Characterization of inorganic phosphate transport in osteoclast-like cells

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OSTEOCLASTS ARE THE PRIMARY CELLS responsible for bone resorption. They arise by the differentiation of osteoclast precursors of the monocyte/macrophage lineage. These cells are required not only for the development of the skeleton but also for mineral homeostasis and normal remodeling of bone in adult animals (28, 36). Bone resorption depends on the ability of the osteoclast to generate an acid extracellular compartment between itself and the bone surface (1). An acidic pH is essential for solubilization of the alkaline salts of bone minerals as well as for digestion of the organic bone matrix by acid lysosomal enzymes that are secreted by osteoclasts (6, 22). The primary cellular mechanism responsible for this acidification is active secretion of protons by the vacuolar-type H+-adenosine triphosphatase (V-type ATPase), which is localized in the ruffled border of the osteoclasts (1).

Inorganic phosphate (Pι) is the major anionic component of bone, and Pι release from bone may be transported into the osteoclast through a Pι transport system (8, 9). Pι influx has been reported to require extensive V-type ATPase activity and thus a large amount of energy. Pι influx in osteoclasts possesses Naπ-dependent phosphate cotransport activity. Thus, osteoclasts are capable of transporting Pi through the type IIa NaPi cotransporter. It is also possible that osteoclasts possess inorganic phosphate (Pi) transport systems to take up Pi from the extracellular compartment or for production of the large amounts of energy necessary for resorption and remodeling.

Characterization of inorganic phosphate transport in osteoclast-like cells. Am J Physiol Cell Physiol 288: C921–C931, 2005. First published December 15, 2004; doi:10.1152/ajpcell.00412.2004.—Osteoclasts possess inorganic phosphate (Pι) transport systems to take up external Pι during bone resorption. In the present study, we characterized Pι transport in mouse osteoclast-like cells that were obtained by differentiation of macrophage RAW264.7 cells with receptor activator of NF-κB ligand (RANKL). In undifferentiated RAW264.7 cells, Pι transport into the cells was Naπ-dependent, but after treatment with RANKL, Naπ-independent Pι transport was significantly increased. In addition, compared with neutral pH, the activity of the Naπ-independent Pι transport system in the osteoclast-like cells was markedly enhanced at pH 5.5. The Naπ-independent system consisted of two components with Km of 0.35 mM and 7.5 mM. The inhibitors of Pι transport, phosphonoformic acid, and arsenate substantially decreased Pι transport. The proton ionophores nigericin and carbonyl cyanide p-trifluoromethoxyphenylhydrazone as well as a Kπ ionophore, valinomycin, significantly suppressed Pι transport activity. Analysis of BCECF fluorescence indicated that Pι transport in osteoclast-like cells is coupled to a proton transport system. In addition, elevation of extracellular Kπ ion stimulated Pι transport, suggesting that membrane voltage is involved in the regulation of Pι transport activity. Finally, bone particles significantly increased Naπ-independent Pι transport activity in osteoclast-like cells. Thus, osteoclast-like cells have a Pι transport system with characteristics that are different from those of other Naπ-dependent Pι transporters. We conclude that stimulation of Pι transport at acidic pH is necessary for bone resorption or for production of the large amounts of energy necessary for acidification of the extracellular environment.

Naπ-dependent phosphate cotransporter; RAW264.7; phosphate uptake

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MATERIALS AND METHODS

Materials. The mouse monocyte/macrophage cell line RAW264.7 was obtained from the Riken Cell Bank (Tokyo, Japan). Dulbecco’s modified Eagle’s medium and MEM (H9251) were obtained from Invitrogen (Carlsbad, CA). Recombinant human RANKL extracellular region (amino acids 137–316) fused to glutathione-S-transferase was expressed in Escherichia coli using the vector pGEX-3T (Amersham Biosciences, Piscataway, NJ) and purified by performing affinity chromatography using a glutathione-Sepharose column (Amersham Biosciences) (31). Recombinant murine macrophage colony-stimulating factor (M-CSF) was obtained from Genzyme/Techne (Cambridge, MA). The anion transporter inhibitor 4,4'-dinitrostilbene-2,2'-disulfonic acid disodium salt (DNDS) was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Bone particles derived from calf femur were a gift from Dr. Y. Ohba (University of Tokushima Graduate School, Tokushima, Japan) (25).

RAW264.7 cell culture. RAW264.7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-activated fetal bovine serum (FBS). Osteoclasts were generated from RAW264.7 cells with recombinant RANKL (160 ng/ml) without M-CSF. The cells were plated at 5 x 10^3/cm^2 in MEM without phenol red supplemented with 10% charcoal-stripped FBS to remove endogenous steroids as described previously (31). The medium was changed on day 3 and replaced with fresh medium and mediators. After 7 days, multinucleated osteoclasts were identified using TRAP histochemical staining, and calcitonin receptor (CTR) mRNA was detected using reverse transcription polymerase chain reaction (RT-PCR) (39).

