Mechanism and role of high-potassium-induced reduction of intracellular \( \text{Ca}^{2+} \) concentration in rat osteoclasts

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Kajiya, Hiroshi, Fujio Okamoto, Hidefumi Fukushima, Keisuke Takada, and Koji Okabe. Mechanism and role of high-potassium-induced reduction of intracellular \( \text{Ca}^{2+} \) concentration in rat osteoclasts. Am J Physiol Cell Physiol 285: C457–C466, 2003.—Osteoclasts are multinucleated, bone-resorbing cells that show structural and functional differences between the resorbing and nonresorbing (motile) states during the bone resorption cycle. In the present study, we measured intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]) in nonresorbing vs. resorbing rat osteoclasts. Basal [\( \text{Ca}^{2+} \)], in osteoclasts possessing pseudopodia (nonresorbing/motile state) was around 110 nM and significantly higher than that in actin ring-forming osteoclasts (resorbing state, around 50 nM). In nonresorbing/motile osteoclasts, exposure to high K\(^+\) reduced [\( \text{Ca}^{2+} \)], whereas high K\(^+\) increased [\( \text{Ca}^{2+} \)], in resorbing state osteoclasts. In nonresorbing/motile cells, membrane depolarization and hyperpolarization applied by the patch-clamp technique decreased and increased [\( \text{Ca}^{2+} \)], respectively. Removal of extracellular \( \text{Ca}^{2+} \) or application of 300 \( \mu \text{M} \) La\(^{3+}\) reduced [\( \text{Ca}^{2+} \)]; to ~50 nM in nonresorbing/motile osteoclasts, and high-K\(^+\)-induced reduction of [\( \text{Ca}^{2+} \)] could not be observed under these conditions. Neither inhibition of intracellular \( \text{Ca}^{2+} \) stores or plasma membrane \( \text{Ca}^{2+} \) pumps nor blocking of L- and N-type \( \text{Ca}^{2+} \) channels significantly reduced [\( \text{Ca}^{2+} \)]. Exposure to high K\(^+\) inhibited the motility of nonresorbing osteoclasts and reduced the number of actin rings and pit formation in resorbing osteoclasts. These results indicate that in nonresorbing/motile osteoclasts, a \( \text{La}^{3+}\)-sensitive \( \text{Ca}^{2+} \) entry pathway is continuously active under resting conditions, keeping [\( \text{Ca}^{2+} \)] high. Changes in membrane potential regulate osteoclastic motility by controlling the net amount of \( \text{Ca}^{2+} \) entry in a “reversed” voltage-dependent manner, i.e., depolarization decreases and hyperpolarization increases [\( \text{Ca}^{2+} \)].

membrane depolarization; resorbing and motile activities; bone resorbing cycle

OSTEOCLASTS are tartrate-resistant acid phosphatase (TRAP)-positive, multinucleated cells that alter their structure and function during the bone resorption cycle. In the resorbing state, osteoclasts adhere to the bone surface via integrin receptors and form ruffled borders and clear zones with a highly polarized cytoplasmic organization. They release protons and secrete lysosomal enzymes across the membrane of this ruffled border and digest both inorganic and organic components of the bone matrix (1, 5, 17). This process increases local \( \text{Ca}^{2+} \) concentration in the resorbed area and, subsequently, intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]). As a result, resorbing activity gradually declines and the osteoclasts turn into nonresorbing/motile cells, which migrate and settle on a new surface area of the bone.

Thus elevation and decline of [\( \text{Ca}^{2+} \)], is very closely related with osteoclastic function. Bizzarri et al. (4) and Moonga et al. (22) demonstrated that calcitonin and interleukin-4, known inhibitors of bone resorption, increase [\( \text{Ca}^{2+} \)] in mouse and rat osteoclasts. Extracellular high-potassium (high K\(^+\)) or extracellular high-calcium (high Ca\(^{2+}\)) solution was also reported to increase [\( \text{Ca}^{2+} \)], (2, 4, 8, 18, 21, 34), suggesting the importance of cell membrane potential and \( \text{Ca}^{2+} \) transport across the membrane for [\( \text{Ca}^{2+} \)] regulation. On the other hand, Shankar et al. (27) reported conflicting results, in that both membrane depolarization induced by high-K\(^+\) solution and hyperpolarization induced by valinomycin increased [\( \text{Ca}^{2+} \)]. Furthermore, Miyauchi et al. (21) demonstrated that high-K\(^+\) solution reduced [\( \text{Ca}^{2+} \)], in osteoclasts attached to bone particles.

