Two Types of $K^+$ Currents Underlying Inward Rectification of Rat Osteoclast Membrane: A Single-Channel Analysis

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Abstract: 1) Electrical membrane properties of spread type-osteoclasts were examined using the whole-cell and cell-attached configurations of the patch clamp technique. The membrane potential of the osteoclasts was measured and grouped into two populations: hyperpolarized and depolarized (around −80 and 0 mV, respectively). The current-voltage relation was N-shaped, showing the property of inward rectification which was produced by $K^+$ conductance. Either $Na^+$ or $Ca^{2+}$ inward currents were not observed.

2) The single-channel recordings were made with osteoclasts of the hyperpolarized population. Two types (slow and fast) of the current were analyzed, which appeared predominantly and had large unit conductances. The reversal potential for both types shifted according to the equilibrium potential for $K^+$.

3) The slow type had high open probabilities (≈0.85) at all potentials examined. The mean open time was 349 ms at the resting potential (RP) and had a tendency to become shorter with hyperpolarization of the membrane. The closed time histogram was fitted with a double exponential function, yielding closed times of 8.3 and 55.2 ms at RP, which were not changed by hyperpolarization. The unit conductance was 32.0 pS. No outward currents were recorded at depolarized potentials to RP.

4) The open probability of the fast type was 0.25 at RP and increased with hyperpolarization of the membrane (0.55 at −80 mV to RP). The mean open time was short (2.6 ms at RP), which became longer with hyperpolarization. The channel had two closed times, 2.8 and 15.3 ms at RP, which were not voltage dependent. The unit conductance was 38.8 pS in the range of hyperpolarization from RP. The amplitude of outward currents evoked by voltage pulses beyond RP was small, probably due to rapid flickering of the current.

5) The two types of single-channel $K^+$ currents showed the inwardly rectifying property and may play an important role in the inward rectification seen in the whole-cell configuration. [Japanese Journal of Physiology, 46, 231–241, 1996]

Key words: inward rectification, $K^+$ channel, single-channel recording, osteoclast, rat.

Osteoclasts are multinuclear giant cells which play an important role in resorption and remodeling of bone. The cells recognize the Arg-Gly-Asp sequence of the bone matrix protein with the vitronectin receptor expressed on the plasma membrane [1–3] and are supposed to possess the receptor or sensor which are activated by extracellular divalent cations [4, 5]. After recognition, the osteoclasts adhere to the bone surface, and develop a tightly sealed microenvironment, to which they secrete protons [6, 7] and lysosomal cathepsins [8, 9] through the ruffled border to degrade the bone matrix. Silver et al. [10] measured the pH and $Ca^{2+}$ concentration in the microenvironment formed by osteoclasts attaching on glass Petri dishes and reported that the pH was reduced to as low as 3 and that the $Ca^{2+}$ concentration increased up to 40 mM.

Investigators have described several types of ionc
currents through the cytoplasmic membrane of the osteoclasts, which may help maintain the function of the cell and the intracellular ion condition. The reported currents include: three classes of voltage-activated $K^+$ currents [11], an inwardly rectifying $K^+$ current [12, 13], inwardly rectifying $K^+$ and outwardly rectifying $Cl^-$ currents [14], a $Ca^{2+}$-activated $K^+$ current [15], and an outwardly rectifying $Cl^-$ current [16]. Furthermore, an inward $Ca^{2+}$ current has been suggested with experiments using fluorescent dye [5, 17–19]. Although several authors have emphasized the importance of the inward-rectifying $K^+$ current in osteoclast functions, detailed analyses at the single-channel level have never appeared. In the present study, we confirmed the absence of the inward $Na^+$ and $Ca^{2+}$ currents and the presence of the inwardly rectifying property in the cytoplasmic membrane of the rat osteoclasts. We then analyzed, at the single-channel level, the kinetics of two types of $K^+$ currents which underlie the inward rectification observed in the whole-cell configuration.