Primary osteoclastogenesis from murine bone marrow cells. Six-week-old C57BL/6 male mice were obtained from SLC (Shizuoka, Japan). The preparation of mouse bone marrow cells and the formation of osteoclasts were performed as described previously (31). Briefly, bone marrow cells were isolated from both the tibiae and femurs of mice and cultured in α-MEM with 10% FBS for 24 h. Nonadherent cells were isolated and enriched using a Ficoll density gradient and cultured at 9 x 10^4/cm^2 in phenol red-free MEM supplemented with 10% charcoal-stripped FBS (31). For stimulation of osteoclast formation, cells were incubated with 10 ng/ml M-CSF and 300 ng/ml recombinant RANKL. The medium was refreshed on day 3, and osteoclast formation was assayed by counting the number of TRAP-positive cells per well between days 7 and 10.

Immunostaining. RAW264.7 cells were plated on glass coverslips at a density of 5 x 10^3/cm^2 and analyzed by immunostaining after 24 h, 48 h, 96 h, and 7 days. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated with 1:50 anti-rat CTR affinity-purified antibody (gift from Asahikasei Parma, Tokyo, Japan) (14, 15), anti-Glvr-1 antibody (1:50) (gift from Y. Taketani, University of Tokushima Graduate School, Tokushima, Japan), or an antibody to the COOH terminus of NaPi type IIA antibody by affinity purification (1:50 dilution) (30). The coverslips were incubated, followed by 1:200 Alexa Fluor 568-conjugated rabbit immunoglobulin G (IgG) combined with 1:200 Alexa Fluor 488 phallidin (Molecular Probes, Eugene, OR), to detect actin filaments. The coverslips were mounted with Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Confocal images were obtained using a Leica TCS-SL (Wetzlar, Germany) laser scanning microscope equipped with a ×40 oil-immersion lens objective.

RT-PCR analysis. Total RNA was prepared using Isogen (Wako Pure Chemical, Tokyo, Japan) as described in the manufacturer’s manual. A first-strand synthesis kit (Invitrogen) was first used to generate full-length cDNA from 1 μg of total RNA. The primers for amplification are shown in Fig. 1A.

PCR Southern blot hybridization analysis. DNA was extracted from cultured cells and analyzed by PCR with oligonucleotide primers.

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**A**

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**B**

![Diagram](ATG.png)

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Fig. 1. Detection of the Na^+-dependent P_i (NaPi) cotransporter. A: sequences of various primers used for detection of reverse transcriptase-polymerase chain reaction (RT-PCR). B: primer positions and combinations of type IIA NaPi, cotransporter used for PCR amplification.
specific for a portion of the NaPi type IIa cotransporter. The PCR primers used were S3 and AS1, S4, and AS3 as described in Fig. 1A. PCR products were separated on a 2% agarose gel and transferred to a nylon membrane Hybond-N+ (Amersham Biosciences). Hybridization was performed with digoxigenin (DIG)-labeled PCR probe using S2 and AS3 primers and incubated for overnight in DIG Easy Hyb solution (Roche Diagnostics, Indianapolis, IN) at 42°C. Anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics) and a DIG detection kit (Roche Diagnostics) were used for probe detection. Further details of the PCR Southern blot hybridization method are described in the manufacturer’s technical manual.

P influx and d-glucose transport measurements. P influx transport was studied in monolayers of RANKL-differentiated RAW264.7 cells in 12-well dishes. Cell densities were \( \sim 1.6 \times 10^4 \) cells per well. The measurement of P influx transport was performed using a modification of a previously described procedure (27). Briefly, cell monolayers were gently washed three times with 0.5 ml of prewarmed (37°C) uptake solution (in mM: 137 NaCl, 5.4 KCl, 2.8 CaCl2, 1.2 MgCl2, and 10 HEPES-Tris, pH 7.4) in the presence or absence of various inhibitors. For pH 5.5 uptake solution, the HEPES-Tris buffer was replaced with MES-Tris buffer. Plates were incubated for 15 min at 37°C before the uptake assay was performed. The cells were preincubated with various inhibitors for 10 min. P influx transport was initiated by the addition of 0.7 ml of prewarmed (37°C) uptake solution containing 0.1 mM KH2PO4 and 1 \( \mu \)Ci/ml \( \text{32P} \), (PerkinElmer, Bridgeport, CT) in the presence or absence of various inhibitors. For kinetic analysis, cells were incubated with 1 \( \mu \)Ci/ml \( \text{32P} \) and increasing concentrations (0.05–12.8 mM) of \( K_2\text{HPO}_4/\text{KH}_2\text{PO}_4 \), such that the concentration of \( K^+ \) was kept constant by the addition of KCl. Cells were incubated for 10 min at 37°C, and transport was terminated by the addition of 1 ml of ice-cold stop solution (in mM: 137 NaCl, 10 Tris-HCl, pH 7.2).

