Membrane Currents of Murine Osteoclasts Generated from Bone Marrow/stromal Cell Co-culture

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Summary

Whole cell clamp recordings were conducted to examine the general behavior of ion channels in osteoclasts which were generated from bone marrow/stromal cell co-culture. In the resting state, inwardly and outwardly rectifying (IR and OR) currents were observed in the osteoclasts cultured for 5 - 18 days and the whole cell currents were classified into three types according to the conductance ratio between the OR and IR currents, such as, the IR type (OR/IR conductance\(\leq 0.2\)), the IR-OR type \(0.2<\text{OR/IR}\leq 5\), and the OR type \(\text{OR/IR}>5\). The IR type was found in 73% of cells \(n=78\), the IR-OR type in 26% and the OR type in 1%. No relation was found between the current types and the number of nuclei or cultured days. The conductances varied among cells, but the IR conductance was larger than the OR conductance. The IR current was characterized by voltage-dependent kinetics, a negative conductance region and Ba\(^2+\)-sensitivity. The reversal potential depended on the extracellular K\(^+\) concentration, indicating that K\(^+\) mediates the IR current. On the other hand, the OR current was reversibly reduced by decreasing extracellular Cl\(^-\) concentration but not affected by K\(^+\), suggesting that the OR current depends on Cl\(^-\). High intracellular Ca\(^{2+}\) \(1 - 10 \mu\text{M}\) transiently activated a different class of outward current. These electrophysiological features resemble those in freshly isolated osteoclasts. We suggest that the ion channels involved
in osteoclastic functions are expressed in the *in vitro*-generated osteoclasts and that the channels develop early in differentiation and are maintained for at least up to 18 days. Thus the co-culture system provide a useful model to examine roles of ion channels in osteoclastic functions.

**Introduction**

Osteoclasts play an important role in calcium homeostasis and bone remodeling in all ages. However, it is difficult to obtain a large number of mammalian osteoclasts from bone tissue, which restricts intensive investigations. Recently, a co-culture system of bone marrow cells and a marrow-derived stromal cell line (ST2) was established and osteoclastic cells have been obtained in large amounts (22). The osteoclastic cells generated in this system satisfy the criteria of osteoclasts, such as positive tartrate resistant acid phosphatase (TRAP) activity, multinuclei and specific morphological appearances and have been employed in molecular and biochemical studies (1, 6, 16, 25, 26). This system also provides a useful experimental preparation for various pharmacological specification and examination of differentiation process, as microscopic environments in the culture system are easily altered.

The importance of ion permeabilities of the plasma membrane in osteoclast functions is unquestionable. Osteoclasts transport acid and many other kinds of ions in resorbing bone (3, 4, 5, 29), and are exposed to drastic changes in both intracellular and extracellular environments. Ionic channels of the cell membrane are considered to adjust cell functions directly or indirectly, via regulation of the membrane potential, pH, and cell volume (9, 11, 12, 14). However, the ion channels have been studied only in freshly-isolated osteoclasts so far, and channel properties of *in vitro*-generated osteoclasts remain to be clarified. In this study, we first recorded channel activities in osteoclasts generated from the co-culture system using the patch clamp technique. We found that the major conductance was mediated by an inwardly rectifying (IR) K⁺ channel and that the properties resembled those reported in freshly-isolated osteoclasts. In addition, the investigation on the relation between the current types and culture days and the number of nuclei revealed that expression of the ion channels was completed even in the early phase of differentiation. The *in vitro*-generated osteoclasts will aid to examine roles of ion channels in osteoclastic functions.
**Materials and Methods**