These reports lead us to hypothesize that the change in [\( \text{Ca}^{2+} \)], may be state dependent (i.e., differing in resorbing vs. nonresorbing osteoclasts). To test this hypothesis, we examined the effect of high-K\(^+\) solution in resorbing vs. nonresorbing osteoclasts, because high-K\(^+\) stimulation was demonstrated to induce both increases and decreases of [\( \text{Ca}^{2+} \)].

MATERIALS AND METHODS

Osteoclast preparation. The procedure for isolation and culture of osteoclasts was slightly modified from the original methods published by Chambers et al. (6). Briefly, neonatal Wistar rats (1–2 days old) were anesthetized with diethyl ether and killed by cervical dislocation. The femora and tibiae were isolated. The procedures were approved by the Council on Animal Care of the Fukuoka Dental College. Adherent soft tissues were removed, and the bones were cut across their epiphyses in culture medium [\( \alpha \)-minimum essential medium (\( \alpha \)-MEM), GIBCO BRL, Grand Island, NY] con-
containing 15% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Osteoclasts were mechanically disaggregated by cueing bone fragments into α-MEM and agitating the suspension with a pipette. The supernatant (10^6–10^7 cells/coverslip) was then dispersed onto 5 × 5-mm glass coverslips, and cells were allowed to settle and attach for 30 min at 37°C. In some experiments, the supernatant was dispersed onto dentine slices (4-mm diameter) to assess bone resorption activity. Contaminating cells were removed by rinsing the coverslips with α-MEM. Osteoclasts were easily identified by their large size and multinucleated shape under a phase-contrast microscope. The cells were incubated with culture medium in 5% CO2-95% air at 37°C (pH 7.4). Under these conditions, >60% of osteoclasts formed actin rings on glass coverslips or dentine slices after culture for 4 h. All experiments were performed within 4–12 h after cell isolation.

[Ca^{2+}]i measurements. [Ca^{2+}]i was measured in single osteoclasts by a dual excitation ratiometric method using the fluorescent calcium indicator fura 2. Osteoclasts adhering to glass coverslips were incubated for 40 min at 25°C with 2 μM fura 2-AM in a physiological salt solution (PSS) containing (in mM) 140 KCl, 3 NaCl, 10 HEPES, 2.5 CaCl2, 0.5 MgCl2, and 10 glucose, pH adjusted to 7.3 with Tris. Ca^2+ was determined to pass through a dicroic mirror (510 nm), and the emitted light was filtered at 520–560 nm detected using photomultiplier (SPEX Industries). The fluorescence intensities at 340 and 493 nm excitation were displayed. At the end of the experiments, cells were stained by TRAP to conclusively identify them as osteoclasts.

Actin microfilament and TRAP assay. Actin microfilaments were detected by binding of rhodamine-conjugated phalloloid (R-PHD) to F-actin. Osteoclasts, cultured in α-MEM on coverslips (5 × 5 mm) for 6 h after isolation, were incubated for 15 min in PSS or test solution. Osteoclasts on coverslips were washed with PSS and fixed with 10% formaldehyde at room temperature for 20 min. After being washed in PSS for 3 min, cells were permeabilized with 0.1% Triton X-100 for 10 min and rinsed with PSS.

To positively identify osteoclasts, cytochemical staining for TRAP was performed. TRAP-stained osteoclasts were then incubated for another 20 min at 37°C with 5 U/ml R-PHD to stain for F-actin. Coverslips were then washed and inspected under a fluorescence microscope (Nikon, Tokyo, Japan). To obtain rhodamine fluorescence, the emitted fluorescence was deflected to pass through a dicroic mirror (550 nm), and the transmitted light was filtered at 580 nm. The number of osteoclasts having actin rings was expressed relative to the total number of TRAP-positive multinucleated cells (MNCs; cells with three or more nuclei).

Bone resorption assay. Resorption activity was assessed by counting the pit formations on dentine slices. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2-95% air. To examine the effect of high K+ on resorption activity, 50 mM KCl was added to the culture medium. After 48 h, the osteoclasts on the dentine slices were fixed with 10% formaldehyde and stained with TRAP, and the number of MNCs was counted. The cells were then peeled from the dentine slices by ultrasonication in 0.25 M ammonium hydroxide, and resorption pits were stained with Mayer's hematoxylin solution. Finally, the number of pits was counted under a phase-contrast microscope. The ratio of resorption pits per total number of MNCs was used as an indicator of osteoclast resorption activity.