**MATERIALS AND METHODS**

1. **Cell preparation.** Osteoclasts were dissociated from Wistar rats with the method described by Chambers and Magnus [20]. Briefly, femora and tibias were dissected from neonatal rats (2–5 d old) under ether anesthesia. Soft tissue and epiphyseal cartilage were removed from the bones in modified minimum essential medium ($\alpha$-MEM) containing 10% heat-inactivated fetal calf serum and antibiotics. The bones were split longitudinally and the bone marrow was curetted out in the medium and gently pipetted with a glass pipette for about 30 s. A few minutes after pipetting the bone marrow in the medium, the cell suspension was plated onto 3.5-cm plastic dishes and then incubated in 5% CO$_2$/95% air at 37°C for 30 min, during which period osteoclasts were allowed to adsorb on the bottom of the dishes. Nonadherent cells were gently washed out with fresh $\alpha$-MEM. The osteoclasts were incubated for another 1.5 to 10 h. For identification of osteoclasts, we used morphological features, retraction in response to calcitonin and tartrate-resistant acid phosphatase (TRAP) staining. All the cells used were spread with lamellipodia (see Arkett et al. [21]).

2. **Electrophysiological measurements and analysis.** The whole-cell and cell-attached configurations of the patch clamp technique were used [22]. Patch electrodes were pulled from 1.5 mm outside diameter and 0.9 mm inside diameter borosilicate glass tubing (Narishige, Tokyo, Japan) on a Narishige PB-7 microelectrode puller. The electrode resistance was 5–8 MΩ in the whole-cell configuration and 10–15 MΩ in the cell-attached mode, when filled with the pipette solution described below. The seal resistance was in the range of 10 to 50 GΩ.

The currents and voltages were measured with an Axopatch 1-C amplifier (Axon Instruments, Burlingame, USA), filtered with 4-pole low-pass Bessel filter (~3 dB at 1 kHz), and displayed on both a digital storage oscilloscope (CS8010, Kenwood, Tokyo) and a thermal array recorder (RTA 1200M, Nihon Kohden, Tokyo) and stored on magnetic tapes with DAT data recorder (RD-120TE, TEAC, Tokyo). The software package P-clamp version 5.1 and MacLab version 3.1 were used for off-line data analysis at a sampling frequency of 1 kHz. Amplitude histograms were fitted with Gaussian curves using a least-squares algorithm, yielding the mean value of the current and the open probability of the channel. The open and closed time histograms were formed in patches where only one channel was active and fitted with the least-squares method to obtain the mean open time and the mean closed time.

3. **Composition of solutions.** The normal external solution (physiological salt solution; PSS) composed of (mM): NaCl, 150; KCl, 5; CaCl$_2$, 2; MgCl$_2$, 0.5; glucose, 10; HEPES, 10. The pH was adjusted to 7.4 with Trisbase. In some experiments, NaCl was partly replaced with KCl to change the equilibrium potential for $K^+$. The TEA-Ba external solution consisted of (mM): tetraethyl ammonium Cl, 140; BaCl$_2$, 10; CsCl, 5; glucose, 10; HEPES, 10; pH 7.4 with Trisbase. The pipette solution contained (mM): KCl, 150; MgCl$_2$, 2; Na$_2$ATP, 2; EGTA, 5; CaCl$_2$, 0.25; HEPES, 10; pH 7.2 with Trisbase.

In some experiments with the cell-attached configuration for recording of the single-channel currents, the external solution composed of (mM): KCl, 150; CaCl$_2$, 1; HEPES, 10; pH 7.4 with Trisbase. The pipette solution in the cell-attached mode contained (mM): KCl, 150; CaCl$_2$, 1; HEPES, 10; pH 7.4 with Trisbase. In some experiments it contained (mM): NaCl, 100; KCl, 50; CaCl$_2$, 1; HEPES, 10; pH 7.4 with Trisbase. The external solutions were exchanged using the Y-tube method [23]. All experiments were carried out at room temperature of around 23°C. Statistical significance was assayed with Student's t-test when appropriate. Significance was taken at $p<0.05$. 

