TRPM7 Is Essential for RANKL-Induced Osteoclastogenesis

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The transient receptor potential melastatin type 7 (TRPM7) channel is a widely expressed non-selective cation channel with fusion to the C-terminal alpha kinase domain and regarded as a key regulator of whole body Mg2+ homeostasis in mammals. However, the roles of TRPM7 during osteoclastogenesis in RAW264.7 cells and bone marrow-derived monocyte/macrophage precursor cells (BMMs) are not clear. In the present study, we investigate the roles of TRPM7 in osteoclastogenesis using methods of small interfering RNA (siRNA), RT-PCR, patch-clamp, and calcium imaging. RANKL (receptor activator of NF-κB ligand) stimulation did not affect the TRPM7 expression and TRPM7-mediated current was activated in HEK293, RAW264.7, and BMM cells by the regulation of Mg2+. Knock-down of TRPM7 by siTRPM7 reduced intracellular Ca2+ concentration ([Ca2+]i) increases by 0 mM [Mg2+], in HEK293 cells and inhibited the generation of RANKL-induced Ca2+ oscillations in RAW264.7 cells. Finally, knock-down of TRPM7 suppressed RANKL-mediated osteoclastogenesis such as activation and translocation of NFATc1, formation of multinucleated cells, and the bone resorptive activity, sequentially. These results suggest that TRPM7 plays an essential role in the RANKL-induced [Ca2+]i oscillations that triggers the late stages of osteoclastogenesis.

Key Words: Calcium signaling, Osteoclastogenesis, RANKL, TRPM7

INTRODUCTION

Calcium (Ca2+) plays a critical role in many cellular processes from differentiation to death in cells. Ca2+ entry into cells mediates by store-operated Ca2+ channels (SOCs) and transient receptor potential (TRP) channels [1]. TRP channels have been proposed to operate as SOCs. TRP channels consist of six transmembrane spanning domains (S1-6) with a pore-forming loop between S5 and S6, and include intracellular N- and C-terminal regions. According to the degree of amino acid homology, the TRP family can be subdivided into seven subgroups (TRPC, TRPV, TRPM, TRPA, TRPP, TRPM1-TRPM8 [2]). Apart from other TRP channels, the distinct characteristic of TRPM does not contain N-terminal ankyrin repeat motifs but contains functional proteins in C-termini. TRPM7, for example, contain functional α-kinase segments, a type of serine/threonine-specific protein kinase [3,4] that is essential for modulating channel activity [5,6]. Due to its structural features, TRPM7 is considered as both a kinase, which is capable of phosphorylating itself and other substrates, and a cation channel, which conducts cations (highly permeable to Ca2+ and Mg2+) into the cell [7]. TRPM7, as a cation channel, is constitutively opened and mediates capacitative Ca2+ entry, which is tightly regulated by intracellular Mg2+ concentration such as Mg-ATP and other Mg-nucleotides [8].

RANKL (receptor activator of nuclear factor-κB ligand) is expressed in osteoblastic/stromal cells and is critical importance for osteoclast differentiation. In our previous works, it has been reported that RANKL-induced oscillations by intracellular Ca2+ concentration ([Ca2+]i) increases are related with the extracellular Ca2+ influx through SERCA (sarcoplasmic/endoplasmic reticulum Ca2+ ATPase) and SOCs, and intracellular ROS (reactive oxygen species) increases [9,10]. Extracellular Ca2+ influx for maintaining [Ca2+]i, oscillations trigger the late stage in osteoclast differentiation [11]. However, Ca2+ entry pathway via the plasma membrane in osteoclastogenesis is not clearly known. The activity of TRPM7, as a Ca2+ permeable cation channel, is indispensable part of maintaining cell homeostasis including cell growth, proliferation and differentiation [4]. TRPM7 also can be activated by ROS and regulated intracellular Mg2+ level

ABBREVIATIONS: RANKL, receptor activator of NF-κB ligand; TRP, transient receptor potential; SOCs, store-operated Ca2+ channels; [Ca2+]i, intracellular Ca2+ concentration; ROS, reactive oxygen species; MNCs, multi-nucleated cells.
activity of the RANKL-induced Ca\textsuperscript{2+} oscillations and osteopo-
meable cation channel in osteoclasts and regulate the
activity of the RANKL-induced Ca\textsuperscript{2+} oscillations as a Ca\textsuperscript{2+}
permeable channel and the role in physiological activities of
osteoclasts.