**Cell culture.** Osteoclast-like cells were generated from co-culture of bone marrow cells of male, 5 - 8 week-old mice (C3H/HEN) with a marrow-derived stromal cell line (ST2) (Riken Cell Bank, Tukuba, Japan) following the method previously described (22). The mice were killed by cervical dislocation. Bone marrow cells were obtained from the femurs and tibias, centrifuged at 1500 rpm for 7 min at 4°C in α-MEM supplemented with 10% fetal calf serum (FCS), streptomycin (0.1 mg/ml) and penicillin (100 U/ml). After incubation at 37°C in a 95% air-5% CO₂ atmosphere over night, non-adherent cells were collected, centrifuged at 1500 rpm at 4°C for 7 min and then incubated in the phosphate buffered saline containing 0.02% pronase and 1.5 mM ethylenediaminetetraacetic acid (EDTA) for 15 min at 37°C. The pronase reaction was stopped by heat-inactivated horse serum (0.2 ml/10 ml pronase solution), and the cell suspension was layered on cold horse serum. After 15 min of sedimentation at unit gravity for 15 min on ice, the uppermost part of the layers was collected, transferred on cold horse serum and then centrifuged at 2000 - 2500 rpm at 4°C for 10 min. The cell pellet was suspended in fresh α-MEM supplemented with 10% FCS at 1×10⁶ cells/ml and co-cultured with ST2 cells (1×10⁵ cells/ml) in the presence of 10⁻⁸ M 1α, 25-dihydroxyvitamin D₃ (1α, 25(OH)₂D₃) and 10⁻⁷ M dexamethasone at 37°C in a 95% air-5% CO₂ atmosphere. Total medium was changed twice a week. Multinucleated cells with a unique osteoclastic morphology and tartrate resistant acid phosphatase (TRAP) activity were identified at 5 - 7 days after the start of co-culture and were maintained for up to 18 days. It was confirmed that these cells made resorptive hemivacuoles when plated on bone slices ST2 cells were removed by incubation with 0.1% collagenase/0.1% bovine serum albumin (BSA) in α-MEM for 20 min - 2 hrs at 37°C before recordings.

**Electrophysiological recordings.** Current signals were recorded under the whole cell clamp configuration at room temperature (18 - 24°C). The reference electrode was at Ag-AgCl wire connected to the bath solution through a Ringer-agar bridge. The standard pipette solution contained; 150 mM K-glutamate, 7 mM MgCl₂, 1 mM ethenylenglycol-bis-(β-aminoethyl ether) N, N, N'-tetraacetic acid (EGTA), 1 mM Na₂-ATP, 10 mM N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid] (HEPES)-KOH (pH 7.3). In some experiments, K-glutamate was replaced by CsCl, Cs-methanesulfonate or mixture of both, to block K⁺ currents. The standard external solution contained: 145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.1% BSA and 10 mM HEPES-NaOH (pH = 7.3). The high K⁺ (30 and 150 mM) solutions were made by replacing NaCl with KCl to compensate for osmolarity. The zero current potential before formation of gigaseal was taken as
Pipette resistance ranged between 5 - 8 MΩ. The series resistance compensation (~70%) was conducted to reduce the voltage error. Data without an adequate voltage control in some cells with large currents were not quantitatively analyzed in this study.

Currents were recorded by an amplifier (Axopatch 200A). After digitizing at 1 KHz through a analog-digital converter (Digidata 1200), data were stored and analyzed by a personal computer program (pClamp 6.02). Whole cell capacitance was estimated using the capacitance compensation circuit of the amplifier. Voltage ramp (0.4 mV/ms) was applied at the holding potential of 0 mV or −60 mV. Leak current was determined from the linear portion of the current-voltage (I-V) relation when either inward or outward current was absent or when the currents were blocked by blockers. The inward and outward conductances were determined from the I-V relation at between −100 and −80 mV and at between +80 and +120 mV, respectively, after subtraction of the leak current. Data are expressed as mean ± SD.

**Results**

**Morphological identification of in vitro-generated osteoclasts**

Cells with three or more nuclei were found later than 5 - 7 days in culture. Morphological features of the cells were classified into two types, according to freshly isolated osteoclasts, that is, the “spread” and “rounded” types (2, 8). The spread type had a flattened large cell body, developed lamellipodia and retraction fibers. The rounded type cells were characterized by a dome shaped small cell body and scarce lamellipodia. These multinucleated cells generated in vitro were positive in TRAP staining, regardless of either rounded or spread type. More than 90% of the cells had the spread type appearance.

Generally osteoclasts became larger and had more nuclei in association with an increase in days in culture, due to repeated fusion of mononuclear cells. The number of nuclei was roughly proportional to the cell size (Fig. 1). Nuclei were first concentrated in the center of the cells, and tended to disperse to the edge as the cells grow. Considerably enlarged cells with thin cell body were not used because stable recordings were difficult to maintain and because inadequate space clamp might distort the current amplitude. The cell size was also estimated from the cell capacitance. In the present study, recordings were made from spread type cells containing 3 - 10 nuclei with cell capacitance of 101.4 ± 56.1 pF (n=125).