RESULTS

1. Electrical membrane properties at whole-cell level

At first, we measured the membrane potential with the whole-cell configuration of the patch clamp technique. Figure 1 illustrates the histogram of the membrane potential, which shows two populations: hyperpolarized (around -80 mV) and depolarized (around 0 mV). Figure 2 depicts the current-voltage relation obtained from a cell under voltage-clamp in various external solutions. In PSS, the resting membrane potential (RP) was -84 mV and the cell was held at the potential. The inward currents elicited by hyperpolarizing steps were much larger than the outward currents evoked by depolarizations. No active inward currents were evoked by depolarizing voltage steps (Fig. 2Aa). The I-V curve was N-shaped, indicating an inward rectification (Fig. 2B, open circles). Ramp pulse experiments gave similar results (not shown). In TEA-Ba solution, both inward and outward currents almost disappeared (Fig. 2Ab) and the I-V relation became linear (Fig. 2B, open triangles). This was also the case when K⁺ in the pipette solution were replaced with Cs⁺ (not shown). When the K⁺ concentration of the external solution was increased to 50 mM under current clamp, the membrane potential shifted to -27 mV, that is, ten-fold increase in the K⁺ concentration shifted the membrane potential by 57 mV. The cell was held at this voltage and clamp pulses were applied. The inward rectification was clearly observed also in depolarized cells (Fig. 2B, closed circles).

Existence of active inward currents was further examined in Fig. 3. An osteoclast was voltage-clamped at -80 mV and square pulses of 40 mV were applied (Fig. 3A). The outward current induced at the holding potential was decreased by clamp pulses to -40 mV, reflecting the negative resistance of the rectifier. Neither Na⁺-free solution nor tetrodotoxin (TTX) changed the whole-cell currents (Fig. 3Ab and Ac). As for the Ca²⁺ current, osteoclasts were voltage-clamped at -60 mV and square pulses of 20 and 40 mV were applied. Either nicardipine, an L-type

![Fig. 2. Current-voltage (I-V) relationships of the osteoclast membrane in various external solutions. A-a: An osteoclast was voltage-clamped at an RP of -84 mV and square pulses were applied stepwise from the holding potential to -120, -100, -60, -40, -20, 0, and 20 mV. Hyperpolarizing command pulses elicited a large inward current, whereas depolarizing pulses generated little outward current. Note the absence of fast inward currents. A-b: The inward and outward currents were greatly reduced by replacing PSS with TEA-Ba solution. No inward currents were evoked by depolarizing pulses. B: The I-V relationship obtained in PSS was N-shaped with inward rectification (open circles). In TEA-Ba solution the relation became linear with a large membrane resistance (open triangles). In 50 mM K⁺ external solution, the osteoclast was depolarized to -27 mV, where the cell was held and square pulses were applied. The inward rectification was clearly observed also in the depolarized osteoclast (closed circles). The lines were drawn by eye.](image)
Ca\(^{2+}\) channel blocker, or Bay K 8644, an L-type Ca\(^{2+}\) channel agonist, did not affect the current (Fig. 3Bb and Bc). The current evoked by clamp pulses to −20 mV was not affected by these drugs either.

2. Two types of inward rectifier channel

Single-channel data were obtained with those cells where the single-channel current was recorded at a pipette potential of 0 mV in PSS, that is, with the cells of hyperpolarized population described in Fig. 1. Several types of single-channel currents were recorded with the cell-attached configuration. Figure 4 shows two lines of current record from different patches with the pipette potential at +40 mV (equivalent to 40 mV hyperpolarization of the patch membrane to RP). Inward currents of various amplitudes and kinetics are seen. Among these currents, predominantly occurring two (shown by f and s) were examined in detail. They were named in the present study f-type current and s-type current for their fast and slow open-close kinetics, respectively.

Figure 5 depicts the two types recorded in 150 mm K\(^+\) external solution, where the osteoclast was depolarized to 0 mV, namely, to the practical \(E_k\) for the patch membrane in this condition. Step pulses were applied to the pipette in order to examine the channel activity at various membrane potentials. The s-type current of the patch was recorded at hyperpolarized potentials to \(E_k\), whereas the current was not observed at depolarized potentials (Fig. 5Aa). The calculated conductance was 34.4 pS.

On the other hand, the f-type current was recorded at both hyperpolarized and depolarized potentials to \(E_k\) (Fig. 5Ab). Depolarization of the membrane patch, however, made the amplitude much smaller probably due to flickering of the current. The conductance was calculated to be 43.5 pS in the range of the membrane hyperpolarization and 7.7 pS in the range of depolarization, where the figure might be inaccurate due to flickering and the sampling frequency of 1 kHz. In single-channel patches, the currents were never seen
to switch between s and f modes.

