na⁺]₀ from 15.5 to 150 mM, further establishing that acid extrusion in pattern-1 cells is mediated by Na-H exchange.

### Table V

| [Na⁺]₀ (mM) | Drug status | Experiments in which 50 μM EIPA was applied | Experiments in which 50 μM HMA was applied |
|-------------|-------------|-------------------------------------------|------------------------------------------|
|             | dpHᵢ/dt 10⁻⁴ pH/s | pH at dpHᵢ/dt | dpHᵢ/dt 10⁻⁴ pH/s | pH at dpHᵢ/dt |
| 0 Absent    | -8.3 ± 3.0 | 6.35 ± 0.04 | | | 6.57 ± 0.05 |
| 0 Present   | 10 ± 2.32 | 6.35 ± 0.04 | | | 22 ± 22 |
| 15.5 Present| 11 ± 1.4 NS¹ | 6.46 ± 0.04 | | | 16 ± 3 NS¹ |
| 150 Present | 8.8 ± 2.3 NS¹ | 6.57 ± 0.04 | | | 6.79 ± 0.07 |
| 150 Removed | 1.5 ± 2.6 NS² | 6.64 ± 0.04 | | | -14 ± 2 NS² |

*The protocols for these experiments were those for Fig. 6 B (EIPA added) and Fig. 6 C (HMA added). The first row ([Na⁺]₀ = 0, drug absent) represents data taken just before point a in the figures. The second row represents data taken just after point b, and so on. The fifth row represents data taken just before e.

¹The value is not significantly different from the one immediately above it (paired t test).

²The value is significantly greater than the one immediately above it (P = 0.002).

The intrinsic buffering power in pattern-1 cells is mediated by Na-H exchange.

### Intrinsic Buffering Power in Pattern-1 Cells

To convert rates of pHᵢ change to acid-base fluxes, we determined the pHᵢ dependence of intrinsic intracellular buffering power, βᵢ. Our approach was to expose acid-loaded osteoclasts to a Na⁺-free solution containing 20 mM NH₃/NH₄⁺, and then decrease [NH₃/NH₄⁺]₀ to 0 mM in a step-wise fashion. This caused pHᵢ to also decrease in a step-wise fashion, as shown in Fig. 7 A. For each step, we computed βᵢ from the ΔpHᵢ and the computed Δ[NH₄⁺]ᵢ, as described in Methods. To exclude
interference from background acid-loading or -extruding processes, we discarded steps for which the initial or final pH baseline drifted more rapidly than \( \pm 5 \times 10^{-4} \) pH/s. Fig. 7 A shows one of three experiments in which the absolute value of \( \frac{d \text{pH}}{d t} \) at all seven pH values was \( 5 \times 10^{-4} \) pH/s or less, indicating the absence of substantial acid-loading or -extruding processes. Fig. 7 B summarizes the results averaged from a total of 19 osteoclasts, and shows that \( \beta \) falls with increasing pH, values, from a high of 25 mM/pH at a pH of 6.41 to a low of 6.2 mM/pH at a pH of 7.36. The solid curve drawn through the points is the result of a fit to a second-order polynomial.

The broken curve represents the results of a fit to the equation describing the buffering power of a single monoprotic weak acid, as described by Wilding, Cheng, and Roos (1992). The best-fit values are a total buffer concentration of 47 ± 4 (SD) mM and an apparent pK\(_a\) of 6.34 ± 0.09 (SD).

**Figure 7.** The intrinsic buffering power \( (\beta_0) \) of rat osteoclasts falls with increasing pH, (A) pH, record from a pattern-1 osteoclast. The cell was acid loaded by means of an NH\(_2\)/NH\(_3\) prepulse (solution 2), and then exposed to a nominally Na\(^+\)-free saline (solution 5) during application and subsequent step-wise reduction of extracellular NH\(_2\)/NH\(_3\). The solutions containing the indicated concentrations of NH\(_2\) were prepared by mixing solutions 5 and 6. Restoration of [Na\(^+\)]\(_o\) to 150 mM caused pH to increase, indicating the activity of Na-H exchange. From these data, \( \beta \) is calculated as described in Materials and Methods. (B) Summary of the data obtained from 19 osteoclasts submitted to the solution changes shown in A. The average \( \frac{d \text{pH}}{d t} \) value observed at the time pH measurements were made was \( -1.5 \pm 0.25 \times 10^{-4} \) pH/s (n = 82). The 64 (pH, \( \beta \)) pairs calculated from these pH, data were divided into eight sets of eight and averaged for \( \beta \) and pH, Vertical and horizontal bars indicate SEM, and are omitted if the SEM is not at least 25% greater than the size of the symbol. A second-order polynomial function, \( \beta = -523 + 177 \times \text{pH} - 14.3 \times (\text{pH})^2 \), was fitted to the data (solid curve). The theoretical \( \beta \) vs pH relationship of a weak monoprotic acid with a total concentration 47 ± 3.9 (SD) mM and an apparent pK\(_a\) of 6.34 ± 0.09 (SD) was also fitted to the data (broken curve).

Osteoclast Na-H Exchangers Are Highly Sensitive to HMA in Pattern-1 Cells

So far, we showed that, in the nominal absence of CO\(_2\)/HCO\(_3\)^{-}, the majority of osteoclasts recover from acute acid loads in a pH, dependent fashion, and that the recovery rates are reduced by lowering [Na\(^+\)]\(_o\) or by applying amiloride or its derivatives. These data are consistent with the presence of Na-H exchangers in the plasma membrane of rat osteoclasts. Based on the sensitivity of Na-H exchangers to amiloride analogues, these transporters can be grouped into three categories: (a)
Na⁺-dependent, CO₂/HCO₃⁻-independent acid-extruders that appear to be Na-H exchangers, but are insensitive to amiloride analogues. These have been reported in cultured rat hippocampal neurons (Raley-Susman, Cragoe, Sapolsky, and Kopito, 1991) and freshly isolated rat hippocampal pyramidal neurons (Schwiening and Boron, 1994). (b) Na-H exchangers that are mildly sensitive to amiloride analogues. These are typified by exchangers found at the apical membranes of epithelia (Clark and Limbird 1991). (c) Na-H exchangers that are highly sensitive to amiloride and its derivatives. By the definition of Clark and Limbird (1991), these exchangers have a 50% inhibitory concentration (IC₅₀) for 5-amino-substituted amiloride analogues that is <1 μM in the presence of physiological amounts of Na⁺. These exchangers are found in many nonepithelial cells and at the basolateral membrane of epithelia, and at least some are encoded by NHE1 cDNA.