**Relation between current types and differentiation.**

Sizable membrane currents were observed in more than 90% of cells tested.
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Fig. 1. Relation between cell size and number of nuclei of TRAP-positive osteoclasts generated in vitro during the culture period of 5-16 days. Cell size was roughly estimated from cell diameters.

The whole cell currents were classified into three types according to the ratio between inwardly rectifying (IR) and outwardly rectifying (OR) conductances (Fig. 2A-C). The histogram in Fig. 2D shows that the IR type (see A; OR/IR conductance ≤0.2) was found in 73% of 78 cells, the IR-OR type (B; 0.2<OR/IR≤5) in 26%, and the OR type (C; OR/IR>5) in 1%. As multinucleated cells were generated by repeating cell fusion, the number of nuclei could be used for an index of differentiation. The relation between the current type and the number of nuclei was shown in Fig. 2E. In the cells with nuclei ≤ 5, the IR type was seen in 78%, and the IR-OR type, in 22%. In the cells with nuclei ≥ 6, the IR type was 73%, and the IR-OR type was 23%. When the relation between the current type and days in culture was examined (Fig. 2F), the high incidence of the IR type was consistent over the culture period (73 - 82%). Thus there is no relationship between the current types and progress of fusion in culture. It is likely that ion channels in the resting state are expressed even in the early phase of differentiation once the cell has satisfied the criteria as an osteoclast (TRAP+, multinuclei and specific morphology). The membrane potential recorded in the resting state varied from cell to cell (-60 - +2.5 mV).
Classification of whole cell current types. A-C, sample records of three different current types based on the conductance ratio between inwardly rectifying (IR) and outwardly rectifying (OR) conductances. A-C were obtained from three different cells cultured for 12 (A) or 9 days (B and C), showing the IR type (OR/IR conductance ≤ 0.2), the IR-OR type (0.2 < OR/IR ≤ 5), and the OR type (OR/IR > 5), respectively. D, number of cells exhibiting the three current types in cells developed between 7 and 18 days. E, percent incidence of the current types in cells with nuclei ≤ 5 and ≥ 6. F, percent incidence of the current types and the culture period.

Fig. 2. Classification of whole cell current types. A-C, sample records of three different current types based on the conductance ratio between inwardly rectifying (IR) and outwardly rectifying (OR) conductances. A-C were obtained from three different cells cultured for 12 (A) or 9 days (B and C), showing the IR type (OR/IR conductance ≤ 0.2), the IR-OR type (0.2 < OR/IR ≤ 5), and the OR type (OR/IR > 5), respectively. D, number of cells exhibiting the three current types in cells developed between 7 and 18 days. E, percent incidence of the current types in cells with nuclei ≤ 5 and ≥ 6. F, percent incidence of the current types and the culture period.

An inwardly rectifying IR current

As the IR current was recorded in most cells, we studied properties of the IR current in detail. The IR conductance normalized by the cell capacitance was 87.0 ± 72.8 pS/pF (n=78), showing a variety of the channel density among cells. The IR conductance tended to decrease with an increase in the number of nuclei (Fig. 3) although the relative coefficient was small. Figure 4A shows a superimposed IR currents evoked by hyperpolarizing voltage pulses in −10 mV increments at a holding potential of 0 mV. At higher negative potentials the IR current was activated faster and declined after the peak (Fig. 4A). In Na⁺-free K⁺-rich (150 mM)
solution, the inactivation was totally eliminated (Fig. 4B). In the 120 mM Na\(^+\) plus 30 mM K\(^+\) solution, the inactivation was smaller than that in the standard solution (data not shown). Namely as the extracellular K\(^+\) concentration ([K\(^+\)]\text{\textsubscript{o}}) was elevated and the extracellular Na\(^+\) concentration was decreased, the amount of inactivation was reduced.

In some cells, a negative conductance region was prominent at potentials more positive to the reversal potential (−80 − −7 mV) (Fig. 5A arrow). When [K\(^+\)]\text{\textsubscript{o}} was increased from 5 to 150 mM, the reversal potential was positively shifted to near 0 mV with an enhancement of the IR current (Fig. 5B). The IR current was inhibited by 1 mM Ba\(^{2+}\) (Fig. 5C). These observations indicate that the IR current is mediated by K\(^+\). Voltage-dependent activation and inactivation kinetics, a negative conductance region and Ba\(^{2+}\)-sensitivity are features common to the inwardly rectifying K\(^+\) (IRK) current in freshly-isolated osteoclasts (8, 10, 18, 22, 31).