METHODS

Cell culture and reagents

RAW264.7 (Korean Cell Line Bank, South Korea) and primary cultured BMMs were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) and minimum essential medium alpha (\(\alpha\)-MEM) supplemented with 10% fetal bovine serum (FBS, Invitro-
gen) and incubated in 5% CO\textsubscript{2} incubator. M-CSF and RANKL were treated at 50 ng/ml concentration in \(\alpha\)-MEM. RANKL and M-CSF were purchased from KOMA Biotech
(Seoul, Korea). HEK293 cells were cultured in DMEM con-
aining 10% FBS, and 100 units/ml penicillin and strepto-
mycin. Fura-2/AM was purchased from Teflabs (Austin, TX, USA). Gadolinium chloride (Gd\textsuperscript{3+}) and adenosine triphos-
phate (ATP) were from Sigma Aldrich (St Louis, MO, USA).

Monoclonal antibody (mAbs) for NFATc1 and polyclonal an-
bodys for TRPM7 were obtained from Santa Cruz Biotech-
tology (Santa Cruz, CA, USA).

Preparation of BMMs

The femur and tibia were isolated from 4
\(\sim\) 6 weeks old
\(\sim\) 6 weeks old
mice as described previously [14]. Whole cells derived from
bone marrow of femur and tibia was collected and cultured in \(\sim\) 6 weeks old
\(\sim\) 6 weeks old
\(\alpha\)-MEM medium containing 10% FBS and 10 ng/ml
\(\alpha\)-MEM medium containing 10% FBS and 10 ng/ml
M-CSF. The following day, non-adherent cells in media
were collected and seeded on adequate plates and treated
with M-CSF (50 ng/ml). After 2 days non-adherent cells
were washed out and adherent cells were used as BMMs.

RT-PCR (reverse transcription polymerase chain reaction)

Total RNA was isolated from each cell using Trizol re-
agents (Invitrogen). Total isolated RNA was amplified ac-
cording to the manufacture’s protocol using AccuPower\textsuperscript{®} RT
PreMix (BIONEER, Daejeon, Korea). cDNA was amplified by PCR with HiFi\textsuperscript{™} Thermostable DNA polymerase (Elpis,
Seoul, Korea). The primer sequences of genes were as fol-
lows: TRPM7 (531 bp), 5’-AGG AGA ATG TCC CAG AAA TCC-3’ (forward) and 5’-TCC TCC ATG TAA CAT CCA AGC-3’ (reverse); \(\beta\)-actin (514 bp), 5’-TGT GAT GGT GGG
AAT GGG TCA G-3’ (forward) and 5’-TCC-3’ (forward)
AAT GGG TCA G-3’ (forward) and 5’-TCC-3’ (forward)
and emitted fluorescence 510 nm (Ratio=F\textsubscript{340}/F380) was col-
lected and monitored at 2 s intervals using a CCD camera
(University Imaging Co., Downingtown, PA, USA) as de-
scribed previously [15]. Images were digitized and analyzed
through MetaFluor software (Universal Imaging).