Glucose uptake measurements were performed to determine whether the changes in osteoclast-like cells were specific to P influx transport. For these experiments, osteoclast-like cells were mixed with 0.5 mM d-glucose and tracer amounts of 1 \( \mu \)Ci/ml \([14C]\)d-glucose. After 10 min (which is within the linear rate phase), the reaction was terminated by addition of 1 ml of ice-cold stop solution.

In each experiment, after three additional washes with 1 ml of ice-cold stop solution, the cells were solubilized by the addition of 0.25 ml of 0.1 N NaOH at room temperature. The cell lysates were added to 2.5 ml of Aquasol-2 (Packard Instruments, Meriden, CT), and \( \text{32P} \), or \( [14C] \)radioactivity was determined using liquid scintillation counting. The protein concentration in lysates was determined using the BCA protein assay kit (Pierce Chemical, Rockford, IL). P, and glucose transport were calculated as nanomoles of \( \text{32P} \), or \([14C] \)per milligram of protein taken up in 10 min. To test the ion dependence of P transport, the NaCl in the uptake buffer was replaced with ChCl.

Finally, for experiments to examine the effect of bone, cells were incubated for 1 h with 600 \( \mu \)g/ml bone particles (8). After cells were washed four times with prewarmed uptake solution, \( \text{32P} \) uptake was assessed. All experiments were performed in triplicate and repeated two to four times.

Optical measurement of intracellular pH. Osteoclasts derived from RAW264.7 cells were washed three times with HEPES buffer (in mM: 153 NaCl, 5 KCl, 5 glucose, and 20 HEPES, pH 7.4) and then incubated with 5 \( \mu \)M BCECF-AM (Dojindo, Kumamoto, Japan) for 30 min at 37°C. The cells were then washed three times with the same buffer to remove unincorporated BCECF-AM. Single-cell measurements of intracellular pH (pHi) were performed using a fluorescence spectrophotometer (model 650-10MS; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 500 and 530 nm as described previously (21). pHi was measured at 5-min intervals after addition of 2 mM K2HPO4/KH2PO4, pH 7.4, in HEPES buffer. Time 0 measurements were performed just before the addition of K2HPO4/KH2PO4. The medium was replaced with P-free medium after 15 min. The resulting fluorescence-to-excitation ratios were converted to pH values using the high-K+/quinercin technique (37). For Na+/K+-free conditions, the NaCl in HEPES-loading buffer was replaced with an equivalent concentration of ChCl.

Statistical analysis. Results are reported as means ± SE for at least three samples. The statistical significance of differences between measured values was determined using ANOVA and InStat software (GraphPad, San Diego, CA). Kinetic parameters of uptake were determined using linear regression analysis of Lineweaver-Burk plots.

RESULTS

Characterization of osteoclast-like cells derived from RAW264.7 cells. To characterize the osteoclast-like cells, we first performed histochemical analysis of RANKL-treated RAW264.7 cells. After 7 days of RANKL treatment, the number of TRAP-positive and multinucleated cells was markedly increased compared with cells treated for 24 h (Fig. 2A). TRAP-positive cells were not detected in untreated RAW264.7 cells after 7 days (data not shown). RT-PCR for the CTR, which mediates calcitonin-stimulated bone resorption by mature osteoclasts, demonstrated ~90% differentiation to osteoclasts (Fig. 2B). Immunostaining of the osteoclast-like cells using a CTR-specific antibody showed staining at the cell surface (Fig. 2C).
Expression of Na⁺-dependent Pᵢ transporters in osteoclast-like cells. We investigated the expression of Na⁺-dependent Pᵢ transporter transcripts in osteoclast-like cells. As shown in Fig. 3, RT-PCR analysis did not detect transcripts for the NaPi type I, type IIA, type IIB, or type IIC cotransporter in either the untreated or the RANKL-treated RAW264.7 cells. However, mRNA for the NaPi type III cotransporter (the amphotropic murine retrovirus, Ram-1, and the gibbon ape leukemia virus, Glvr-1) were expressed in both cells, but the levels of the transcript were the same in treated and untreated cells. Similar results were also found in primary osteoclast cells derived from mouse bone marrow cells (Fig. 3A). To detect of the type IIA NaPi cotransporter transcript in osteoclasts, we performed PCR Southern blot analysis using different combination of primers (Fig. 3B). The expression of type IIA transporter was detected in both primary and RAW264-derived osteoclast cells, but the levels of the transcript were the same in treated and untreated cells. Immunohistochemical analysis revealed the mouse type III transporter in the plasma membrane of the osteoclast-like cells, but the type IIA NaPi cotransporter was not detected (Fig. 3C). We further investigated whether the immunoreactive signals for the type IIA NaPi cotransporter could be observed in the plasma membrane using Western blot analysis. The mouse type IIA NaPi cotransporter did not react with the plasma membrane fraction when the antibodies to the NH₂ or COOH terminus of type IIA NaPi cotransporter were used (data not shown).