To classify the Na-H exchangers of rat osteoclasts, we determined the IC₅₀ for HMA by monitoring pHᵢ recovery rates from acute acid loads after raising [Na⁺]₀ from 0 to 150 mM in the presence of 0–10 μM HMA. To permit determination of dpHᵢ/dt over a wide range of pHᵢ values, we increased the duration of the NH₃/NH₄⁺ prepulse, such that washout of the NH₃/NH₄⁺ into a Na⁺-free solution drove pHᵢ to values between 6.25 and 6.50. In the absence of HMA, subsequently raising [Na⁺]₀ from 0 to 150 mM caused the pHᵢ recovery, at a pHᵢ of 6.85, to proceed at a rate of 134 ± 24 × 10⁻⁴ pH/s (n = 12). This value is substantially higher than the comparable dpHᵢ/dt of 36 ± 3.9 × 10⁻⁴ pH/s (n = 15; P = 0.002) observed when the NH₃/NH₄⁺ was washed out directly into our standard HEPES solution containing 150 mM Na⁺. This higher dpHᵢ/dt when the NH₃/NH₄⁺ was washed out into a Na⁺-free solution probably reflects the relatively low [Na⁺]ᵢ, as reported in other cells (Grinstein, Cohen, and Rothstein, 1984; Green, Yamaguchi, Kleeman, and Muallem, 1988; Paradiso, 1992). As is shown in Fig. 8A, HMA caused a dose-dependent decrease in pHᵢ recovery rate. Note that neither the application of 10 μM HMA (Fig. 8A) nor the withdrawal of 10 μM HMA (not shown) produced a discernible alkalinization or acidification, respectively. Thus, the paradoxical alkalinization elicited by HMA (see Fig. 6C) occurs only at doses > 10 μM.

Fig. 8B summarizes the effect of HMA on total net acid-extrusion rate (Jₜₜₜ). The latter was computed as the product of dpHᵢ/dt (determined at pHᵢ 6.60) and β₁ (computed at pHᵢ 6.60 from the regression coefficients of the polynomial describing the pHᵢ dependence of β₁ in Fig. 7B). Using a nonlinear least-squares approach, and assuming a single population of Na-H exchangers, we fitted the Michaelis-Menten equation to the fractional-inhibition-vs-[HMA]₀ data obtained from 47 osteoclasts. For data obtained at a pHᵢ of 6.60, the Kᵢ was 49 ± 13 nM (mean ± SD; Fig. 8B, inset). Similar results were obtained when the fluxes were determined at pHᵢ values of 6.5, 6.7, 6.8, or 6.9.

[Na⁺]₀ Dependence of the Na-H Exchange in Pattern-1 Cells

To estimate the dependence of Na-H exchange on [Na⁺]₀, we determined rates of pHᵢ recovery from acid loads at a pHᵢ of 6.60 in the presence of increasing [Na⁺]₀. We employed a protocol similar to that of Fig. 4C, in which we prepulsed with NH₃/NH₄⁺ for at least 85 s, washed the NH₃/NH₄⁺ out into a Na⁺-free solution, allowed pHᵢ to stabilize, and then exposed the cell to a solution containing either 7.5, 15, 30,
45, 60, 100, or 150 mM Na⁺. Finally, in each case except the last, we switched to a solution containing 150 mM Na⁺. Osteoclasts exhibiting a pHᵢ recovery in the nominal absence of Na⁺ (pattern-2 cells) were excluded from this analysis. Fig. 9A shows representative pHᵢ records. As expected for Na-H exchange, the pHᵢ recovery rate was greater with increasing [Na⁺]₀. Fig. 9B summarizes the [Na⁺]₀ dependence of Jᵗₒₜᵃˡ at a pHᵢ of 6.60 for 88 osteoclasts. Using a nonlinear least-squares curve fitting procedure to fit the Hill equation to these data yielded a Kᵦ of 45 ± 5.0 (SD) mM, an apparent Hill coefficient (nᵦ) of 1.2 ± 0.2 (SD), and a maximal flux 628 ± 165 (SD) µM/s. The best-fit nᵦ is consistent with a Hill coefficient of unity.

**Figure 8.** The Na-H exchanger of rat osteoclasts is highly sensitive to inhibition by HMA. (A) Switching the cells from 0 (solution 5) to 150 mM Na⁺ (solution 1) in the presence of hexamethyleneamiloride (HMA) leads to a dose-dependent decrease in the rate of acid-load recovery. The cytoplasmic acid load was delivered by means of a NH₃/NH₄⁺ prepulse (solution 2) that is not shown. Superimposed pHᵢ transients of eight different osteoclasts at increasing concentrations HMA are shown. The concentration HMA in µM is given after the letters (a,b, . . . , g). The pHᵢ transient labeled a was recorded in the presence of 0.1% (vol/vol) DMSO. Note that the application of HMA does not induce the alkalinization seen with 50 µM of the drug at any of the concentrations used. (B) Summary of the data obtained from 47 osteoclasts submitted to the solution changes shown in A. The number of observations used to determine the average Na-H exchange flux at pHᵢ = 6.60 is shown in parentheses above the vertical bars. (Inset) Fitting the Michaelis-Menten equation to the % inhibition-vs-[HMA]₀ data, yielded an Kᵦ of 49 ± 13 (SD) nM. Vertical bars represent the SEM, and are omitted if the SEM is not at least 25% greater than the radius of the symbol.