Electrophysiology

Whole-cell voltage-clamp recordings were made using the perforated patch-clamp method at room temperature. Cur-
rents were recorded using a MultiClamp 700B amplifier
(Axon Instruments, Union City, CA, USA), subsequently
digitized with a sampling rate of 10 kHz, and analyzed us-
ing pCLAMP10 software (Axon Instruments). The pipette
resistance varied between 3 \(\sim\) 5 MΩ. Whole-cell currents
were elicited by voltage ramps from \(-100 \text{ mV to } +100 \text{ mV}\)
(50-ms duration) applied every 2 s from a holding potential
of 0 mV. Pipettes for recordings of TRPM7 currents were
filled with an internal solution containing (in mM): 140
CaCl\textsubscript{2}, 5 NaCl, 10 EGTA, 10 HEPES, adjusted to pH 7.2
with CsOH [16]. Nystatin was diluted to a final concen-
tration of 250 \(\mu\)g/ml in the internal solution. The external
solution containing (in mM): 140 NaCl, 3 KCl, 1 CaCl\textsubscript{2},
2 MgCl\textsubscript{2}, 10 HEPES, 10 glucose, adjusted to pH 7.2 with
NaOH, and the external solution was replaced by 3 mM
by following the manufacture’s procedure. Cells were seed-
ed on 35 mm dish at a density of \(1 \sim 5 \times 10^5\) cells in anti-
biotics free media. After 24 h, cells were re-plated in ade-
quate dish. Each plasmid and Lipofectamine 2000 (Invitro-
gen) were diluted in 250 \(\mu\)l Opti-MEM respectively and then
mixed. The mixture was incubated for 20 min at room tem-
perature before adding the cell media. Cells were assorted with
pEGFP expression.

Western blot

Whole cell lysates were prepared using RIPA lysis buffer
(20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0,
0.1% Triton-X100, 0.01 mg/ml aprotinin, 5 \(\mu\)g/ml leupeptin,
0.4 mM PMSF, and 4 mM NaVo), and then spun at 12,000
rpm for 10 min to remove insoluble material. Proteins (50
\sim\) 100 \(\mu\)g/well) were subjected to 6 \sim\) 12 SDS-PAGE re-
spectively, and then were separated by size. Separated proteins were electro-transferred to a nitrocellulose membrane, blo-
ced with 6% skimmed milk, and probed with Abs against
TRPM7 (1 : 1,000) and NFATc1 (1 : 3,000). Thereafter,
blots were washed, exposed to horseradish peroxidase-con-
jugated secondary antibodies for 1 h, and finally detected
by chemiluminescence (Amersham Pharmacia Biotech,
Alington Heights, IL, USA).

[Ca\textsuperscript{2+}], measurement

Cells were seeded on cover glass in 35-mm dishes (5\(\times\)10\textsuperscript{4}
cells). After 24 h, cells were stimulated with RANKL (50
ng/ml) for indicated time. Cells in physiological salt solution
(140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10
mM HEPES, 10 mM glucose, 310 mOsm, pH 7.4) were in-
cluded and monitored at 2 s intervals using a CCD camera
(University Imaging Co., Downingtown, PA, USA) as de-
scribed previously [15]. Images were digitized and analyzed
through MetaFluor software (Universal Imaging).
CaCl₂ to change a Mg²⁺-free external solution.

**Immunocytochemistry**

Cells were seeded on coverslips (22×22) and treated with 50 ng/ml RANKL for 48 h. After fixation in 4% paraformaldehyde (PFA) for 5 min, cells were sequentially incubated in blocking solution (0.1% gelatin, 1% BSA, 0.01% sodium azide, 5% goat serum) for 30 min, overnight in blocking solution containing Abs against NFATc1 (1:100), and finally were treated with Alexa 488-labeled anti-mouse IgG antibody (Molecular Probes) in blocking solution for 1 h. Nuclei was separately stained with DAPI.