An outwardly rectifying (OR) current.

The OR current was affected neither by [K\(^+\)]\text{\textsubscript{o}} (Fig. 5B) nor by Ba\(^{2+}\), indicating that K\(^+\) did not mediate the OR current. On the other hand, the OR current was
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Fig. 4. Activation and inactivation kinetics of the IR current. A family of currents was evoked by step hyperpolarizing pulses in -10 mV increments applied at a holding potential of 0 mV in the standard Ringer (A) and the Na⁺-free, high K⁺ (B) solutions.

Fig. 5. Current-voltage (I-V) relations of the IR current. The I-V relations were obtained by voltage ramps applied at a holding potential of 0 mV. A, IR current with a negative conductance region (arrow). B, I-V relations subsequently perfused with solutions containing 5 and 150 mM K⁺. C, Ba²⁺ (1 mM) blocked the IR current. [K⁺]₀ was 30 mM.
Fig. 6. The OR current and the extracellular Cl⁻ concentration. A family of currents evoked by voltage pulses in ±10 mV increments at holding potential of 0 mV was superimposed. Perfusing medium was subsequently changed from the standard Ringer control to the low Cl⁻ (36 and 4 mM) solutions and returned to the Ringer solution. In the low Cl⁻ solutions, NaCl was replaced by Na⁺-isethionate and K⁺ was omitted.
Fig. 7. Ratio of IR and OR conductances at different concentrations of \([K^+]_o\). Data was obtained from different cells at 3 mM (n=17), 5 mM (n=6), 30 mM (n=3) and 150 mM \([K^+]_o\) (n=4).

Fig. 8. A transient outward current recorded with high intracellular \(Ca^{2+}\) (1 \(\mu\)M). A, a time course of the change in the outward conductance following rupture of the patch membrane. B, I-V relations obtained by voltage ramps at times indicated by a - c in A.
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reversibly reduced by decreasing the extracellular Cl⁻ concentration ([Cl⁻]₀) (Fig. 6). In the standard Ringer solution, the OR conductance was 18.4 ± 31.7 pS/pF (n=78) with pipette containing K⁺-glutamate and 36.5 ± 64.8 pS/pF (n = 25) with the Cs⁺-containing pipette solution. In Fig. 7, each point represents ratio between the IR and OR conductances at different [K⁺]₀, indicating that [K⁺]₀ did not influence the current ratio.

With pipette solutions containing high Ca²⁺ concentration (1 - 10 µM), a different class of outward current was often recorded. As shown in the time course in Fig. 8A, the outward current was activated after a short delay following formation of the whole cell clamp configuration by the rupture of the patch membrane and then gradually eliminated. Figure 8B shows I-V relations obtained from voltage ramps at times indicated by a-c in the time course (Fig. 8A). The I-V relation of the Ca²⁺-activated outward current differed from that of the Cl⁻-dependent OR current recorded with the Ca²⁺-chelating pipette solution (Figs. 2B and C, 5B). The maximum conductance was 60.5 ± 44.3 pS/pF (n=9) with 1 µM Ca²⁺ and 117.2 ± 113.6 pS/pF (n=9) with 10 µM Ca²⁺. Further analysis of the Ca²⁺ activated outward current was not done, because the investigation was hampered by the transient nature. Effects of high intracellular Ca²⁺ on the IR current were inconsistent, although a slight increase was observed in some cells.

Discussion

We first described general aspects of ion channel currents, which are activated at different membrane potentials, in osteoclasts generated from bone marrow/stromal cell co-culture. In the resting state, IR current and/or an OR current were dominantly found and the whole cell currents were classified into three types, such as, IR, IR-OR and OR types. Almost all cells exhibited the IR current, and there was no significant relation between the current type and the number of nuclei and also between the current type and the cultured days. The IR current is mediated by K⁺ and shares common features with that in freshly isolated osteoclasts; a voltage-dependent activation and inactivation kinetics, a negative conductance region at a certain range of the membrane potential positive to the reversal potential and sensitivity to Ba²⁺ (8, 10, 18, 23, 31). The IR conductance varied greatly among cells (3.7 - 445 pS/pF), but was in the similar range reported for freshly isolated osteoclasts (less than 282 pS/pF) (10, 24). These findings indicate that there is no significant difference in the IR K⁺ conductance between the in vitro-generated and freshly isolated osteoclasts.