Measurement of osteoclast motility. Coverslips with adherent osteoclasts were placed in a temperature-controlled chamber (volume of 1 ml) and superfused continuously at 1 ml/min with α-MEM with 5 mM HEPES, adjusted to pH 7.3.
with Tris. To assess cellular motility, cell surface areas were recorded on video using a charge-coupled device camera (CS38310; Tokyo Electronic Industry, Tokyo, Japan) mounted to a phase-contrast microscope (TMD-300; Nikon, Tokyo, Japan). Images were stored in a time-lapse videorecorder (KV-7168, Toshiba, Japan) and digitized to a computer (Macintosh Computer 8500/120; Apple Japan) at 2-min intervals over a 60-min period. NIH Image software was used for image acquisition, processing, and measurement of cell surface area. Cell motility was assessed as relative changes in membrane surface area. Membrane surface area \( A(t) \), at time \( t \) (in minutes), was quantitated by tracing the outlines of every osteoclast in the viewing field. Surface area was normalized to the value before high-K\( ^+ \) stimulation (100%; Fig. 3B; \( t = -10 \) min). A second measurement was taken 2 min after intervention, \( A(t+2) \). The nonoverlapping area \( A(t+2)-A(t) \) between these two successive surface areas was also measured, and the relative change in areas \( [A(t+2)-A(t)]/A(t) \) was used as an index of cell motility. The effect of membrane depolarization on cell motility was tested by exposure to high-K\( ^+ \) medium (addition of 50 mM KCl in α-MEM) and assessed by comparing the motility before, during, and after application of high-K\( ^+ \) solution. In some experiments, to prevent the hyperosmolality caused by addition of 50 mM KCl, α-MEM with high K\( ^+ \) was prepared by replacing 50 mM NaCl in α-MEM with an equimolar amount of KCl using a made-to-order salt-deficient α-MEM (Nikkon Biomedical Laboratories, Kyoto, Japan).

Chemicals. Fura 2-AM was obtained from Dojindo (Kumamoto, Japan). Ionomycin was purchased from Calbiochem-Novabiochem International (San Diego, CA). DiBAC\(_4\)(3) was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical.

Drugs were dissolved in dimethyl sulfoxide (DMSO) and later diluted to final concentration in the PSS. Final DMSO concentration was 0.1% or less.

Statistics. Data are expressed as means ± SE. The level of statistical significance was estimated by ANOVA, and \( P \) values <0.05 were considered as statistically significant.

RESULTS

Morphological properties of resorbing and nonresorbing osteoclasts and the effect of high-K\( ^+ \) solution on bone resorption activity. There are two types of MNCs and TRAP-positive cells adherent to glass coverslips after 6 h of cultivation (Fig. 1Aa). These two types of cells are distinguished by their shape and ability to form actin rings (Fig. 1Ab). One type has a large, flat, and round shape and displays F-actin distinctly organized in so-called actin rings (Fig. 1Ab, white arrowhead). The other type has a more bulgy shape with serrated edges, and actin rings are not observed (Fig. 1Ab).

There was an average of 71.9 ± 1.7 (means ± SE) TRAP-positive MNCs per coverslip (73 coverslips examined). Without stimulation (i.e., control), 61.9 ± 2.5% (average 43 cells/coverslip) of these TRAP-positive MNCs possessed an actin ring (Fig. 1Ba). Two days of cell cultivation on dentin slices, instead of glass coverslips, decreased both the number of TRAP-positive MNCs and the proportion of TRAP-positive MNCs displaying actin rings: 41.7 ± 3.8 TRAP-positive MNCs and 13.6 ± 3.1 TRAP-positive MNCs possessed actin rings on average, and 22.4 ± 3.5 pits were counted (10 dentine slice preparations, Fig. 1Bb, left). Exposure to 100 mM K\( ^+ \) solution abolished the ring formation within a few minutes, and only 28.4 ± 3.4% of MNCs