Effect of RANKL on Pᵢ transport in RAW264.7 cells. We next characterized how the differentiation of RAW264.7 cells to osteoclast-like cells by RANKL affected the activity and properties of Pᵢ transport. Pᵢ transport activity was assayed by measuring the uptake of ³²Pᵢ at pH 7.4. As shown in Fig. 4A, Pᵢ transport activity was slightly increased (1.2-fold) in the presence of the Na⁺ ion in the RANKL-induced osteoclast-like cells compared with untreated RAW264.7 cells (control). In contrast, in the absence of Na⁺ ion (i.e., ChCl in place of NaCl), Pᵢ transport activity was increased ~4.9-fold in the RANKL-treated cells compared with the untreated RAW264.7 cells. On the basis of the observation that Na⁺-independent Pᵢ transport activity was increased in the RANKL-induced osteoclast-like cells, we suggest that types I, II, and III NaPi cotransporters are not involved in the upregulation of Pᵢ trans-
port in the osteoclast-like cells. In addition, the results suggest that Na\(^+\)-dependent transport activity in untreated RAW264.7 cells may be contributed by the type III NaPi cotransporter.

In the absence and presence Na\(^+\) ion, P\(_i\) transport activity in osteoclast-like cells was markedly increased at pH 5.5 and gradually decreased as the pH became more alkaline (Fig. 4, B and C). In contrast, in untreated RAW264.7 cells, P\(_i\) transport activity was increased at pH 6.5 and 7.5 in the presence of Na\(^+\)-free solution. P\(_i\) uptake was measured in 137 mM ChCl uptake solution at pH 5.5, 6.5, 7.5, and 8.5. C: pH dependence of P\(_i\) uptake in Na\(^+\)-free solution. P\(_i\) uptake was measured in 137 mM ChCl uptake solution at pH 5.5, 6.5, 7.5, and 8.5. D: typical time course of P\(_i\) uptake in osteoclast-like cells (circles) or untreated RAW264.7 cells (triangles). The P\(_i\) influx into each cell was measured at 37°C in 137 mM NaCl medium, pH 7.5, in the presence of 0.1 mM PO\(_4\). E: effects on osteoclast differentiation on D-glucose uptake. \[^{[14C]}\]D-glucose was measured in osteoclast-like cells in uptake solution containing 137 mM ChCl, pH 5.5, or in untreated RAW264.7 cells in uptake solution containing 137 mM NaCl, pH 6.5. F: stimulation of P\(_i\) transport by RANKL in conjunction with differentiation. RAW264.7 cells were cultured with RANKL, and P\(_i\) transport was measured after 24 h, 48 h, 96 h, and 7 days. *P < 0.05 compared with 24-h measurement. In all experiments, values represent means ± SE; n = 3. P\(_i\) transport was calculated as nm of \(^{32}\)Pi per mg of protein taken up during a 10-min period. *P < 0.05.

Fig. 4. Characterization of P\(_i\) transport in osteoclast-like cells. A: Na\(^+\) independence of P\(_i\) transport. Uptake of P\(_i\) was measured in cells treated with (solid bars) or without RANKL (open bars). The P\(_i\) influx into each cell was measured at 37°C in 137 mM NaCl or ChCl medium, pH 7.5, in the presence of 0.1 mM PO\(_4\). B and C: pH dependence of P\(_i\) uptake was measured in RANKL-treated (solid bars) and untreated (open bars) cells. B: pH dependence of P\(_i\) uptake in the presence of Na\(^+\). P\(_i\) uptake was measured in 137 mM NaCl uptake solution at pH 5.5, 6.5, 7.5, and 8.5. C: pH dependence of P\(_i\) uptake in Na\(^+\)-free solution. P\(_i\) uptake was measured in 137 mM ChCl uptake solution at pH 5.5, 6.5, 7.5, and 8.5. D: typical time course of P\(_i\) uptake in osteoclast-like cells (circles) or untreated RAW264.7 cells (triangles). The P\(_i\) influx into each cell was measured at 37°C in 137 mM NaCl medium, pH 7.5, in the presence of 0.1 mM PO\(_4\), for 30 min and reached a steady state after 40 min (Fig. 4D). As shown in Fig. 4E, uptake of 0.5 mM glucose in the RANKL-treated RAW264.7 cells was increased compared with the untreated cells, although not as substantially as P\(_i\) transport. The enhancement of P\(_i\) uptake by treatment with RANKL was remarkably time dependent (Fig. 4F), showing increases in parallel with the degree of osteoclastic cell differentiation (Fig. 2A).