Size, number of nuclei and morphological appearance vary among cells which
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satisfy criteria for osteoclasts. Freshly isolated osteoclasts are often classified into two types based on their morphology, that is, “spread” and “rounded” types. The spread and rounded types are considered to be phenotypes responsible for motile and resorptive actions, respectively (2). Arkett et al. (2) reported that the incidence of the rounded phenotype depended on the microenvironments, that is, greater when cells were plated on bone slices and lower when cells were plated on glasses. The proportion of the rounded type was very low (≤10%) in culture dishes which we used in this study, so data described herein were obtained from the “spread” type cells.

It is reported that cell to cell contact of pre-osteoclasts and stromal cells is necessary for differentiation and that stromal cells release factors to differentiate pre-osteoclasts into osteoclasts (7, 13, 21, 25, 26, 27). As pre-osteoclasts fuse each other during differentiation, the cell is enlarged and had more nuclei. The resorptive capability is greater in cells with more nuclei (19). We examined whether the channel expression depended on the differentiation process or not, but no relation was found between the current types and the number of nuclei. It is likely that the ion channels in the resting state develop in the early phase of differentiation and are maintained during advances in cellular fusion.

We found that a subpopulation of the in vitro-generated osteoclasts exhibited an OR current in the resting state. The amplitude of the OR conductance was smaller than that of the IR conductance in most cells which possessed both conductances. The OR conductance was not affected by extracellular K⁺ and Ba²⁺, but reduced by decreasing the extracellular Cl⁻ concentration. These findings suggest that the OR conductance is mediated by Cl⁻ current. An outwardly rectifying K⁺ current was reported in the resting state of osteoclasts (20) particularly in the rounded type (1, 8). The resting Cl⁻ conductance has been described in some freshly isolated cells (11, 24). In addition, requirement of a conductive Cl⁻ pathway in acid secretion (5) and a potent inhibition of bone resorption by a Cl⁻ channel blocker, 4, 4'-diisothiocyanato stilbene-2, 2'-disulfonic acid (DIDS) (9, 12, 28), would imply that Cl⁻ is needed to regulate resorption by neutralizing the charge imbalance caused by secretion of a large amount of H⁺ into resorption lacunae.

We detected another outward current when currents were recorded with pipette solutions containing high Ca²⁺ concentrations (1-10 μM). The outward current is distinct from the Cl⁻-dependent OR current, as follows. First, from the I-V relation obtained from voltage ramps, the amplitude of the outward current was reduced at large depolarization. Second, the outward current was activated following a short delay after the whole cell clamp configuration was formed, then decreased gradually and eventually disappeared. A transient activation of a Ca²⁺-activated
K⁺ current was reported in chick osteoclasts (30, 32). As the intracellular free Ca²⁺ level of osteoclasts is elevated during bone resorption (15, 17), the Ca²⁺-activated outward current may be involved in the osteoclastic activities. However further studies are necessary to clarify properties and roles of the outward current.

These results suggest that ion channels specific to osteoclastic functions developed even in the early phase of differentiation in the bone marrow/stromal cell co-culture system. The in vitro-generated osteoclast offers an useful model not only for biochemical and molecular biological studies but for electrophysiological studies. The IR K⁺ current is considered to set the resting membrane potential (10, 11, 31). Here the membrane potential ranged from −60 mV to 2.5 mV. Two levels of the membrane potential, hyperpolarized and depolarized levels, are common with cells exhibiting IR K⁺ channels (15). Ionic channel activities are, also, significant in regulation of cell volume (14). As the spread type is suggested to be phenotype responsible for the motile phase of osteoclasts (1), ion channels presented herein would be involved in regulation of cell morphology during movements. In addition, as a high intracellular Ca²⁺ induces another current, a variety of ion channels may be activated during functional states of osteoclasts. Thus the in vitro-generated osteoclasts would be useful to examine ion channels in pathological microenvironments and also to screen the effects of various medicine on the channels.

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