Fig. 1. A: morphological characteristics of resorbing and nonresorbing/motile state rat osteoclasts. a: tartrate-resistant acid phosphatase (TRAP) activity of osteoclasts on glass. b: the same cell as in Aa stained with rhodamine-conjugated phalloidin (R-PHLD). Arrowheads indicate actin ring formation. B: effect of high K\( ^+ \) on bone resorption activity. a: effect of high K\( ^+ \) in the presence (2.5 mM) or absence (0.1 mM EGTA) of Ca\(^{2+}\) and ionomycin (5 μM) on actin ring formation. Ordinate expresses the percentage of TRAP-positive multinucleated cells (MNCs) displaying actin ring from total number of MNCs. b: relationship between number of actin rings and pits formed in TRAP-positive MNCs. MNCs were cultured for 48 h on dentine slices. In Ba and Bb, each column indicates means ± SE, and number of experiments for each condition is shown in parenthesis. **P < 0.01.
remained actin ring positive after 15 min (glass cover-slips, \( P < 0.05 \); Fig. 1Ba, middle). Actin rings were reorganized after 30- to 60-min superfusion with PSS, and percent values were restored to initial levels.

When the cells were exposed to \( \text{Ca}^{2+} \)-free high-K\(^+\) solution, 50.6 ± 3.4\% of TRAP-positive MNCs possessed actin rings, a value similar to control and significantly higher than that observed in \( \text{Ca}^{2+}\)-containing high-K\(^+\) solution \( (P < 0.05) \). Application of 5 \( \mu \text{M} \) ionomycin, a \( \text{Ca}^{2+} \) ionophore, completely inhibited actin ring formation \((0.16 \pm 0.51\% \text{ of TRAP-positive MNCs})\); \( P < 0.01 \); Fig. 1Ba). When MNCs were cultured in \( \alpha\)-MEM with 50 mM KCl, the number of rings and pits was reduced to 2.75 ± 1.2 and 5.38 ± 2.6 \((n = 19)\), respectively \((P < 0.01) \). Because this solution has increased osmolality, we tested the effect of 50 mM NaCl added and found that cultivation of MNCs within \( \alpha\)-MEM plus 50 mM NaCl did not alter actin ring or pit formation. Thus the inhibition of resorbing activity in MNCs was due to increased extracellular K\(^+\) ([K\(^+\)]\(_o\)) and not caused by hyperosmolality.

For the remainder of this paper, we will refer to TRAP-positive MNCs as osteoclasts.

**Fig. 2.** Effects of high-K\(^+\) solution on intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). A: distribution of basal [Ca\(^{2+}\)]\(_i\) in osteoclasts. B: relationship between basal [Ca\(^{2+}\)]\(_i\) level and changes in [Ca\(^{2+}\)]\(_i\). C: effects of 100 mM K\(^+\) on [Ca\(^{2+}\)]\(_i\) in 2 types of osteoclasts having low and high [Ca\(^{2+}\)]\(_i\) under basal conditions. Video images were recorded before \((a)\) and during \((b)\) application of 100 mM K\(^+\) solution. Each color corresponds to [Ca\(^{2+}\)], as indicated at right. D: time course of [Ca\(^{2+}\)] changes induced by 100 mM K\(^+\) solution in the 2 types of cells. In D, \(a\) and \(b\) mark time of recorded video images of Ca and Cb, respectively.
serrated-edged, nonresorbing state osteoclasts (cell 2) decreased \([\text{Ca}^{2+}]_i\) during superfusion of 100 mM K\(^+\) solution (Fig. 2, Ca and Cb). The time course of \([\text{Ca}^{2+}]_i\) change in the two types of osteoclasts is shown in Fig. 2D. Changes in \([\text{K}^{+}]_o\) were reversible.

Effect of high-K\(^+\)-induced \([\text{Ca}^{2+}]_i\) reduction on osteoclast function. As shown in Fig. 1, round osteoclasts with actin rings were associated with pit formation and elevated \([\text{Ca}^{2+}]_i\), suggesting that \([\text{Ca}^{2+}]_i\) elevation might shift the osteoclasts from the resorbing state to the nonresorbing/motile state. Thus, to investigate the role of \([\text{Ca}^{2+}]_i\) in bulgy osteoclasts with serrated edges, we assessed motile activity, estimated by measuring the two-dimensional change in cell surface area. Replacement of culture medium (\(\alpha\)-MEM) with 5 mM HEPES (pH 7.3) caused retraction of the pseudopodia (Fig. 3Aa and Ab), reduced cellular area by 10\(\%\) (Fig. 3B), and reduced osteoclast motility (Fig. 3C) with maximal effect apparent after 5–10 min. This response was reversible and returned to control values.