3. **Kinetic properties of the s-type channel**

Figures 6 and 7 depict the kinetics of the s-type channel recorded in PSS with 150 mM K⁺ in the pipette. Figure 6A shows the single-channel current obtained from the same patch at various pipette potentials. The open probability was high and the amplitude increased with an increase in the pipette potential. The amplitude histograms at various potentials are illustrated in Fig. 6B. The open probability obtained from 5 experiments is plotted against the pipette potential in Fig. 6C. The probability was high throughout the potential range examined and was voltage-independent. The current amplitude is plotted against the pipette potential in Fig. 6D (open circles), giving a unit conductance of 32.0 pS and the reversal potential at 75 mV depolarization to RP. When the external K⁺ concentration was increased to 150 mM, the data points shifted to the left, changing the reversal potential to −2 mV (closed circles). With the extracellular K⁺ concentration 150 mM and the intrapipette 50 mM, the data points shifted further to the left to yield the reversal potential at 26 mV hyperpolarization to RP (open triangles). The shift of the reversal potential is explained by the Nernst equation assuming K⁺ conductance. Figure 7A shows the open time histograms at various pipette potentials. At all potentials tested, the histograms were fitted with single exponentials, giving mean open times which are plotted against the pipette potential in Fig. 7B. The open time has a tendency to become shorter with hyperpolarization of the membrane without statistical significance. The closed time histograms were fitted with double exponential equations (not shown), yielding mean closed times of 8.3 ± 3.7 (n=5) and 55.2 ± 20.7 (n=5) ms at RP, which were not changed by the membrane potential.

4. **Kinetic properties of the f-type channel**

Figures 8 and 9 show the kinetic properties of the f-type channel in PSS. Figure 8A illustrates the single-channel currents from the same patch at various pipette potentials. Since it was not possible to analyze the small flickering outward currents evoked by depo-
Fig. 6. Analysis of the s-type channel in the cell attached configuration. The external solution was PSS and the pipette solution contained 150 mM K+.
A: The s-type current was recorded at various pipette potentials. Numbers to the right of each trace refer to the pipette potential. Dashed lines indicate the closed level. All traces are from the same patch.

B: The amplitude histograms of the current with pipette potential at 0 (a), +20 (b), +40 (c), and +60 mV (d). The open probabilities were 0.92, 0.91, 0.89 and 0.85, respectively. The curve was fitted by the sum of two Gaussian distributions with a least-squares method.

C: The open probability is plotted against the pipette potential. The open circles and bars give the mean ± SD (n=5). The open probability was not significantly affected by the potential change.

D: The amplitude of the single-channel current was measured in various ionic conditions and plotted against the pipette potential. The reversal potentials for the current through the patch were estimated by extrapolation. Open circles: external solution = 5 mM K+, intrapipette solution = 150 mM K+; the unit conductance was 32.0 pS and the reversal potential was extrapolated to be 75 mV depolarized to RP. Closed circles: external solution = 150 mM K+, intrapipette solution = 150 mM K++; the unit conductance was 34.4 pS and the reversal potential was −2 mV to RP. Open triangles: external solution = 150 mM K+, intrapipette solution = 50 mM K++; the unit conductance was 24.7 pS and the reversal potential was 26 mV hyperpolarized to RP. The symbols and bars represent the mean ± SD from 5 experiments other than those in Fig. 5. Where no bar is shown, SD is smaller than the symbol.

Polarization of the membrane, detailed analyses were limited to the currents at more hyperpolarized potentials to RP. Figure 8B depicts the amplitude histograms. The open probability was rather low and increased at more hyperpolarized potentials (Fig. 8C). The current amplitude is plotted against the pipette potential in Fig. 8D. In PSS (open circles), the unit conductance was 38.8 pS and the reversal potential was suggested by extrapolation to be 70 mV depolarized to RP. When the external K+ concentration was raised to 150 mM, the data points shifted to the left with the reversal potential at −4 mV to RP (closed circles). With the extracellular K+ concentration 150 mM and the intrapipette 50 mM, the data points shifted further to the left giving the reversal potential at 25 mV hyperpolarization to RP (open triangles). The open time histograms are illustrated in Fig. 9A and the mean open time is plotted against the pipette potential in Fig. 9B, showing that it became longer with hyperpolarization of the membrane. The closed
Inward Rectifiers in Osteoclasts

represented a motile phase and expressed an inwardly rectifying K\(^+\) channel; rounded osteoclasts, a resorptive phase and outwardly rectifying K\(^+\) channel. On the other hand, Yamashita et al. [4] recently examined the effects of G-protein on the inward rectifier of rabbit osteoclasts which were plated on glass cover slips, coated or uncoated with vimeonectin, or on plastic culture dishes. They reported that both spread and rounded osteoclasts had inwardly rectifying K\(^+\) current without any evidence of outward rectification. It is, therefore, controversial at present whether the function, morphology and K\(^+\) channel expression are correlated with each other or not.