**TRAP staining**

The method of TRAP staining has been detailed previously [10]. Briefly, cells were seeded on 48 well-plate at a density of 2×10⁴ cells/well and cultured in α-MEM containing 10% FBS with 50 ng/ml RANKL and M-CSF. After 6 days, TRAP (tartrate-resistant acid phosphatase) staining was performed to determine the extent of differentiation. Cytochemical staining of TRAP-resistant cells was performed using the Leukocyte Acid Phosphate Assay Kit (Sigma Aldrich) by following the manufacturer’s procedure. TRAP⁺ multinucleated cells (MNCs, ≥3 nuclei) were counted.

**Pit assay**

Cells were seeded on bone-slice covered plates (OAAS) and maintained with 50 ng/ml M-CSF and sRANKL for 15 days. After that, cells were washed with sodium hypochlorite solution for 1 h at room temperature. Bone slices were imaged, and pits were calculated using MetaMorph software (Molecular Devices).

**Statistics**

All data were expressed as the mean±SEM. Statistical significance was determined by using a paired or unpaired Student’s t-test. Statistical significance was set at p<0.05 level.

**RESULTS**

**Expression and actions of endogenous TRPM7 channels**

TRPM7 has been identified as spontaneously activated Ca²⁺- and Mg²⁺-entry channels and plays a key role for osteoblasts proliferation [17]. In this study, we first examined whether RANKL stimulation affects the expression of TRPM7. In order to confirm the expression of TRPM7, RANKL was respectively treated for 6 days that is predicted time to be differentiated into osteoclast. As shown in Fig. 1A, the expression of TRPM7 in RAW264.7 cells was not altered in the presence of RANKL. mRNA expressions of TRPM7 were reduced in siTRPM7-transfected cells (Fig. 1B). In whole-cell patch-clamp experiments, the removal of Mg²⁺ in extracellular solutions led to activation of TRPM7-
mediated currents that exhibit a highly nonlinear current-voltage (I-V) relationship with noticeable outward rectification at positive potentials in eGFP-transfected cells but not showed these effects in siTRPM7-transfected cells (Fig. 1C and 1D). A strongly outward-rectifying I-V relationship of TRPM7 is very similar features to other previous reports and these results show that TRPM7 is expressed regardless of RANKL stimulation and activated by low-Mg\textsuperscript{2+} concentration [14,15,18].

Effects of deletion of TRPM7 on RANKL-induced Ca\textsuperscript{2+} signaling

RANKL-induced Ca\textsuperscript{2+} increase, which is composed of internal Ca\textsuperscript{2+} release and extracellular Ca\textsuperscript{2+} influx, is essential step for triggering late-stage of osteoclastogenesis by activating NFATc1 [9,10]. To confirm whether TRPM7 is involved in the induction of RANKL-induced Ca\textsuperscript{2+} oscillations as a Ca\textsuperscript{2+} permeable channel, we examined the effects of deletion of TRPM7 on Ca\textsuperscript{2+} responses in the presence or absence of RANKL. Reduced mRNA expression of TRPM7 by siTRPM7 treatment (Fig. 2A) resulted in disappearance of Ca\textsuperscript{2+} oscil-