Because the osmolality of high-K\(^+\) medium (addition of 50 mM K\(^+\) to \(\alpha\)-MEM) is significantly increased, we also tested the effect of \(\alpha\)-MEM with 50 mM KCl replaced for NaCl on osteoclastic cell area and motility. The inhibitory effects of \(\alpha\)-MEM with high K\(^+\) replaced for NaCl were similar to that of the \(\alpha\)-MEM with 50 mM KCl added (area changed to 93\(\%\) of control; motility changed from 7 to 0.3\(\%\); \(n = 2\)). We also tested whether addition of 50 mM NaCl to \(\alpha\)-MEM would affect cell area or motility. We found that this high-Na\(^+\) medium caused a slight cell shrinking (area changed to 95\(\%\) of control) but had no effect on cell motility (\(n = 2\)). These results indicate that the inhibition of cell motility in osteoclasts was due to increased [K\(^+\)], and not caused by hyperosmolality.

The relationship between membrane depolarization and \([\text{Ca}^{2+}]_i\) reduction in nonresorbing osteoclasts. To determine whether the high-K\(^+\)-induced \([\text{Ca}^{2+}]_i\) reduction was caused by membrane depolarization, we simultaneously recorded membrane potential and \([\text{Ca}^{2+}]_i\) with a dual-probe method (Fig. 4A; see MATERIALS AND METHODS). Application of high-K\(^+\) solution to a nonresorbing/motile state osteoclast produced a rapid increase and decrease of fluorescence intensities at 493 (upper trace) and 340 (lower trace) nm, respectively (4 of 5 examined nonresorbing/motile cells). Although absolute values of the membrane potential and \([\text{Ca}^{2+}]_i\) cannot be obtained simultaneously, these results indicate that there is a clear relationship between membrane depolarization and reduction of \([\text{Ca}^{2+}]_i\), by high-K\(^+\) solution.

To obtain direct evidence that membrane depolarization indeed caused this \([\text{Ca}^{2+}]_i\) reduction, changes in \([\text{Ca}^{2+}]_i\) were recorded under voltage-clamp conditions (patch-clamp technique). Membrane potential was kept at 0 mV after the whole cell patch-clamp configuration was established. Next, membrane hyperpolar-
Consideration of the effects of high-K+-induced increases on the intracellular Ca^2+ concentration in osteoclasts. Application of 0.5 mM thapsigargin, an inhibitor of intracellular Ca^2+ stores, reduced the intracellular Ca^2+ concentration in osteoclasts. This decrease was not significant when thapsigargin was applied for 10 min nor when 300 nM CCCP was applied for 10 min. Thapsigargin and CCCP also had no effect on the high-K+-induced decrease in intracellular Ca^2+ concentration and the high-K+ response (data not shown).

Removal of K^+ or application of a K^+ channel blocker did not further reduce the intracellular Ca^2+ concentration, suggesting that the removal of K^+ was essential for maintaining the high intracellular Ca^2+ concentration in osteoclasts. The removal of K^+ was not due to Ca^2+ uptake into cytoplasmic stores or extrusion into the extracellular space. The removal of K^+ in resorbing-state osteoclasts was increased with elevation of K^+ in the K^+ solution. Although the removal of K^+ in resorbing-state osteoclasts abolished high-K+-induced increases in intracellular Ca^2+ concentration, neither L-type nor N-type Ca^2+ channel blockers nor La^3+-sensitive or DHP-insensitive Ca^2+ influx and not due to Ca^2+ uptake into cytoplasmic stores or extrusion into the extracellular space.

Possible pathway of Ca^2+ entry in osteoclasts during the nonresorbing/motile state. Electrophysiological studies have shown that several ion channels are present in osteoclasts; that is, an inwardly rectifying K^+, outwardly rectifying K^+, and outwardly rectifying Cl^- channels (1, 26, 28, 32). In addition, recent evidence showed the presence of nonselective cation conductances in rat and rabbit osteoclasts (9, 13, 23).
Among these ionic conductances, the nonselective cation channels have been considered to be pathways for Ca\(^{2+}\) flux, because electrophysiological experiments did not show evidence for voltage-dependent Ca\(^{2+}\)-selective channels (25, 26, 28).

