Effects of Inositol 1,4,5-triphosphate on Osteoclast Differentiation in RANKL-induced Osteoclastogenesis

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The receptor activator of NF-κB ligand (RANKL) signal is an activator of tumor necrosis factor receptor-associated factor 6 (TRAF6), which leads to the activation of NF-κB and other signal transduction pathways essential for osteoclastogenesis, such as Ca^{2+} signaling. However, the intracellular levels of inositol 1,4,5-trisphosphate (IP_3) and IP_3-mediated cellular function of RANKL during osteoclastogenesis are not known. In the present study, we determined the levels of IP_3 and evaluated IP_3-mediated osteoclast differentiation and osteoclast activity by RANKL treatment of mouse leukemic macrophage cells (RAW 264.7) and mouse bone marrow-derived monocyte/macrophage precursor cells (BMMs). During osteoclastogenesis, the expression levels of Ca^{2+} signaling proteins such as IP_3 receptors (IP_3Rs), plasma membrane Ca^{2+} ATPase, and sarco/endoplasmic reticulum Ca^{2+} ATPase type2 did not change by RANKL treatment for up to 6 days in both cell types. At 24 h after RANKL treatment, a higher steady-state level of IP_3 was observed in RAW 264.7 cells transfected with green fluorescent protein (GFP)-tagged pleckstrin homology (PH) domains of phospholipase C (PLC) δ, a probe specifically detecting intracellular IP_3 levels. In BMMs, the inhibition of PLC with U73122 [a specific inhibitor of phospholipase C (PLC)] and of IP_3Rs with 2-aminoethoxydiphenyl borate (2APB; a non-specific inhibitor of IP_3Rs) inhibited the generation of RANKL-induced multinucleated cells and decreased the bone-resorption rate in dentin slice, respectively. These results suggest that intracellular IP_3 levels and the IP_3-mediated signaling pathway play an important role in RANKL-induced osteoclastogenesis.

Key Words: Inositol 1,4,5-trisphosphate, RANKL, Osteoclastogenesis, Ca^{2+} signaling

INTRODUCTION

Bone is a connective tissue that constructs the skeleton and is composed of the intracellular matrix proteoglycans, osteoblasts, osteoclasts, and osteocytes. It is a dynamic tissue that is constantly renewed by bone remodeling. Bone remodeling is an active process and is regulated by the activity of bone-forming osteoblasts and bone-resorbing osteoclasts [1,2]. In fact, an imbalance in bone remodeling can cause pathological conditions such as rheumatoid arthritis, osteoporosis, and osteopetrosis.

Cells grow and differentiate through cell-to-cell interactions, and extracellular signals lead to specific cell reactions through signal transduction. Cell-to-cell signaling of mesenchymal cells, including osteoblasts, induces the differentiation of osteoclast precursor cells into mature osteoclasts [3,4]. Receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) is expressed on the surfaces of osteoclasts, and an essential factor for osteoclast differentiation. RANKL, a member of the tumor necrosis factor (TNF) family, is expressed in the bone, bone marrow, and lymphoid tissue during osteoclastogenesis. RANKL binds to RANK and transmits a differentiation signal [5]. It is known that RANKL stimulation initially activates tumor necrosis factor receptor-associated factor 6 (TRAF6), and then sequentially activates NF-κB, c-fos, JNK, ERK, and PI3K in osteoclast precursor cells [6,7]. Recent studies of osteoclastogenesis have focused on TRAF6, JNK, and NF-κB signaling pathways triggered by the binding of RANKL to RANK on osteoclast precursors. During osteoclastogenesis, auto-amplification of NFATc1 induces osteoclast-specific genes including AP-1, tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, and cathepsin K [6]. Recently, Ca^{2+} signaling has been recognized as an essential pathway in the differentiation of osteoclasts. In particular, a pre-

ABBREVIATIONS: RANKL, receptor activator of NF-κB ligand; TRAF6, tumor necrosis factor receptor-associated factor 6; IP_3, inositol 1,4,5-trisphosphate; BMMs, bone marrow-derived monocyte/macrophage precursor cells; PLC, phospholipase C.

vious study has shown that treatment with a Ca^{2+} chelator inhibits osteoclastogenesis in osteoclast precursor cells [8], suggesting that Ca^{2+} signaling is important in osteoclastogenesis.