**pHᵢ Dependency of Na-H Exchange in Pattern-1 Cells**

During the recovery of pHᵢ from an acute acid load, Jᵗₒₜᵃˡ is the result of fluxes due to Na-H exchange (Jₙₐ-H) as well as to fluxes due to other acid-loading or extrusion processes resistant to amiloride (Jₘᵢₙ/R). Thus, if we assume that the only action of amiloride is to block Na-H exchange by 100%, Jₙₐ-H is the difference between Jᵗₒₜᵃˡ and Jₘᵢₙ/R. We determined Jₘᵢₙ/R values in two ways. First, we determined rates of pHᵢ decrease produced by the application of 1 mM amiloride under resting conditions (see first application of amiloride in Fig. 5A), and multiplied the dpHᵢ/dt values, obtained at pHᵢ intervals of 0.05, by the corresponding βᵢ. The latter were obtained from the regression coefficients of the second-order polynomial describing the pHᵢ
Figure 9. $[\text{Na}^+]_o$ dependency of Na-H exchange in pattern-1 cells. (A) Osteoclasts were acid loaded by means of a $\text{NH}_3/\text{NH}_4^+$ prepulse (solution 2) and switched to a nominally Na-free solution (solution 5). Next, pH$_i$ was allowed to reach a stable value (not shown) before the cell was exposed to a solution containing the indicated Na concentration in millimolar. Solutions containing $<150$ mM Na$^+$ were prepared by mixing solution 5 and a solution identical to solution 1 except that it contained exactly 120 mM NaCl in addition to $\sim30$ mM NMDG-Cl. The final Na$^+$ concentration of all solutions used was confirmed with a flame photometer. Representative parts of the pH$_i$ traces are shown. (B) Summary of the data obtained from 88 osteoclasts submitted to the solution changes described in A. The number of cells used to determine the average Na-H exchange flux at pH$_i$ = 6.60 as a function Na$^+$ is shown in parentheses. To these data, the Hill equation was fitted. The best fit required a $K_m$ of 45 ± 5.0 (SD) mM, an apparent Hill coefficient ($n_H$) of 1.2 ± 0.2 (SD), and a maximal flux 628 ± 165 (SD) μM/s.

Figure 10. pH$_i$ dependency of Na-H exchange in pattern-1 cells. (A) Na-H exchange fluxes, corrected for the amiloride-resistant flux, as a function of pH$_i$ observed in 13 osteoclasts. (Inset) The amiloride-resistant flux vs pH$_i$ relationship. To these data, a straight line with a slope of 43 ± 6 (SD) μM/(s.pH) and an intercept of 310 ± 40 (SD) μM/s was fitted. (B) Na-H exchange fluxes shown in A normalized for their individual maximal predicted Na-H exchange flux ($J/J_{max}$) as a function of pH$_i$. Dependence of $\beta_1$ (Fig. 7 B). The resultant amiloride-resistant acid-loading fluxes are represented by the negative values in Fig. 10 A (inset). Second, we determined rates of pH$_i$ increase, after an acid load imposed by an $\text{NH}_3/\text{NH}_4^+$ prepulse, while exposing the cell to 1 mM amiloride (see second application of amiloride in Fig. 5 A). These data yielded fluxes due to amiloride-resistant acid-extruding mechanisms, and are represented by the positive values in the inset of Fig. 10 A. In the pH$_i$ range
6.5–7.3, the $J_{\text{amil,}R}$ values (obtained from a total of 21 cells) increased approximately linearly from +30 (acid extrusion) to −10 (acid loading) μM/s.

We obtained the pH dependence of $J_{\text{total}}$ in 13 experiments performed according to the protocol shown in Fig. 8A (record a). The dpH/dt values at pH 6.6 for these cells are summarized in column a of Fig. 8B. For each of the 13 experiments, we computed $J_{\text{total}}$ at pH intervals of 0.05 by multiplying the dpH/dt values by the corresponding $\beta_i$ values. Subtracting the best-fit values for $J_{\text{amil,}R}$ (Fig. 10A, inset) from $J_{\text{total}}$ yielded the 13 $J_{\text{Na-H}}$ vs pHi relationships shown in Fig. 10A. The magnitude of $J_{\text{Na-H}}$ values varied widely from cell to cell, from as little as 250 μM/s at a pHi of 6.5, to as much as 1,000 μM/s. Regardless of its magnitude, $J_{\text{Na-H}}$ decreased markedly with increasing pHi.

Because intracellular protons appear to cooperatively activate Na-H exchange in other cells (Aronson, 1985), we fitted the Hill-equation to our data to estimate the apparent Hill coefficient ($n_H$), as well as the maximal $J_{\text{Na-H}}$. For each of the 13 experiments, we used a nonlinear least-squares curve fitting procedure to estimate $J_{\text{max}}$, and then normalized the $J_{\text{Na-H}}$ data for that experiment by its $J_{\text{max}}$. Fig. 10B shows the normalized $J_{\text{Na-H}}$ vs pHi plots for each of the experiments. The mean $J_{\text{max}}$ was 741 ± 82 μM/s, whereas the mean $n_H$ was 2.9 ± 0.1. Finally, $J_{\text{Na-H}}$ was half maximal at a mean pHi of 6.73 ± 0.02.

Evidence for the Presence of Proton Pumps in Pattern-2 Cells

Incidence, morphology and initial pH of pattern-2 cells. As noted above, we found that, in a minority (~20% overall) of osteoclasts plated on glass, pHi recovered rapidly from acid loads even if we reduced $[\text{Na}^+]_o$ to 15.5 or 0 mM, or if we introduced amiloride. Among cells for which we examined the pHi recovery from acid loads at an $[\text{Na}^+]_o$ of 15.5 mM, 11 out of 29 (38%) had high dpH/dt values, as defined above, and thus are classified as pattern-2 cells. Similarly, 29 of 224 (13%) cells in which the recovery was examined in the absence of Na+ fulfilled the pattern-2 criterion, as did 7 of 29 (24%) cells examined in the presence of amiloride. Interestingly, 55% (6/11) of the cells classified as pattern-2 by the 15.5-mM Na+ protocol were round, as were 86% (25/29) of the osteoclasts classified by the 0-Na+ protocol and 57% (4/7) classified by the amiloride protocol. Even though 74% (35/47) of the pattern-2 cells were round, only slightly less than half (22/53 or 42% by the 0-Na+ protocol) of the round osteoclasts were pattern-2 cells.