In the present study, the predominant membrane current in osteoclasts in the nonresorbing/motile state was the inwardly rectifying K\(^+\) current, which was abolished by Cs\(^+\) in the electrode solution or omission of K\(^+\) in PSS (data not shown). Eight of fifteen cells total exhibited an outwardly rectifying Cl\(^-\) current, which was eliminated by addition of NPPB (50 \(\mu\)M) or DIDS (100 \(\mu\)M) to the bath (data not shown). Voltage-dependent ionic currents such as outwardly rectifying K\(^+\) and voltage-dependent Ca\(^{2+}\) currents were not detected. When both the inwardly rectifying K\(^+\) current and outwardly rectifying Cl\(^-\) currents were eliminated, a remaining background current with slight outward rectification was seen (Fig. 6Aa, labeled Na\(^+\)-rich). Following application of Ca\(^{2+}\)-rich Na\(^+\)-free solution, inward current was reduced with no apparent effect on the outward current (labeled Ca\(^{2+}\)-rich). Application of LaCl\(_3\) (300 \(\mu\)M) produced further inhibition of inward current (Fig. 6Aa; labeled Ca\(^{2+}\)-rich + La\(^{3+}\)), whereas nicardipine (10 \(\mu\)M) did not inhibit this current (data not shown). The current-voltage (I-V) relationship of the La\(^{3+}\)-sensitive current exhibited outward rectification with a reversal potential close to 0 mV (0.8 ± 2.1 mV, \(n = 3\)) (Fig. 6Ab). Slope conductance of the inward current (measured between -100 and 0 mV) in Ca\(^{2+}\)-rich solution (4.04 ± 0.16 pS/pF) was significantly inhibited by addition of La\(^{3+}\) (2.42 ± 0.13 mV, 0.70 ± 0.15 pS/pF, \(n = 3\)).

Fig. 5. Effects of extracellular Ca\(^{2+}\), La\(^{3+}\), thapsigargin, nicardipine, vanadate, and carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on changes in [Ca\(^{2+}\)]\(_i\) induced by 100 mM K\(^+\) solution. A: extracellular K\(^+\) concentration-dependent reduction of [Ca\(^{2+}\)]\(_i\) in osteoclasts in the nonresorbing/motile state (\(n = 3\)). B and C: effects of Ca\(^{2+}\)-free solution (\(n = 20\)) and 300 \(\mu\)M La\(^{3+}\) (\(n = 12\)) on changes in [Ca\(^{2+}\)]\(_i\), induced by 100 mM K\(^+\) solution. D: effects of 5 \(\mu\)M thapsigargin, 1 \(\mu\)M nicardipine, 500 nM vanadate, and 100 nM CCCP on the changes in [Ca\(^{2+}\)]\(_i\), induced by 100 mM K\(^+\) solution. Control indicates the high-K\(^+\)-induced [Ca\(^{2+}\)]\(_i\) decrease (~50 nM in average) before pretreatment with inhibitors and was normalized to 100% for each cell (\(n = 5\); dotted line in D). Cells were then exposed to drugs for 10 min before application of high-K\(^+\) solution. The change in high-K\(^+\)-induced response in the presence of each drug was expressed relative to control (percent of control). Each column indicates means ± SE, and the number of experiments under each condition is shown in parentheses.
brane depolarization and exposure to high K states. Con
stitution in osteoclasts during resorbing and nonresorbing

It was reported that exposure to LaCl₃ from that in Ca²⁺-rich solution
without LaCl₃. B: effects of LaCl₃ on the current recorded in the Ca²⁺-rich solution elicited by a voltage ramp from
−100 to 0 mV. Conductance values are expressed normalized to cell capacitance. Membrane capacitances of
osteoclasts in our experiments ranged from 84 to 106 pF. Data were obtained from 3 cells. **P < 0.01.

DISCUSSION

[Ca²⁺]ᵢ mobilization by high-K⁺-induced depolarization
in osteoclasts during resorbing and nonresorbing states. Conflicting results regarding the effects of mem-
brane depolarization and exposure to high K⁺ on
[Ca²⁺]ᵢ, and Ca²⁺ entry in osteoclasts have been pub-
lished (2, 4, 8, 21, 27). It was reported that exposure to
high K⁺ increases [Ca²⁺]ᵢ in osteoclast-like cells [avian
(21), mouse (4), and rat (27)]. In contrast, Miyachi et
al. (21) reported that high-K⁺ solution reduced [Ca²⁺]ᵢ in
avian osteoclasts after 5 h cultivation if the cells
were incubated in the presence of bone particles. Vali-
nomycin, a K⁺-channel ionophore, increased [Ca²⁺]ᵢ in
rat osteoclasts (27). These observations indicate that
membrane potential changes do not uniformly alter
[Ca²⁺]ᵢ in osteoclasts.