Kinetics of P\(_i\) transport in the osteoclast-like cells. To further understand the mechanism of P\(_i\) transport in the osteoclast-like cells, we investigated the kinetic properties of P\(_i\) transport in these cells. As shown in Fig. 5A, we measured P\(_i\) transport at pH 5.5 in Na\(^+\)-free solution using various P\(_i\) concentrations. Kinetic analysis revealed two components of P\(_i\) transport with high and low affinities for P\(_i\). The K\(_m\) for the high-affinity component was 0.35 mM (0–1.6 mM P\(_i\)) (Fig. 5B), and the K\(_m\) for the low-affinity component was 7.5 mM (1.6–12.8 mM P\(_i\)) (Fig. 5C). These transport systems were not found in untreated RAW264.7 cells (data not shown).
Monovalent cation selectivity for activation of $P_i$ transport.

Figure 6A shows the $P_i$ influx into the osteoclast-like cells in the presence of various monovalent cations at equal Cl$^-$ concentrations. When the cation was 137 mM Ch, K$^+$, Na$^+$, Li$^+$, Rb$^+$, or Cs$^+$, K$^+$ was able to activate the influx relative to the other cations. Therefore, we investigated the dependence of $P_i$ transport on K$^+$. As shown in Fig. 6B, the extracellular K$^+$ concentration varied from 0 to 150 mM. The K$^+$ concentration was kept constant by maintaining the sum of KCl and K$^+$-gluconate concentrations at 150 mM. Values represent means ± SE; n = 3.

Fig. 5. Kinetics of $P_i$ transport in osteoclast-like cells. The influx medium contained 137 mM ChCl, and the $P_i$ concentration was varied from 0 to 12.8 mM by addition of K$_2$HPO$_4$/KH$_2$PO$_4$, pH 5.5. A: Michaelis-Menten curve of $P_i$ uptake from 0 to 12.8 mM; inset: Michaelis-Menten curve of $P_i$ uptake from 0 to 1.6 mM. B: Lineweaver-Burk plot for $P_i$ concentrations between 0 and 2 mM. C: Lineweaver-Burk plot for $P_i$ concentrations between 2 and 12.8 mM.

Fig. 6. Activation of $P_i$ influx by monovalent cations in osteoclast-like cells. A: monovalent cation activation of $P_i$ influx was measured in an uptake solution, pH 5.5, wherein NaCl was replaced by ChCl, LiCl, RbCl, CsCl, or KCl. *P < 0.05, statistically significant difference vs. uptake in ChCl uptake solution. B: dependence of $P_i$ uptake in osteoclast-like cells on the extracellular K$^+$ concentration. The K$^+$ concentration varied from 0 to 150 mM. B: dependence of $P_i$ uptake in osteoclast-like cells on Cl$^-$. The concentration of Cl$^-$ was varied from 0 to 150 mM. The K$^+$ concentration was kept constant by maintaining the sum of KCl and K$^+$-gluconate concentrations at 150 mM. Values represent means ± SE; n = 3.
K\(^+\) ion stimulated Pi transport in a concentration-dependent manner. Furthermore, as shown in Fig. 6C, Pi transport activity was not affected by changes in the Cl\(^-\) concentration. These results suggest that Pi transport activity in the osteoclast-like cells is stimulated in the presence of increasing concentrations of K\(^+\) ions.

**Effect of Pi transport inhibitors on Pi transport activity in the osteoclast-like cells.** To further investigate the characteristics of Pi transport in osteoclast-like cells, we examined the effect of Pi transport-specific inhibitors. As shown in Fig. 7A, phosphonoformic acid (PFA; 1 mM), a competitive inhibitor of Pi transport, significantly inhibited Pi uptake in the osteoclast-like cells in the presence and absence of Na\(^+\) (56.3 and 32.6% inhibition, respectively). In the kidney and intestine, inhibition of Pi transport by PFA requires the presence of Na\(^+\) ion (19, 35). However, in the osteoclast-like cells, PFA affected Pi transport activity in the absence of Na\(^+\). We next examined the effect of arsenate (2 mM), a competitive inhibitor of Pi, (5, 35), which also inhibited Pi uptake (89.3% inhibition) in the osteoclast-like cells (Fig. 7B). Furthermore, phosphonoacetic acid (PAA; 1 mM) inhibited (90% inhibition) Pi transport in the osteoclast-like cells (Fig. 7C). In contrast, PAA is only a weak inhibitor of the type IIA NaPi cotransporter (38). Similar effects were shown in the cells preincubated with these inhibitors for 30 min to 2 h before uptake (data not shown). Thus the effects of PAA and PFA on Pi inhibition in osteoclast cells are distinct from their effects on the type IIA NaPi cotransporter in kidney and intestine.