Hormone and neurotransmitter-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) regulate gene expression, growth, differentiation, muscle contraction, memory, and learning [9]. Particularly, in osteoclast differentiation, Ca^{2+} was reported to play an important role by sequentially activating calcineurin and NFATc1 [5,8,10]. In this study, Ca^{2+} signals were shown to exist in a unique form, such as Ca^{2+} oscillations, which are a recurring phenomena of increases and decreases in $[Ca^{2+}]_i$. Generally, in non-excitable cells, the increase in $[Ca^{2+}]_i$ occurs through the activation of Ca^{2+} channels or Ca^{2+} release from the ER into the cytoplasm, and is then followed by activation of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and the plasma membrane Ca^{2+} ATPase (PMCA) that remove Ca^{2+} from the cytosol [11,12]. In most cells, extracellular signals such as hormones and neurotransmitters stimulate phospholipase C (PLC) and then hydrolyze a plasma membrane lipid phosphatidyl-inositol bisphosphate (PIP_2) to generate inositol 1,4,5-trisphosphate (IP_3) in the cytosol. Increased levels of IP_3 promote Ca^{2+} release into the cytosol by changing Ca^{2+} permeability of the IP_3Rs in the ER, which is the intracellular Ca^{2+} store. Ca^{2+} release from the ER can change the conductance of Ca^{2+} release-activated Ca^{2+} channels. It induces Ca^{2+} influx from an environment of high concentration of extracellular Ca^{2+} [12]. An induced increase in $[Ca^{2+}]_i$ is removed by the Ca^{2+} pump. Nevertheless, there are no reports on the intracellular levels of IP_3 in osteoclast differentiation. In the present study, we assessed the levels of IP_3 and the associated IP_3-induced osteoclast differentiation and activity during RANKL-mediated osteoclastogenesis in osteoclast precursor cells.

METHODS

Cell cultures and reagents

The mouse monocyte cell line RAW264.7 (Korean Cell Line Bank, South Korea) and primary cultured, bone marrow-derived monocyte/macrophage precursor cells (BMMs) were respectively cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) and α-minimum essential medium (α-MEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics, in a 37^oC incubator (5% CO_2). To maintain BMMs, α-MEM was supplemented with 50 ng/ml macrophage colony-stimulating factor (M-CSF). RANKL and M-CSF were purchased from Koma Biotech, Inc. (Seoul, Korea). U73122, U73343 and 2-aminoethoxydiphenyl borate (2-APB) were from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies for IP_3R1, IP_3R2, and IP_3R3 were generous gifts from Dr. Akihiko Tanimura (Hokkaido University, Japan), and the monoclonal antibody for PMCA (5F10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The vector containing the PLC δ-PH-GFP construct was a generous gift from Dr. Shmuel Muallem (NIH/NIDCR, USA).

Preparation of BMMs

Four-week-old male mice (Koatec, Pyeongtaek, Korea) were sacrificed, and the femur and tibia were separated.

Bone marrow cells derived from the femur and tibia were collected and cultured in α-MEM containing 10% FBS and 10 ng/ml M-CSF. The next day, non-adherent cells in the media were collected and seeded on adequate number of plates with 50 ng/ml M-CSF. After 2 days, non-adherent cells were washed out with fresh media, and the adherent cells were used as BMMs.

Western blot analysis

Whole cell proteins were prepared in lysis buffer [20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO4), and the extracts were centrifuged at $1,100 \times g$ for 10 min to remove insoluble material. Cleared extracts ($50 \sim 100\,\mu g$ of protein/well) were subjected to $6 \sim 12\%$ SDS-PAGE, and the proteins were electrotransferred to a nitrocellulose membrane, blocked with 6% skimmed milk, and probed with antibodies against IP_3R1 (1 : 1,000), IP_3R2 (1 : 5,000), IP_3R3 (1 : 1,000), PMCA (1 : 5,000), SERCA2b (1 : 5,000). The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech, Schenectady, NY, USA).

Expression of IP_3 Sponge and PLC δ-PH-GFP expression in RAW264.7 cells

RAW264.7 cells were transiently transfected with a green fluorescent protein (GFP)-tagged high affinity (R441Q) or low affinity (K508A) IP_3-sequestering sponge [13], or were transfected with PLC-δ1-PH-GFP construct provided by Dr. Shmuel Muallem (NIH/NIDCR, USA) using Lipofectamine 2000 reagent (invitrogen), according to the manufacturer's instructions. Cells were incubated for 48 h at 37^oC, in a 5% CO_2 atmosphere with saturated humidity to allow expression of each protein was confirmed by GFP fluorescence.

Measurements of PLC δ-PH-GFP expression in RAW 264.7 cells

After RANKL treatment, GFP-positive cells were identified, and the GFP density in PLC δ-PH-GFP cDNA-transfected RAW264.7 cells was measured using a confocal laser scanning microscope (Leica, Buffalo, NY, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

TRAP stain assay

BMMs were seeded in 48-well plates at a concentration of 1×10^4 cells per well and pretreated with indicated compounds. Cells were then stimulated with 50 ng/ml sRANKL. After 6 days, a TRAP stain assay was performed to confirm the cell differentiation rate. TRAP-positive cells were stained using a Leukocyte Acid Phosphate Assay Kit (Sigma-Aldrich) by following the manufacturer's procedure. TRAP-positive multinucleated cells (containing ≥ 3 nuclei) were then counted.