It is well known that osteoclasts differ in their mor-
phological and functional properties between the
resorbing and nonresorbing/motile states of the bone
resorption cycle (15, 17, 18). Resorbing osteoclasts ex-
hibit actin rings without pseudopodia, whereas non-
resorbing/motile osteoclasts have pseudopodia but not
actin rings. In our present experiments, 61% of MNCs
possessed actin rings when cultured on glass coverslip.
As shown in Fig. 2, resorbing state osteoclasts (possess-
ing actin rings) showed low [Ca²⁺]ᵢ, and nonresorbing/
 motile state osteoclasts showed high [Ca²⁺]ᵢ at rest.
High-K⁺ solution increased [Ca²⁺]ᵢ in resorbing osteo-
clasts but reduced [Ca²⁺]ᵢ in nonresorbing osteoclasts.
These results demonstrate the state dependence of
basal [Ca²⁺]ᵢ, and Ca²⁺ response.

Possible mechanisms of high-K⁺-induced [Ca²⁺]ᵢ mo-
bilization in nonresorbing osteoclasts. In nonresorbing
osteoclasts, high K⁺ produced membrane depolariza-
tion and at the same time decreased [Ca²⁺]ᵢ. Because
this decrease of [Ca²⁺]ᵢ, could be observed simulta-
neously with membrane depolarization and mimicked
by direct membrane depolarization via whole cell
patch-clamp method (Fig. 4), a hyperpolarization-de-
pendent Ca²⁺ entry pathway is likely to be present in
osteoclasts during the nonresorbing/motile state.

Miyachi et al. (20) and Yamakawa et al. (33) re-
ported that Arg-Gly-Asp reduced [Ca²⁺]ᵢ, in avian oste-
oclast precursors. This reduction was suppressed by
vanadate, a plasma Ca²⁺ pump inhibitor, but not by
modulators of intracellular Ca²⁺ stores. However, in
our present study, neither vanadate, thapsigargin, nor
CCCP modified the high-K⁺-induced [Ca²⁺]ᵢ reduction,
suggesting either species differences or stimulation-
dependent differences in Ca²⁺ mobilization. In rat os-
teoclasts, either removal of extracellular Ca\textsuperscript{2+} or application of La\textsuperscript{3+}, a nonspecific Ca\textsuperscript{2+} influx blocker, inhibited high-K\textsuperscript{-}-induced reduction in [Ca\textsuperscript{2+}]\textsubscript{i}. These results indicate that in nonresorbing osteoclasts, membrane depolarization switches off a La\textsuperscript{3+}-sensitive Ca\textsuperscript{2+} influx pathway rather than activating Ca\textsuperscript{2+} transport to the extracellular space or into intracellular Ca\textsuperscript{2+} stores.

As shown in Fig. 6, a voltage-dependent Ca\textsuperscript{2+} channel could not be detected, but a La\textsuperscript{3+}-sensitive current component was identified in the background current of our cells. In a preliminary report, mRNAs for TRP-like channels (TRP3 and 6) have been detected in mouse osteoclasts (3). In TRP channel-expressing mammalian cells, TRP conductances in general are nonselective, permeable to Ca\textsuperscript{2+} (or Mg\textsuperscript{2+}) > monovalent cations, show linear (or slightly outwardly rectified) I-V relationship under physiological conditions, and are inhibited by La\textsuperscript{3+} (19). If this background current were mostly carried through TRP channels, then the reversal potential should be more positive because Ca\textsuperscript{2+} was the main charge-carrying cation under the conditions of our experiments. Therefore, the background current seems to be due to other divalent cation conductances. Whereas TRP channels may be latent in the background current, other channels such as La\textsuperscript{3+}-sensitive Ca\textsuperscript{2+}-permeable conductance, or possibly other divalent cation conductances including TRP channel variant phenotypes, are more likely to play a role in the hyperpolarization-induced [Ca\textsuperscript{2+}]\textsubscript{i} entry mechanism. Additional experiments, ideally using single-channel analysis, will be required to clarify these questions.