**Effect of proton ionophore on Pi transport in the osteoclast-like cells.** To further investigate the connection between the Pi transport system and osteoclast function, we used several inhibitors of ion channels or transporters that are associated with osteoclast function (Fig. 7D). Pi transport activity in the osteoclast-like cells was not inhibited by acetazolamide, a specific inhibitor of carbonic anhydrase II. In addition, Pi uptake was inhibited by N-ethyl maleimide (NEM) but not by bafilomycin A1, which are specific inhibitors of the V-type ATPase. This suggests that the effects of NEM on Pi transport were mediated by components other than the V-type ATPase. Amiloride and omeprazole, which block epithelial proton-dependent transport, such as the Na\(^+\)/H\(^+\) exchanger, slightly affected Pi transport activity. Furthermore, the anion transport inhibitors, 4,4'-disulfonic acid (DIDS) and DNDS, did not affect Pi transport activity. In contrast, the proton ionophores, nigericin and FCCP, and the K\(^+\) ionophore, valinomycin, significantly decreased Pi transport activity. These observations suggest that a proton gradient is necessary to transport Pi into the osteoclast-like cells.

**Proton-dependent Pi transport in osteoclast-like cells.** To further investigate whether the Pi transport system in the

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Fig. 7. Effect of specific inhibitors of Pi transporters. A: effects phosphonoformic acid (PFA) on osteoclast-like cells. Pi uptake was measured in the presence of 0–5 mM PFA in uptake solution. Experiments were conducted at pH 5.5 in the presence of 0.1 mM PO\(_4\) and 137 mM ChCl or NaCl. B: inhibition of Pi uptake by arsenate. Various concentrations of arsenate were added to uptake solution containing ChCl, and Pi uptake was measured at pH 5.5. C: inhibition of Pi uptake by phosphonoacetic acid (PAA). Various concentrations of PAA were added to uptake solution containing ChCl, and Pi uptake was measured at pH 5.5. D: pharmacology of inhibition of Pi influx in osteoclast-like cells. The ability of various channel and transporter inhibitors to inhibit the Na\(^+\)-free Pi uptake was assessed in osteoclast-like cells. Drug treatment included 0.2 mM N-ethyl maleimide (NEM), 1 μM bafilomycin A1, 0.5 mM acetazolamide, 0.2 mM 4,4'-disulfonic acid (DIDS) and DNDS, 1 mM amiloride, 1 mM 4,4'-dinitrostilbene-2,2'-disulfonic acid disodium salt (DNDS), 5 μM nigericin, 1 μM valinomycin, 0.5 mM FCCP, and 0.5 mM omeprazole for 10 min before uptake and upon uptake. The values of Pi uptake are indicated as the percentage of uptake in the absence of inhibitors. Values represent the means ± SE; n = 3. *P < 0.05. **P < 0.01.
osteoclast-like cells is mediated by a proton-coupled transport system, we measured the pH changes induced by Pi. For this purpose, we used the BCECF fluorescence technique (21, 37) to determine pH changes in monolayers of RANKL-induced RAW264.7 cells. As shown in Fig. 8A, after an initial period of stabilization in NaCl buffer at pH 6.5, the addition of 2 mM K2HPO4 induced a slight decrease in pH within 5 min. When the initial external pH was 5.5, the changes in pH were larger. In both cases, upon removal of K2HPO4, the pH returned to the initial value. Although the decline of pH in the presence of 2 mM K2HPO4 was Na+/H10O1 independent, the recovery to the initial pH 6.5 was not as fast in the absence of Na+/H10O1 (Fig. 8B).

These results suggest that H+/H10O1 flowed into osteoclast-like cells along with Pi in a manner that was unaffected by Na+/H10O1.

Fig. 8. Proton-dependent Pi transport. To measure H+/H10O1-dependent Pi uptake, intracellular pH (pHi) changes were measured in osteoclast-like cells at pH 5.5 (squares) or 6.5 (circles) in Na+/H10O1-containing (153 mM NaCl; A) and Na+/H10O1-free (153 mM ChCl; B) HEPES-buffered solution. pHi was measured at 5-min intervals. The cells were first bathed in a Pi-free HEPES-buffered solution and then incubated in 2 mM Pi-containing HEPES-buffered solution. Time 0 represents the measurement before addition of the Pi-containing solution. After 15 min, the medium was replaced with fresh Pi-free HEPES-buffered solution.