For the 47 pattern-2 cells identified on the basis of either the 15.5-mM Na+ protocol, the 0-Na+ protocol, or the amiloride protocol, the mean initial pHi was 7.47 ± 0.03. This is 0.16 higher than the average initial pHi values of the entire osteoclast population, which includes the 47 pattern-2 cells.

Recovery of pHi from acid loads in pattern-2 cells in the absence of Na+. 29 pattern-2 cells were identified by their recovery from an acid load in the absence of Na+. An example is shown in Fig. 11A. In these osteoclasts, the maximal recovery rates averaged 38 ± 5 $\times$ $10^{-4}$ pH/s, as determined at an average pHi = 7.09 ± 0.05. This dpH/dt value is substantially higher than the value of $-1.3 \times 10^{-4}$ pH/s observed in pattern-1 cells under similar 0-Na+ conditions (see Table III, protocol 3). In 16 of the 29 experiments, we permitted the cell to recover from the acid load until pHi
stabilized. The average final pH$_i$ for these 16 osteoclasts was 7.27 ± 0.05, which was 0.25 lower than the initial pH$_i$ (7.52 ± 0.05) for these cells ($P = 5 \times 10^{-5}$).

**Effect of Na$^+$ readdition in pattern-2 cells.** In 9 of the 16 cells for which we allowed pH$_i$ to stabilize in the absence of Na$^+$ (see above), increasing [Na$^+$]$_o$ from 0 to 150 mM had no discernible effect on pH$_i$. An example is the first NH$_4^+$ pulse in Fig. 11 A (arrow). The mean final pH$_i$ for these nine cells in which Na$^+$ failed to elicit a further pH$_i$ recovery was 7.35 ± 0.06, which was 0.20 ± 0.05 lower than the initial pH$_i$, for

![Figure 11](image)

**Figure 11.** In pattern-2 cells, pH$_i$ recovery from acid loads is independent of Na$^+$, insensitive to amiloride, but blocked by NBD-Cl. (A) Recovery of pH$_i$ from acid loads in a pattern-2 cell exposed to a nominally Na$^+$-free solution. The osteoclast was twice acid loaded by means of a NH$_3$/NH$_4^+$ prepulse (solution 2), and exposed to a 0-Na$^+$ saline (solution 5). Despite the absence of Na$^+$, pH$_i$ recovered briskly both times. Restoring the extracellular Na$^+$ did not elicit any further pH$_i$ increase (arrow). (Inset) Example of a pattern-2 cell in which restoring extracellular Na$^+$ to 150 mM (solution 1) led to a further pH$_i$ increase. (B) Recovery of pH$_i$ from acid loads in a pattern-2 cell exposed to either a low-Na$^+$ solution, or a 150-mM Na$^+$ solution containing 1 mM amiloride. The osteoclast was twice acid loaded by means of a NH$_3$/NH$_4^+$ prepulse (solution 2). The pH$_i$ recovery from the first acid load proceeded rapidly, even though [Na$^+$]$_o$ was only 15.5 mM (solution 4), and the recovery from the second acid load was brisk despite the presence of 1 mM amiloride (dissolved in solution 1). The solutions and the preparation of osteoclasts were the same as used in the experiment shown in Fig. 5 B (a pattern-1 cell). Note that neither increasing [Na$^+$]$_o$ to 150 mM (first pulse) nor removing the amiloride (second pulse) led to a further pH$_i$ recovery. (C) Blockade of the pH$_i$ recovery from an acid load in a pattern-2 cell exposed to 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl), a known vacuolar-type proton pump blocker. The osteoclast was twice acid loaded by means of a NH$_3$/NH$_4^+$ prepulse (solution 2). Despite the nominal absence of external Na$^+$ (solution 5), pH$_i$ recovered from the first acid load; raising [Na$^+$]$_o$ to 150 mM caused pH$_i$ to increase further (arrow). After the second acid load, the pH$_i$ recovery in the nominal absence of Na$^+$ was interrupted by applying 100 µM NBD-Cl to the cell (dissolved in solution 5), which unmasked an acid-loading process. Restoring [Na$^+$]$_o$ to 150 mM caused a further alkalinization (arrow).

these cells ($P = 0.005$). That Na$^+$ failed to increase pH$_i$ in these cells implies that Na-H exchangers were inactive, at least at such an alkaline pH$_i$. In the remaining seven osteoclasts, pH$_i$ after recovery from the acid load in the 0-Na$^+$ solution stabilized at a lower value, 7.20 ± 0.09. In these cells, increasing [Na$^+$]$_o$ from 0 to 150 mM resulted in a further pH$_i$ recovery, consistent with Na-H exchange activity. An example is given in the inset of Fig. 11 A. The initial rate of these Na$^+$-induced pH$_i$ recoveries averaged 71 ± 33 × 10$^{-4}$ pH/s; the total increase in pH$_i$ averaged
0.23 ± 0.08. It is noteworthy that this Na-H exchange activity occurred over a much higher pH range than seen in a typical pattern-1 cell (see Fig. 10). After the Na⁺-induced alkalinization, pH in these seven cells averaged 7.43 ± 0.12. In a paired t test, the final pH after Na⁺ readdition for these seven cells was not significantly different from the initial pH, 7.52 ± 0.07 (P = 0.24).

In eight of the experiments on pattern-2 cells, in which we allowed pH to stabilize in the absence of Na⁺ and then increased [Na⁺]₀ to 150 mM, we acid loaded the osteoclast a second time. An example of such an experiment is shown in Fig. 11 A. The results are summarized in Table VI. We found that, for the second pH recovery, neither the maximal rate of pH recovery nor the final value to which pH recovered was significantly different from the corresponding value for the first pH recovery.