Bizzarri et al. (4) and Miyauchi et al. (21) demonstrated that high-K\textsuperscript{+} solution increased [Ca\textsuperscript{2+}]\textsubscript{i}, by increasing Ca\textsuperscript{2+} influx via a DHP-sensitive pathway. However, our previous reports showed no evidence for the presence of DHP-sensitive Ca\textsuperscript{2+} channels in rat osteoclasts (25, 26, 28), and the resting membrane potential of resorbing state osteoclasts was high (around −25 mV; Okamoto F, Kajiya H, and Okabe K, unpublished observation). Furthermore, Bizzarri et al. (4) showed that valinomycin, a K\textsuperscript{+} ionophore, reduced [Ca\textsuperscript{2+}]\textsubscript{i} when the membrane of osteoclasts was depolarized by high K\textsuperscript{+}; under these conditions, valinomycin would not be expected to hyperpolarize the membrane because the K\textsuperscript{+} equilibrium potential was kept high by high-K\textsuperscript{+} external solution. It is uncertain whether depolarization induced by high-K\textsuperscript{+} solution is sufficient to elevate [Ca\textsuperscript{2+}]\textsubscript{i} in resorbing state osteoclasts. In these cells, both membrane surfaces are polarized by accumulation of various specific proteins, such as vacular H\textsuperscript{+}-ATPase and CIC7, and create a unique extracellular environment (very high [Ca\textsuperscript{2+}]\textsubscript{i} and very low pH) underneath the osteoclast cell membrane. Thus the intracellular milieu does not simply depend on the extracellular milieu.

We cannot speculate about possible mechanisms for [Ca\textsuperscript{2+}]\textsubscript{i} elevation in resorbing-state osteoclasts; however, this process does not appear to utilize the same Ca\textsuperscript{2+} entry pathway employed by nonresorbing osteoclasts, because elevation of [Ca\textsuperscript{2+}]\textsubscript{i} by high-K\textsuperscript{+} solution in resorbing osteoclasts is abolished by superfusion of Ca\textsuperscript{2+}-free solution, but not La\textsuperscript{3+} (H. Kajiya, unpublished observations). Further studies are required to clarify these issues.

Effects of membrane depolarization on osteoclastic resorbing activity and motility. Many investigators have reported that osteoclasts exhibit actin rings during the bone-resorbing state (14, 15, 30, 35). Actin ring formation in osteoclasts is regulated by signaling pathways mediated by PKA (16, 29), phosphatidylinositol 3-kinase (PI 3-kinase) (24), and intracellular Ca\textsuperscript{2+} (14, 21, 31). In our experiments, high-K\textsuperscript{+} stimulation inhibited actin ring and pit formation on dentine slices. This disruption of ring formation induced by high K\textsuperscript{+} was prevented by using a Ca\textsuperscript{2+}-free extracellular solution. Furthermore, ionomycin, a Ca\textsuperscript{2+} ionophore, also completely inhibited the ring formation in a [Ca\textsuperscript{2+}]\textsubscript{i}-dependent manner. Therefore, we conclude that high-K\textsuperscript{+} solution depolarizes the membrane and inhibits actin ring formation due to elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, through activation of Ca\textsuperscript{2+} entry.

On the other hand, exposure to high K\textsuperscript{+} in nonresorbing/motile osteoclasts reduced motility. It is likely that high-K\textsuperscript{+}-induced reduction of [Ca\textsuperscript{2+}]\textsubscript{i} contributes to suppressing motility. Changes in [Ca\textsuperscript{2+}]\textsubscript{i}, induced by hormones and cytokines regulate cytoskeletal structure, chemotactic migration, and motility of osteoclasts: Grano et al. (10) reported that hepatocyte growth factor increases [Ca\textsuperscript{2+}]\textsubscript{i}, and stimulates chemotactic migration in osteoclast-like cells obtained from human giant cell tumors. Colucci et al. (7) reported that soluble laminin-2 (merosin), which is expressed in basement membranes and binds integrin receptors, increases [Ca\textsuperscript{2+}]\textsubscript{i}, and stimulates the chemotactic migration of human osteoclast-like cells. Miyauchi et al. (20) reported that reduction of cytosolic Ca\textsuperscript{2+} favors actin ring formation. In nonresorbing/motile osteoclasts, higher basal [Ca\textsuperscript{2+}]\textsubscript{i} keeps motile activity high, and inhibition of the La\textsuperscript{3+} sensitive Ca\textsuperscript{2+} influx by membrane depolarization promotes anchoring of the cells onto the bone surface and formation of actin rings.

In conclusion, membrane depolarization in nonresorbing/motile state osteoclasts decreases [Ca\textsuperscript{2+}]\textsubscript{i} via inhibition of La\textsuperscript{3+}-sensitive Ca\textsuperscript{2+} influx. Nonselective cation channels are good candidates for this Ca\textsuperscript{2+} entry system.

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DISCLOSURES

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