Effect of bone particles on Pi transport in the osteoclast-like cells. Because it was previously shown that Na+/H10O1-dependent Pi uptake was increased by adding bone particles (8), we next investigated whether H+/H10O1-dependent Pi transport activity also would be increased by incubation with bone particles. As shown in Fig. 9, bone particles stimulated Pi transport activity in the osteoclast-like cells. Further analysis showed that the stimulation by bone particles was dose dependent, and a time course study demonstrated that Pi transport increased twofold within 10 min after the addition of the bone particles (data not shown).

DISCUSSION

In the present study, we have characterized the Pi transport properties of osteoclast-like cells that were derived from RAW264.7 cells by treatment with RANKL. The Pi transport system in osteoclast cells has not been well characterized, owing to the difficulty in obtaining sufficient numbers of mature, freshly isolated osteoclasts. In addition, osteoclastogenesis is influenced by a variety of factors that could differ between preparations, resulting in variability in cell cultures. We found that RANKL causes the time-dependent increase in the activity of the H+/H10O1-dependent and voltage-sensitive Pi transport system. PFA, arsenate, and PAA, specific inhibitors of Pi transport, also inhibited the transport system in the osteoclast-like cells. In addition, these cells expressed two components (high and low affinity for Pi) of H+/H10O1-dependent Pi transport. The values for the dissociation of Pi into H2PO4− and HPO42− are 2.1 and 7.2 pK, respectively. Therefore, below pH 6.0, most of the Pi is present as the monovalent H2PO4− species.

In the present study of the pH dependency of Pi uptake into the osteoclast-like cells, we have demonstrated that transport rates are the highest between pH 5.0 and 5.5, where the H2PO4− species is predominant. This suggests that Pi is taken up as H2PO4− across the plasma membrane in the osteoclast-like cells.

Fig. 9. Stimulation of Pi uptake activities by bone. Osteoclast-like cells were exposed to bone particles for 1 h before Pi uptake was measured. Cells that were not exposed to bone served as the untreated control. Pi uptake was measured for 10 min at pH 5.5 in ChCl uptake solution. Values represent the means ± SE; n = 3. *P < 0.05.
Some initial findings were reported by Gupta and colleagues (8, 9), who showed that the type Iia NaPi cotransporter is expressed in various primary osteoclasts and osteoclast-like cells. In the present study, we examined the expression of the type Iia NaPi cotransporter in osteoclast-like cells that were generated by treatment of mouse RAW264.7 cells with RANKL. Expression of the type Iia NaPi cotransporter was detected in these cells or in murine bone marrow-derived cells. However, the subcellular distribution of type Iia NaPi cotransporter detected using immunohistochemical analysis and protein expression examined using Western blotting were negative. We do not know the reason for this apparent discrepancy with the previous work of Gupta and colleagues (8, 9). One possibility may be due to the culture conditions used in the present study (i.e., charcoal-stripped FBS). On the other hand, Npt2a−/− mice showed several skeletal abnormalities, including reduced osteoclast number. These results suggest that the type Iia NaPi cotransporter gene plays a role in osteoclasts (10, 16). Npt2a−/− mice showed several skeletal abnormalities, including reduced osteoclast number. Because PTH increases osteoclast number and activity, reduced serum PTH levels in Npt2a−/− mice may contribute to compromised osteoclast function and a consequent bone-remodeling defect. Further studies are needed to clarify the role of Npt2 in the mouse osteoclast.

In contrast to the type Iia NaPi cotransporter, the type III NaPi cotransporter may play a role in osteoclast-like cells (17). We also found that Na⁺-dependent Pi transport in the untreated RAW264.7 cells may be mediated by the type III NaPi cotransporter (Ram-1 and Glvr-1). However, the type III Na⁺-dependent Pi transporter was not increased by treatment of the RAW264.7 cells with RANKL. The present studies have indicated that the H⁺-dependent Pi transporter, but not the type III NaPi cotransporter, is upregulated in the osteoclast-like cells.