![Fig. 2. Effects of deletion of TRPM7 on [Ca\textsuperscript{2+}] oscillation mediated by RANKL stimulation and removal of extracellular Mg\textsuperscript{2+}. (A) Cells were transfected with siTRPM7 using lipofectamine 2000. After 72 h of transfection, whole mRNA was collected using Trizol method. Beta-actin is used as loading control. (B) Control and siTRPM7 treated cells were treated with RANKL. After 48 h of RANKL stimulation, [Ca\textsuperscript{2+}] in single cell was measured using Fura-2 fluorescence dye. To confirm cell’s viability, 1 mM ATP was used. (C) Application of 0 mM [Mg\textsuperscript{2+}] induced [Ca\textsuperscript{2+}] increases and it was repeated by the second application of 0 mM [Mg\textsuperscript{2+}] in HEK293 cells. [Ca\textsuperscript{2+}] increases also inhibited by 100 \mu M Gd\textsuperscript{3+} and transfected siTRPM7. (D) Application of 0 mM [Mg\textsuperscript{2+}] induced [Ca\textsuperscript{2+}] increases in the absence or presence of RANKL and it was inhibited by 100 \mu M Gd\textsuperscript{3+} and transfected siTRPM7 in RAW264.7 cells. (E) The degree of [Ca\textsuperscript{2+}] increases by 0 mM [Mg\textsuperscript{2+}] in HEK293 and RAW264.7 cells. Data were expressed as the mean±SEM. ***p<0.001 compared with 0 mM [Mg\textsuperscript{2+}] treated control. n.s., not significant.](image-url)
lutions in response to RANKL stimulation (Fig. 2B), which indicates that activation of TRPM7 is related with the induction of Ca\(^{2+}\) oscillations in osteoclastogenesis. We also observed that siTRPM7 inhibited Ca\(^{2+}\) signaling by 0 mM [Mg\(^{2+}\)]\(_{e}\) in the presence or absence of RANKL of RAW264.7 cells (Fig. 2D and 2E). The generation of Ca\(^{2+}\) oscillations are related with Ca\(^{2+}\) entry and Ca\(^{2+}\) release from intracellular IP\(_{3}\)-sensitive Ca\(^{2+}\) stores, and Ca\(^{2+}\) entry is mediated through the Mg\(^{2+}\)-sensitive pathway in polarized cells [19,20]. In HEK293 cells, Ca\(^{2+}\) signaling by 0 mM [Mg\(^{2+}\)]\(_{e}\) was completely blocked by 100 \(\mu\)M Gd\(^{3+}\), a known blocker of Ca\(^{2+}\) release activated Ca\(^{2+}\) entry (Fig. 2C and 2E). Interestingly, the [Ca\(^{2+}\)]\(_{i}\) increases by Mg\(^{2+}\) free solution were not observed in TRPM7 knock-down cells. These results suggest that TRPM7 is playing an essential role in RANKL-induced Ca\(^{2+}\) oscillations as mediating an extracellular Ca\(^{2+}\) entry which is regulated through the Mg\(^{2+}\)-sensitive pathway.

Effects of deletion of TRPM7 in late-stage of osteoclastogenesis

It has been known that RANKL-induced Ca\(^{2+}\) oscillations is a key factor to determine the late-stage of osteoclastogenesis by sequentially activating calmodulin, calcineurin, and NFATc1 [11]. Activated NFATc1 gets translocated into

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**Fig. 3.** Effects of deletion of TRPM7 on NFATc1 and bone resorptive activity. (A and B) Cells were transfected with sicontrol and siTRPM7 in the presence or absence of RANKL for 48 h. To confirm the localization and expression of NFATc1, cells were fixed with 4% paraformaldehyde (PFA) and whole lysates were collected using RIPA lysis buffer respectively. Localization inside the cell and NFATc1 expression in whole lysates was confirmed with antibody for NFATc1. (C) To verify the formation of MNCs, each sample was incubated for 6 days in the presence of RANKL. TRAP staining was performed as described in methods. And then MNCs in each well was counted. (D) To determine the bone resorptive activity, cells were seeded on bone-slice covered plate in the presence of RANKL for 15 days. After incubation, whole image of each well was taken to calculate mineralized area as described in methods. Data were normalized to the expression level in RANKL treated control and expressed as the mean±SEM. **p < 0.01, *p < 0.05 compared with RANKL treated control.
the nucleus and function as a transcription factor, which induces the expression of proteins such as TRAP, Src kinase, INF-γ, calcitonin, etc [21]. Over these series of protein expression, precursor cells get fused into MNCs, which have abilities of mineralizing the bone. According to previous results, we hypothesized that abolished Ca^{2+} oscillations by deletion of TRPM7 would affect on the activities of bone resorption. To confirm our hypothesis, the expression and translocation of NFATc1 in response to deletion of TRPM7 was examined in RAW264.7 cells and BMMs. As a result, lack of TRPM7 diminished RANKL-induced NFATc1 translocation into nucleus and reduced NFATc1 expression by 60% in RAW264.7 cells (Fig. 3A) and BMMs (Fig. 3B). Furthermore, deletion of TRPM7 significantly reduced RANKL-induced responses, formation of MNCs in BMMs (Fig. 3C) and bone resorptive activity in RAW264.7 cells (Fig. 3D). Taken together, these results suggest that abolished Ca^{2+} oscillations by deletion of TRPM7 affected on NFATc1 activities, fusion into MNCs, and bone resorption.