**Na⁺ independence and amiloride insensitivity of the pH recovery in the same pattern-2 cells.** When we initially distinguished between pattern-1 and -2 cells, we identified 11 pattern-2 cells (five according to protocol 1, and six according to protocol 2 of Table III) solely on the basis of a relatively rapid dpHᵢ/dt in the presence of 15.5 mM Na⁺. We identified an additional four pattern-2 cells (according to the protocol of Table IV) solely on the basis of a relatively rapid pHᵢ recovery in the presence of 1 mM amiloride and 150 mM Na⁺. To determine whether the pHᵢ recovery is both Na⁺ independent and amiloride insensitive in the same cells, we subjected cells to the protocol shown in Fig. 11 B. We first examined the recovery of pHᵢ from an acid load in the presence of 15.5 mM Na⁺, after which we increased [Na⁺]₀ to 150 mM. Next, we acid loaded the osteoclast a second time, and examined the pHᵢ recovery in the presence of 1 mM amiloride. As is shown in the figure, the same osteoclast that had a rapid pHᵢ recovery at reduced [Na⁺]₀ also had a rapid recovery in the presence of amiloride. In three cells, the average maximal pHᵢ recovery rate was 28 ± 8.3 × 10⁻⁴ pH/s (pHᵢ = 7.08 ± 0.14) at low [Na⁺]₀, and 24 ± 2.6 × 10⁻⁴ pH/s (pHᵢ = 7.03 ± 0.12) in the presence of amiloride.

**Sensitivity of the pHᵢ recovery to NBD-Cl in pattern-2 cells studied in the absence of Na⁺.** These above data indicate that a subgroup of osteoclasts express a robust,

### Table VI

| Parameter                      | First pulse | Second pulse | n  | Significance |
|-------------------------------|-------------|--------------|----|--------------|
| Minimum pHᵢ                   | 7.08 ± 0.07 | 6.88 ± 0.05  | 8  | 0.001        |
| Initial vs minimum pHᵢ        | -0.54 ± 0.03| -0.55 ± 0.07 | 8  | NS           |
| Max dpHᵢ/dt (10⁻⁴ pH/s)       | 33 ± 7      | 37 ± 5       | 8  | NS           |
| pHᵢ at Max dpHᵢ/dt            | 7.12 ± 0.08 | 6.96 ± 0.07  | 8  | 0.003        |
| Final pHᵢ                      | 7.35 ± 0.07 | 7.27 ± 0.07  | 8  | NS           |

*The experiments followed the protocol of Fig. 11 A. Minimum pHᵢ is the lowest pHᵢ achieved after washout of NH₃/NH₄⁺. Max dpHᵢ/dt is the maximal rate of pHᵢ recovery, measured at pHᵢ at max dpHᵢ/dt; this pHᵢ is slightly higher than the minimum pHᵢ. dpHᵢ/dt at pHᵢ 6.9 is the pHᵢ recovery rate measured at a pHᵢ of 6.9. Initial vs minimum pHᵢ is the difference between the pHᵢ value prevailing before application of the NH₃/NH₄⁺ and the minimum pHᵢ. Final pHᵢ is the value prevailing after recovery of pHᵢ from the acid load in the absence of Na⁺ was complete, or nearly so. n is the number of observations. Significance was judged on the basis of paired t tests, comparing values for the first and second pulses.
Na⁺-independent, amiloride-insensitive acid-extrusion mechanism. To test the hypothesis that the pHᵢ recovery from acid loads under nominally Na⁺-free conditions is brought about by a plasma membrane H⁺ pump, we examined the effect of 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl), a potent inhibitor of vacuolar-type proton pumps (Forgac, 1989). In the experiment shown in Fig. 11 C, we twice monitored the recovery of pHᵢ from acid loads. The recovery from the first acid load occurred in the absence of Na⁺, indicating the presence of a potent, Na⁺-independent acid-extrusion mechanism. After the readdition of Na⁺, which led to a further pHᵢ recovery (first arrow in Fig. 11 C), the osteoclast was acid loaded a second time. However, we interrupted the pHᵢ recovery from this second acid load with 100 μM NBD-Cl, which not only blocked the further pHᵢ recovery, but unmasked a slow background acidification. In three such experiments, as well as eight similar ones in which the first NH₄⁺ pulse was omitted, NBD-Cl greatly decreased the rate of pHᵢ recovery. The drug was applied after the pHᵢ recovery had proceeded from an average pHᵢ of 7.00 ± 0.12 to 7.14 ± 0.11. At the latter pHᵢ value (i.e., before the addition of NBD-Cl), dpHᵢ/dt was 30 ± 7 x 10⁻⁴ pH/s (n = 11). In three of the 11 cells, addition of NBD-Cl caused dpHᵢ/dt to approach zero; for these cells, the mean dpHᵢ/dt in the presence of NBD-Cl was +3.7 ± 1.9 x 10⁻⁴ pH/s (n = 3). The paired dpHᵢ/dt before the addition of NBD-Cl was +7.7 ± 1.7 x 10⁻⁴ pH/s (n = 3). In the other eight cells, NBD-Cl actually caused pHᵢ to decline (as in Fig. 11 C). For these cells, the mean dpHᵢ/dt in the presence of NBD-Cl was −74 ± 31 x 10⁻⁴ pH/s (n = 8); dpHᵢ/dt averaged 39 ± 8 x 10⁻⁴ pH/s (n = 8) before the application of the blocker. This NBD-Cl-induced acidification is consistent with the unmasking of an acid-loading process, possibly Na-H exchange operating in reverse in the absence of external Na⁺. In addition, it suggests that the rate of pHᵢ recovery observed in the absence of NBD-Cl underestimates the contribution of the H⁺ pump. From the mean dpHᵢ/dt in the absence of NBD-Cl (30 x 10⁻⁴ pH/s; n = 11), and the mean dpHᵢ/dt in the presence of the drug (−58 x 10⁻⁴ pH/s; n = 11), we calculate that the rate of pHᵢ recovery due to the H⁺ pump is 83 x 10⁻⁴ pH/s at a mean pHᵢ value of 7.14.