On the other hand, the H⁺-dependent Pi transport system has been reported in sheep and goat intestine (13, 32). In ruminants, the salivary glands are the major organs responsible for endogenous Pi secretion entering the gut. The daily secretion rate is 10–16 L/day for sheep and 30–50 L/day in cows, containing 16–40 mmol/l orthophosphate, that is, ~200–300 mmol/day (13). This amount of Pi greatly exceeds that supplied by the diet (50–60 mmol/day), and therefore an efficient scavenging system for the absorption of Pi, in the gut and secretion via saliva in ruminants is essential and of major significance (13). In these ruminants, the physiological relevance of the H⁺-dependent Pi transport system could be reflected by the lower pH values in the digesta of the upper small intestine (13). The H⁺-dependent Pi transport system is essential for efficiency in Pi intake. In the duodenum of goat, the transporter affinity for Pi (Kₐ 0.32 ± 0.33 mM) was very similar to that of mouse osteoclasts (13). The existence of the H⁺ rather than Na⁺ coupling for Pi transport in the osteoclast may represent the use of an alternative cation substrate. Hirayama et al. (11) showed that H⁺ can substitute for Na⁺ in driving sugar transport through the intestinal Na⁺-glucose cotransporter (SGLT1). Therefore, it could be that the difference in the cation specificity of the Pi transporter in kidney (Na⁺ dependent) and osteoclast (H⁺ dependent) is a mechanistic adaptation and a structural modification in the cation binding sites of Pi transporters in response to extracellular pH (13).

What is a role of the H⁺-dependent Pi transport system in the osteoclast-like cells? One of the most important roles of the transport system is ATP production for the secretion of protons (28, 36). The osteoclast consumes large amounts of energy to drive HCl secretion, which produces Ca²⁺, water, and Pi, from the strongly basic CaPO₄ salt hydroxyapatite, [Ca₅(P0₄)₃]·Ca(OH)₂, which comprises bone minerals. The source of the energy for this secretion is the oxidative phosphorylation of glucose in the mitochondria. Protons are derived from carbonic acid, a process facilitated by high expression of carbonic anhydrase II in osteoclasts. In addition, the extensive proton secretion in osteoclasts is mediated by high expression levels of the V-type ATPase (Fig. 10). Because this process is energy intensive, osteoclasts contain large numbers of mitochondria. Therefore, some of the Pi released by bone resorption may be taken up through osteoclast Pi transport processes and used for ATP production.

The low-affinity Pi transport system that we have identified in the present study may be involved in the removal of degraded products. The basic building blocks of bone are proteins, such as collagen, and hydroxyapatite, which is dissociated to Ca²⁺ and HPO₄²⁻ under acidic conditions. Recent reports have indicated that the products of acidic bone degradation are trafficked by the osteoclast (24, 29, 34). Vesicular

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Fig. 10. Hypothesis regarding Pi transporters in osteoclast-like cells. The osteoclast consumes large amounts of energy to drive HCl secretion, which produces Ca²⁺, water, and phosphate from the strongly basic CaPO₄ salt hydroxyapatite, [Ca₅(P0₄)₃]·Ca(OH)₂, that comprises bone minerals. The primary cellular mechanism responsible for this acidification is active secretion of protons by the vacuolar-type H⁺-adenosine triphosphatase (V-type ATPase), which is localized in the ruffled border of the osteoclasts. Pi influx has been reported to require extensive V-type ATPase activity and thus a large amount of energy. Pi influx may also help maintain the ATP content during the cyclical processes of migration, attachment, and resorption. At least three NaPi transporters (type Iia, type III, and H⁺ dependent) are expressed in the plasma membrane of RANKL-induced osteoclast-like cells.
transcytosis is important in bone-resorbing osteoclasts. In contrast, during bone resorption, a large amount of Ca\(^{2+}\) (up to 40 mM) and Pi ion is generated within the osteoclast hemivacuole (26, 33). The precise mechanisms involved in the disposal of Ca\(^{2+}\) are not clear. Recently, Berger et al. (2) demonstrated that the Ca\(^{2+}\) produced in the resorption hemivacuole is continuously transported out of the resorptive site. Moreover, Stenberg and Horton (34) reported a critical role of the microtubule network in transport for trafficking events. These in situ studies suggest that in a bone-resorbing osteoclast, a relatively large amount of Ca\(^{2+}\) enters from the resorption hemivacuole into the cell and is continuously released at the basolateral plasma membrane. There are likely to be three routes of Ca\(^{2+}\) disposal: leakage, bulk transcytosis, and selective disposal involving channels and transporter (2, 7, 26, 29). We suspect that the low-affinity Pi transport system may be involved in the transcellular Pi transport system, providing Pi and degraded products for release as well as movement of Pi through the cytoplasm.

Finally, in the present study, we have demonstrated the characteristics of the Pi transport system in osteoclast-like cells. This transport system is activated at acidic pH and has \(K_m\) for Pi of \(\sim 0.35\) mM and 7.5 mM. The activity of the two transport components is enhanced by RANKL and bone particles. Further studies must be performed to determine the molecular identity of these transporters and their roles in Pi transport in the osteoclast.

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