In the present study, we plated the dissociated osteoclasts on plastic culture dishes and most osteoclasts were spread. Several hours after plating, however, a few cells became rounded. All the osteoclasts examined in the present experiments were spread type and all the cells studied with the whole-cell configuration showed the typical inward rectification without evidence of outward rectification. The inward rectification has been reported in various preparations including both excitable and non-excitable cells such as: cardiac ventricular cells [24–26], cultured myotubes [27, 28], tunicate egg cells [29], tumor mast cells [30], macrophages [31, 32], retinal glial cells [33] and Schwann cells [34]. The inward rectification is known to be produced by K\(^+\) conductance, which was also the case in rat osteoclasts as shown by the findings that the reversal potential shifted according to the K\(^+\) equilibrium potential and that blockade of the K\(^+\) channels by external TEA-Ba or internal Cs\(^+\) completely abolished the property (Fig. 2).

The bimodal distribution of the membrane potential (Fig. 1) is consistent with those reported in rat and rabbit osteoclasts [4, 13]. This may reflect different functional states in a similar morphology, which may result from the activation or blockade of the inward rectifier. Recent reports have shown that the inward rectifying K\(^+\) channels are regulated by a G-protein-coupled pathway, thereby suggesting that the channel is functionally important [4, 35].

Lack of the Na\(^+\) channel was suggested by the absence of the inward current evoked by depolarizing pulses from a holding potential of \(-84\) mV, where the Na\(^+\) channels might not be inactivated (Fig. 2). Furthermore, neither Na\(^+\)-free solution nor TTX affected the currents evoked by depolarizing pulses from \(-80\) mV (Fig. 3A). Lack of Ca\(^{2+}\) channels was shown by the absence of inward currents in TEA-Ba solution, where Ba\(^{2+}\) are known to pass through the Ca\(^{2+}\) channels more effectively than Ca\(^{2+}\) (Fig. 2A). This idea is supported by the results in Fig. 3B, that neither

Fig. 7. Mean open time of the s-type channel. A: The open time histograms were fitted with single exponentials and gave the mean open time at different pipette potentials in the same patch (a, 349 ms at 0; b, 310 ms at +20; c, 292 ms at +40 and d, 249 ms at +60 mV). The bath solution was PSS and the pipette solution contained 150 mm K\(^+\). B: The mean open time is plotted against the pipette potential (n=5). The bars give ±SD of the mean. The mean open time tended to decrease at more hyperpolarized potentials of the patch membrane, with no statistical significance.

**DISCUSSION**

1. Whole cell currents. Arkett et al. [21] suggested a correlation among function, morphology and the type of K\(^+\) channel expressed on the cell membrane of the rat osteoclasts which were plated on dentine, type I collagen or glass: spread osteoclasts
time histograms were fitted with double exponential functions (not shown), giving the closed times of 3.4±1.2 (n=5) and 15.0±4.7 (n=5) ms at RP. The closed time did not vary with the membrane potential.
nicardipine nor BAY K 8644 affected the currents evoked by square-pulse depolarizations to -40 and -20 mV. These findings indicate that rat osteoclasts do not possess Na\(^+\) and Ca\(^{2+}\) channels to generate active inward currents. This notion is consistent with other electrophysiological studies with osteoclasts of the rat [11], rabbit [12], and chick [13]. A fluorometric study with Ca\(^{2+}\) indicator fura-2, however, suggested the existence of voltage-gated Ca\(^{2+}\) channels in cultured chicken osteoclasts plated on glass coverslips [17].