**DISCUSSION**

As tools for communication, intracellular Ca^{2+} concentration determine various cellular responses such as proliferation, movement, differentiation, etc [1]. Two separated Ca^{2+} sources, internal and external Ca^{2+} sources, are virtually being used for triggering and maintaining all the Ca^{2+} responses including osteoclastogenesis. The property as a Ca^{2+} conducting channels raised the possibility that TRPM7 may be involved in osteoclastogenesis, based on the requirement for Ca^{2+} responses. In present study, we examined the potential use of TRPM7 in modulating the bone resorptive activities of osteoclast. The first finding of this study is that TRPM7 is being expressed in osteoclast precursor cells and the constitutive activities are being negatively regulated by intracellular Mg^{2+} concentration. In osteoclastogenesis, precursor cells present long-lasting Ca^{2+} oscillations in response to RANKL stimulation. Signals leading differentiation clearly require sustained extracellular Ca^{2+} influx through certain Ca^{2+} permeable channels. Hence, our result suggests that TRPM7 must be considered as a candidate for mediating the long-lasting Ca^{2+} oscillations. Notably, RANKL-induced Ca^{2+} oscillations and extracellular Ca^{2+} influx mediated by reduction of intracellular Mg^{2+} concentration were completely diminished by deletion of TRPM7. In our previous work, ROS, which is generated by RANKL stimulations, induce Ca^{2+} oscillations through activating the enzymatic reaction such as PLC [9,22]. Based on its characteristics as a kinase and channel, our results strongly suggest that function of TRPM7 as a kinase and cation channel is tightly linked with RANKL-induced ROS generation. Taken together, constitutive expression of TRPM7 would help precursor cells immediately sense the ROS generation and then ROS mediated activation of TRPM7 contributes the induction of Ca^{2+} oscillations by allowing extracellular Ca^{2+} influx. In terms of Mg^{2+} homeostasis in blood plasma, it is well established that bone is also considered as Mg^{2+} store in a body. Negative regulation of TRPM7 by Mg^{2+} concentration may explain the regulation of osteoclast activation. Our present study strongly proposes that Mg^{2+} concentration in blood plasma is one of determinants for osteoclast activity by modulating the TRPM7 conductance.

It has been reported that NFATc1 expression is enhanced in response to RANKL stimulation at transcriptional level.

Considering our main focus of this study, which is on identifying the possibility as a candidate for modulating the bone metabolism, we investigated the effects of deletion of TRPM7 on late-stage of osteoclastogenesis like forming MNCs and bone resorption. Only if TRPM7 has roles in a process of being differentiated into fully-activated osteoclasts, we may use it as a target molecule to treat bone diseases. Our results indicate that extracellular Ca^{2+} influx through TRPM7 is regarded as a nodal point which sequentially regulates in the activity of NFATc1, forming MNCs, and physiological activity of osteoclasts. These findings address missing links between generation of ROS and induction of Ca^{2+} oscillations, which are required for triggering late-stage of osteoclastogenesis. TRPM7, which is activated by sensing both intracellular ROS generation and extracellular Mg^{2+} concentration, is acting as a mediator of extracellular Ca^{2+} entry in RANKL-induced osteoclastogenesis. Based on our results, adjusting the activities of TRPM7 by genetic modification and Mg^{2+} ingestion has to be considered as a novel way to treat bone diseases.

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