In the pHᵢ recovery from the second NH₄⁺ pulse in Fig. 11 C, simultaneously restoring [Na⁺]₀ to 150 mM and removing the NBD-Cl caused pHᵢ to recover rapidly. We made similar observations in seven other experiments in which the Na⁺ was returned in the continuous presence of NBD-Cl. The mean Na⁺-dependent dpHᵢ/dt in these seven experiments was 65 ± 28 x 10⁻⁴ pH/s (mean pHᵢ 6.94 ± 0.07). These results suggest that the Na-H exchangers were unaffected by NBD-Cl.

pHᵢ Dependency of the H⁺ pump fluxes. For experiments in which we monitored the recovery of pHᵢ in the absence of Na⁺ (as in the recovery from the first NH₄⁺ pulse in Fig. 11 A), we computed total acid-extrusion rates (J_total) by multiplying rates of pHᵢ change by the βᵢ value prevailing at the appropriate pHᵢ. The latter was determined from the regression coefficients of the second-order polynomial (Fig. 7 B), obtained from experiments on pattern-1 cells. Thus, we have assumed that βᵢ is the same in pattern-1 and pattern-2 cells. In cells recovering from acute acid loads in the absence of Na⁺, J_total is the result of fluxes due to the H⁺ pump (J_H-ATPase) and other acid loading or extrusion processes resistant to NBD-Cl (J_NBD-Cl/R). We determined J_NBD-Cl/R values from rates of pHᵢ change in the eight experiments in which the application of 100 μM NBD-Cl in a Na⁺-free solution caused pHᵢ to
decrease, indicating maximum blockage of the H^+ pumps. The pHi dependence of \( J_{\text{NBD-CI/R}} \) from these experiments is summarized in Fig. 12A (inset), where the negative \( J_{\text{NBD-CI/R}} \) values indicate net acid loading. In the pHi range 6.9–7.5, \( J_{\text{NBD-CI/R}} \) increased approximately linearly from \(-25\) to \(-5\) \( \mu \text{M/s} \). By subtracting \( J_{\text{NBD-CI/R}} \) from \( J_{\text{total}} \), we arrived at \( J_{\text{H-ATPase}} \), assuming that the only action of NBD-Cl is to block the H^+ pumps 100%. We calculated \( J_{\text{H-ATPase}} \) for 13 cells in which the proton pumps were active in the same pHi range for which we had \( \beta_i \) values. As shown in Fig. 12A, the magnitude of \( J_{\text{H-ATPase}} \), as well as its pHi dependency, varied considerably from cell to cell. We found H^+ pump activity at pHi values as low as \( \approx 6.75 \) (Fig. 12A) and as high as \( \approx 7.80 \) (not shown). For all 13 cells, \( J_{\text{H-ATPase}} \) decreased with increasing pHi over at least some range of pHi values. In three of the 13 cells, \( J_{\text{H-ATPase}} \) appears to have reached a maximal value. As shown in Fig. 12B, the average \( J_{\text{H-ATPase}} \) decreased from 200 to 11 \( \mu \text{M/s} \) in the pHi range 6.8–7.45 in an approximately linear fashion. We found a qualitatively similar \( \text{d} \text{pHi/dt} \) vs pHi relationship for seven other cells (not shown) over a more alkaline pHi range.

**DISCUSSION**

**Overview**

To characterize acid-base transporters in osteoclasts, we examined the mechanisms by which freshly isolated rat osteoclasts, plated on glass and studied in the nominal absence of CO\(_2\)/HCO\(_3\)\(^-\), defend themselves from acute acid loads. Using digital imaging to monitor the fluorescence of BCECF, we found that the cells had a mean initial pHi of \( \approx 7.31 \) and recovered rapidly from acid loads. In most cells (i.e.,
pattern-1 cells), this recovery was due exclusively to Na-H exchange, which proved to be very sensitive to inhibition by the amiloride analogue HMA. In the remainder of the cells (i.e., pattern-2 cells), which represented a portion of a morphologically distinguishable subgroup of osteoclasts, the pHi recovery was due at least in part to a Na⁺-independent acid extruder that was blocked by NBD-Cl, an inhibitor of vacuolar-type H⁺ pumps. In at least some pattern-2 cells, both the Na-H exchanger and the H⁺ pump contributed to the pHi recovery. In addition, we found that the intrinsic intracellular buffering power (βi) decreased with increasing pHi values. From the βi and amiloride data, we deduced that the Na-H exchanger in pattern-1 cells is maximally active at low pHi values, but is virtually inactive at a pHi of ~7.1. Finally, H⁺ pumps were found active in the pHi range 6.7-7.8. On average, H⁺ pump fluxes tended to decrease with increasing pHi. Hence, freshly isolated rat osteoclasts express basolateral-type Na-H exchangers, vacuolar-type H⁺ pumps, or both, in their plasma membranes.

**The Buffering Power of Rat Osteoclasts**

The application/withdrawal of weak bases and acids is a common method for determining βi (Roos and Boron, 1981). The stepwise reduction in [NH₃]o offers the added advantage of producing the pHi dependence of βi, and has been applied in studies of mesangial cells (Boyarsky et al., 1988), gastric parietal cells (Wenzl and Machen, 1989), cardiac Purkinje fibers (Vaughan-Jones and Wu, 1990) and renal epithelial cells (Kraut, Hart, and Nord, 1992). The approach requires that the only mechanism for modulating pHi be the passive flux of NH₃. Therefore, we conducted our experiments in the nominal absence of Na⁺, avoided cells with H⁺ activity (i.e., pattern-2 cells), and excluded data in which the pHi drifted more rapidly than 5 × 10⁻⁴ pH/s. Removal of Na⁺ has been shown not to influence βi (Vaughan-Jones and Wu 1990; Wilding et al., 1992). In osteoclasts, we found that βi decreases from ~25 mM at pHi 6.4 to ~6.0 mM at pHi 7.3, in a curvilinear fashion (Fig. 7). Thus, the intrinsic buffers are expected to be more effective at defending pHi from acid than alkali loads. Qualitatively similar βi-pHi relationships also are found in the aforementioned cell types. Such βi-pHi profiles probably reflect the actions of several intracellular buffers, each with its own pKa and concentration. However, it is of interest to note that, for rat carotid-body glomus cells, Wilding et al. (1992) were able to approximate their βi vs pH data by assuming the presence of a single intracellular buffer having a pKa of 6.41 and a total concentration of 41 mM. Using a similar approach, we found that the intracellular buffers of osteoclasts behave as if they were a single buffer with a pKa of 6.34 and a total buffer concentration of 47 mM.