2. Single-channel currents. Single-channel recordings with the cell-attached configuration showed the existence of several types of channels (Fig. 4). We carried out kinetic analysis of two types, f and s, among them, because they appeared predomi-
Inward Rectifiers in Osteoclasts

Fig. 9. Mean open time of the f-type channel. A: The open time histograms were fitted with single exponentials and gave the mean open time at different pipette potentials in the same patch (a, 2.6 ms at 0; b, 2.9 ms at +20; c, 3.9 ms at +40 and d, 7.5 ms at +60 mV). The bath solution was PSS and the pipette solution contained 150 mm K⁺. B: The mean open time is plotted against the pipette potential (n=4 to 5), which had a tendency to become longer at more hyperpolarized potentials of the patch. The open circles and bars give the mean±SD. The asterisk indicates a significant increase from the value at RP.

3. Kinetics of the s-type channel. The s-type current was observed in about 80% of the patches examined in the cell-attached configuration. Most of the inward rectifiers reported so far have slow open-close kinetics, including those described in osteoclasts by Kelly et al. [12] and Sims et al. [14]. The unit conductance was 32.0 pS in the present study, which is similar to those reported in other inwardly rectifying K⁺ channels: 31 pS in rabbit osteoclasts [12]; 25 pS in rat osteoclasts [14]; 27 pS in guinea-pig ventricular cells [26]; and 26.3 pS in skeletal muscle cells [27]. The outward current at more depolarized potentials than E_K was never observed (Fig. 5), probably due to blockage of the channel by divalent cations at the cytoplasmic side of the channel, as described in cardiac ventricular cells [24]. Recent studies have reported the importance of the blockade by intracellular polyamines for formation of the inward rectification [36, 37]. There have been no descriptions of the open probability of the rectifier of osteoclasts. In the present study, it was found to be high (≥0.85 at all potentials recorded) and was not changed by the membrane potential. This high open probability agrees with that of the skeletal muscle cells [11] but not with that of the ventricular cells [24]. The mean open time was 349 ms at RP. It has a tendency to become shorter with hyperpolarization of the patch membrane, as was reported in the guinea-pig ventricular cells and skeletal muscle cells. Two closed times were estimated: 8.3 and 55.2 ms at RP. Matsuda and Stanfield [27] described three closed times in cultured muscle cells: 0.88, 17.3 and 104.7 ms at −40 mV to RP. In the present study, the shortest one may have been missed due to the low time resolution of the recording system.

4. Kinetics of the f-type channel. The single-channel current of the f-type channel showed quick opening and closing. Outward currents were recorded but the amplitude was much smaller, giving a decreased conductance at depolarized potentials of the patch membrane to E_K (Fig. 5). The kinetic analysis in this potential range could not be performed due to the low signal-noise ratio. Mukai et al. [30] reported a similar flickering-type K⁺ current in rat basophilic leukemia cells without describing the open-close kinetics. They further showed that current had the inward rectification at the single-channel level probably due to blockade by intracellular Ca²⁺. The f-type channel in the present study may also have been blocked by Ca²⁺, Mg²⁺ and/or polyamines at the internal orifice of the channel. We obtained a unit conductance of 38.8 pS, which is much larger than that of the leukemia cells (24.5 pS). The open probability was small and appeared to be weakly voltage-dependent, increasing with hyperpolarization of the patch mem-
brane. The mean open time was short (2.6 ms at RP) and became longer at hyperpolarized potentials. Whereas, the two closed times (2.8 and 15.3 ms at RP) were not voltage-dependent.

5. Implications in the cell function. The stoichiometry of mobilization of bone mineral was reported to be around 2H⁺/Ca²⁺, which requires a transport of massive quantities of protons into the resorption microenvironment [38]. The protons are released actively via vacuolar H⁺-ATPase, which is electrogenic and hyperpolarizes the cell [39]. The hyperpolarization would activate the inward rectifier K⁺ channel, which provides counter ions and clamps the membrane at EK⁺.

In conclusion, spread-type osteoclasts do not possess active inward currents carried by Na⁺ and Ca²⁺. The inward rectification of these cells is based on at least two classes of K⁺ channel, which have an inward-rectifying property at the single-channel level.

The authors thank Dr. K. Hata, 2nd Department of Oral Anatomy, Faculty of Dentistry, Kyushu University, for techniques to dissociate the osteoclasts.

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Inward Rectifiers in Osteoclasts


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