**Evidence for Na-H Exchange in Rat Osteoclasts**

Evidence for Na-H exchange in pattern-1 and pattern-2 cells. We defined pattern-1 cells as osteoclasts in which the pHi recovery from acid loads (Fig. 4) is due exclusively to Na-H exchange. Pattern-1 cells accounted for ~80% of all osteoclasts in our study, and included all cells with extensions and a polygonal and/or elongated shape, as well as ~45% of round cells. We identified Na-H exchange in pattern-1 cells on the basis of two observations: first, the pHi recovery rate was inhibited ~70% by reducing
[Na⁺]₀ from 150 to 15.5 mM (Figs. 4 and 5 B), and inhibited completely by removing all Na⁺ (Figs. 4 C, 6 A, 7 A, and 8 A). Second, the pHᵢ recovery rate was reduced more than 80% by amiloride and by two 5-N-substituted amiloride analogues, EIPA and HMA (see Figs. 5 A, 6, B and C, and 8). Pattern-2 cells are those in which pHᵢ recovered from an acid load in the total absence of Na⁺ or in the presence of Na⁺ with amiloride. In ~60% of these, the subsequent readdition of external Na⁺ led to a substantial increase in pHᵢ, consistent with the presence of some Na-H exchange activity.

Na-H exchange rates and Jₘₐₓ values (Fig. 10) varied widely among osteoclasts. Although it is possible that the explanation for these differences is trivial, such as cell-to-cell differences in cell surface-to-volume ratio, it is intriguing to speculate that the differences reflect the variability in the activity of individual Na-H exchangers or in the surface density of exchangers. It is interesting to note that Na-H exchangers in pattern-2 cells are active at pHᵢ values that are substantially higher than in pattern-1 cells. For example, Fig. 10 B shows that, in almost all pattern-1 cells, Na-H exchange activity approaches zero at a pHᵢ of 7.1–7.2. On the other hand, Figs. 11 A (inset) and C show that, in pattern-2 cells, Na-H exchange was substantial at pHᵢ values above 7.3. These observations suggest either that Na-H exchange activity may be regulated by the osteoclast over a wide range, or that pattern-2 cells express a different isoform of the Na-H exchanger. Indeed, our data indicate that H⁺ pumps in individual osteoclasts, at least when plated on glass, can be virtually inactive (pattern-1 cells) or extremely robust (pattern-2 cells).

Dependence on pHᵢ. Fig. 10 summarizes the pHᵢ dependence of the Na-H exchange rate, corrected for the minor degree of amiloride-insensitive background acid loading that we observed in rat osteoclasts. The two hallmarks of the J₉ₐ-H vs pHᵢ profile in rat osteoclasts are: (a) a rather low J₉ₐ-H near the average initial pHᵢ, and (b) a rather steep dependence of J₉ₐ-H on pHᵢ as pHᵢ falls below this initial value. By contrast, in unstimulated UMR-106 cells, an osteoblastic cell line, J₉ₐ-H is fairly high at the initial pHᵢ and increases only modestly as pHᵢ falls below this value (Gupta, Schwiening, and Boron, 1994). On the other hand, the pHᵢ dependence of J₉ₐ-H in UMR-106 cells increases markedly after stimulation with agents such as calcitonin gene-related peptide. The steepness of the J₉ₐ-H vs pHᵢ relationship can be described by the Hill coefficient (n₉ₐ), which averaged 2.9 in our experiments. This suggests that three or more protons interact at the intracellular face of the exchanger, consistent with the presence of an allosteric modifier site (Aronson, Nee, and Suhm, 1982; Aronson, 1985). Although, as noted above, maximal Na-H exchange rates varied two- to threefold from cell to cell, the pHᵢ values that yielded half-maximal activation of the exchanger (pHᵢ/₂) fell within a rather narrow range, from 6.64 to 6.78 (Fig. 10 B). By analogy with other cells (see, e.g., Grinstein and Rothstein, 1986), one might expect signals from cell surface receptors to affect the pHᵢ dependency of the osteoclast Na-H exchanger by modulating Jₘₐₓ, n₉ₐ and/or pHᵢ/₂.

Inhibition by amiloride and 5-N-substituted amiloride analogues. The Na-H exchangers of osteoclasts were largely blocked by 1 mM amiloride, 50 μM EIPA, or 10–50 μM HMA. In contrast to the blockade produced by amiloride, that produced by EIPA and HMA were poorly reversible, probably reflecting the greater affinity of these two compounds for Na-H exchangers (Kleyman and Cragoe, 1988). The apparent K₁/₂ for
inhibition by amiloride analogues varies widely among Na-H exchangers, though the values generally are far lower for Na-H exchangers in nonepithelial cells and at the basolateral membranes of epithelia than they are for their apical epithelial counterparts (for review see Clark and Limbird, 1991). For example, working with epithelial LLC-PK1/Cl4 cells, Haggerty, Agarwal, Reilly, Adelberg, and Slayman (1988) found a $K_{1/2}$ for EIPA of 44 nM for the basolateral, but 13 μM for the apical exchanger. Our value of 49 nM for inhibition of the rat osteoclast Na-H exchanger by HMA in the presence of 150 mM Na⁺ is in the range expected for a nonepithelial/basolateral Na-H exchanger.

The paradoxical alkalinization caused by high levels of EIPA or HMA. At concentrations of 50 μM, both EIPA and HMA elicited pHᵢ increases, even in the absence of Na⁺. Conversely, withdrawal of these compounds caused at least a transient decrease in pHᵢ. Paradoxical alkalinizations induced by 50 μM EIPA also have been observed in NIH-3T3 fibroblasts (Kaplan and Boron, 1994) and rat forebrain astrocytes (Boyarsky, Schue, Davis, Ransom, and Boron, 1993). At least in the case of HMA, we observed no such paradoxical alkalinization in our osteoclast experiments at concentrations of 10 μM or less. Thus, the effect is restricted to relatively high doses of HMA, doses that are well above those necessary to block the osteoclast Na-H exchanger. As to the mechanism of the alkalinization, our data, and that of others, suggest that it is not due to a dye-HMA interaction. We found that the apparent pHᵢ increase elicited by 50 μM HMA was paralleled by a reversible decrease in the $I_{440}$ signal, with little change in $I_{490}$. However, the apparent pHᵢ (i.e., $I_{490}/I_{440}$) in osteoclasts is unaffected by 50 μM HMA when we clamped pHᵢ to 7.00 using a high-K⁺/nigericin solution. Moreover, in experiments in which pHᵢ was monitored with BCECF in mesangial cells (Boyarsky et al., 1988) or renal proximal-tubule cells (Geibel, Giebisch, and Boron, 1989), 50 μM EIPA failed to elicit a paradoxical pHᵢ increase. Thus, a dye-HMA interaction is an unlikely explanation for the paradoxical pHᵢ increase. A second explanation is that EIPA and HMA, which are weak bases, enter the cell by nonionic diffusion and become protonated in the cytoplasm. Although there is evidence that the neutral weak-base form of the compounds can indeed enter cells (Kleyman and Cragoe, 1988; Nasri-Sebdami, Cragoe, Cognard, Potreau, and Raymond, 1989), calculations⁴ show that equilibration of these weak bases across the membrane would lead to only trivial changes in pHᵢ. Thus, transport of EIPA or HMA could lead to sizable pHᵢ increases only if the cell had a mechanism for actively transporting the protonated forms of these amiloride analogues out of the cell. A third explanation for the pHᵢ increases elicited by EIPA and HMA is that the compounds indirectly produce a change in acid-base transport, or in cellular biochemistry, that would lead to a sustained intracellular alkaline load. Thus, it is

⁴ For example, if we assume that the pKₐ for HMA is 8.5 (Kleyman and Cragoe, 1988), then at a pH₀ of 7.4 and a total HMA concentration of 50 μM, the concentration of the unprotonated, neutral form of HMA would be 3.7 μM. Upon equilibration of this unprotonated HMA across the cell membrane, the intracellular concentration of neutral HMA also would be 3.7 μM. At a pHᵢ of 6.5, as in Fig. 6 C, this 3.7 μM of intracellular neutral HMA would be in equilibrium with 570 μM of protonated HMA, which would have been formed at the expense of 370 μM cytoplasmic H⁺. Given an intracellular buffering power of 24 mM/pH at a pHᵢ of 6.5, this alkali load would have produced a pHᵢ increase of only 0.015, far less than the alkalinization of ~0.4 shown in Fig. 6 C.
likely that, in susceptible cells, high levels of EIPA or HMA produce an increase in pH, that is real but unexplained.

Evidence for Plasma-Membrane Proton Pumps in Isolated Rat Osteoclasts

Of the round osteoclasts, which lacked cytoplasmic extensions, ~40% (i.e., pattern-2 cells) expressed a Na+-independent acid-extrusion mechanism that appeared to be an H⁺ pump. This conclusion is supported by three observations: first, pHᵢ recovery from acid loads was brisk, despite the reduction (Fig. 11 B) or complete removal of external Na⁺ (Fig. 11, A and C). Second, cells recovered from acid loads in the presence of 1 mM amiloride (Fig. 11 B). Finally, the recovery of pHᵢ from acid loads was blocked rapidly by NBD-Cl (Fig. 11 C), an alkylating agent that is rather specific for vacuolar-type H⁺ pumps (Blair et al., 1989; for review see Forgac, 1989). Similar criteria have been used by others to infer the presence of plasma-membrane H⁺ pumps in macrophages (Bidani, Brown, Heming, Gurich, and Dubose, 1989; Swallow, Grinstein, and Rotstein, 1988, 1990; Tapper and Sundler, 1992).

Osteoclasts are known to alternate between a nonresorbing, migratory phase, in which they exhibit cytoplasmic extensions (lamellipodia), and a resorbing, less motile phase, in which they do not display lamellipodia (Kanehisa and Heersche, 1988; for review, see Tetti, Marchisio, and Zambonin-Zallone, 1991) and have a round shape. Several studies indicate that osteoclasts can acidify the bone surface (Baron et al., 1985) and express vacuolar-type H⁺ pumps in their ruffled-border membrane during resorption (Blair et al., 1989; Vaananen et al., 1990; Chatterjee et al., 1992). In addition, immunocytochemical studies have shown that osteoclastlike cells generated in vitro stain positively for the vacuolar-type H⁺ pump-specific antibody E11 (Kurihara, Gluck, and Roodman, 1990; Wang, Hemken, Menton, and Gluck, 1992). Taken together with these earlier findings, our observation that only 20% of the osteoclasts we studied express functional vacuolar-type H⁺ pumps suggests three possibilities. First, only some of the osteoclasts we studied had been involved in bone resorption at the time of isolation. According to this scenario, active osteoclasts would use vacuolar-type H⁺ pumps to both resorb bone and regulate pHᵢ. Nonresorbing osteoclasts, lacking functional H⁺ pumps, would use Na-H exchangers to regulate pHᵢ. Cells in an intermediate state would express both H⁺ pumps and Na-H exchangers. The second possibility is that all cells had expressed H⁺ pumps in vivo, but most stopped expressing them in their plasma membranes after being plated on glass, where they are not capable of forming a ruffled border. Finally, it is possible that after seeding the osteoclasts on an artificial substratum the H⁺ pumps remain inserted in the plasma membrane but are inactivated in the majority of cells. Our data do not address the issue of whether the H⁺ pumps we observed were located in a specialized membrane region, as is the case in active cells in situ. Neither do we have direct evidence that the H⁺ pumps of freshly isolated osteoclasts are the same as those responsible for bone resorption in vivo.

In conclusion, freshly isolated rat osteoclasts can express two H⁺-extruding systems in their plasma membranes: a Na-H exchanger, probably of the ubiquitous basolateral type, and an H⁺ pump of the vacuolar type.

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