Measurement of bone-resorption rate (pit assay)

BMMs were collected from 4-week-old mice, as described

previously [14]. Collected BMMs were seeded on 48-well bone-slice covered plates, coated with an osteoclast activity assay substrate (OAAS^{TM}; OCT, Cheonan, Korea). BMMs in bone-sliced plates were maintained with 50 ng/ml M-CSF and sRANKL for 15 days. After 15 days, cells were washed out with sodium hypochlorite solution for 1 h at room temperature. The bone slices were imaged, and the pits in the bone slices were identified based on pixel area calculations. The data were expressed relative to the pit areas in RANKL-stimulated BMMs by using the MetaMorph software (Molecular Devices).

Statistical analyses

Data from at least three independent experiments were expressed as mean±SD. Statistically significant differences between groups were determined using the Student t-test.

RESULTS

RANKL-induced expression of Ca^{2+} signaling proteins

To assess the effect of RANKL stimulation on the expression levels of Ca^{2+} signaling proteins such as IP$_3$R, SERCA, and PMCA, RAW264.7 cells and BMMs were treated with RANKL for 24 h, 48 h, 72 h, and 6 days. There were no differences in the expression levels of these Ca^{2+} signaling proteins after RANKL treatment (Fig. 1, n=3).

RANKL-induced [Ca^{2+}]$_i$ oscillations depend on Ca^{2+} release from the IP$_3$-sensitive stores

Previous studies show that Ca^{2+} oscillations were generated by RANKL stimulation [15]. To confirm the generation of RANKL-induced Ca^{2+} oscillations, we treated RANKL for 48 h on BMMs. As a results, RANKL-induced Ca^{2+} oscillations were observed at 48 h (Fig. 2A, n=3). To determine the role of PLC/IP$_3$-dependent pathway in RANKL-induced Ca^{2+} oscillations, cells were pretreated with 10 μM U73122, a specific blocker of PLC, or its inactive analog, U73343. U73122 prevented RANKL-induced Ca^{2+} oscillations, whereas 10 μM U73343 had no effect

Fig. 1. Effect of receptor activator of NF-κB ligand (RANKL) on the expression of Ca^{2+} signaling proteins. RAW264.7 cells and mouse bone marrow derived monocyte/macrophage precursor cells (BMMs) were treated with RANKL (50 ng/ml). Levels of three types of inositol 1,4,5-trisphosphate receptors (IP$_3$Rs), plasma membrane Ca^{2+} ATPase (PMCA), and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2b were measured at different time points from 24 h to 6 days after RANKL treatment.

(Fig. 2B, n=3) and inhibition of IP$_3$R with the IP$_3$R inhibitor 75 μM 2APB inhibited RANKL-induced Ca^{2+} oscillations in BMMs (Fig. 2C, n=3). In addition, we also observed that expression of the IP$_3$ sponge completely abrogated RANKL-induced Ca^{2+} oscillations although the expression of low affinity IP$_3$ sponge did not prevent the RANKL-induced Ca^{2+} oscillations (Fig. 2D, n=3).

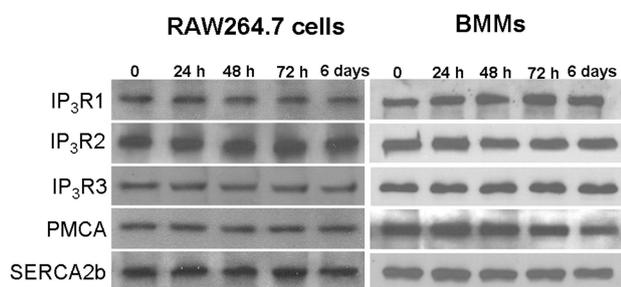### RANKL-mediated changes in intracellular IP$_3$ levels

Because there were no differences in expression levels of Ca^{2+} signaling proteins after RANKL treatment (Fig. 1), we hypothesized that RANKL-mediated activation of PLC induced an increase in intracellular IP$_3$ levels, and then sequentially generated Ca^{2+} oscillations. To test our hypothesis, we transfected RAW264.7 cells with the PLC δ-PH-GFP domain. The localization of PLC δ-PH-GFP domain was confirmed using confocal laser microscopy after 24 h of RANKL treatment. In the steady state, the PLC δ PH-domain was located in the cell membrane in the absence of RANKL treatment; however, after 24 h of RANKL treatment, the PH-domain bound to IP$_3$ and then moved into the cytoplasm (Fig. 3, n=3).

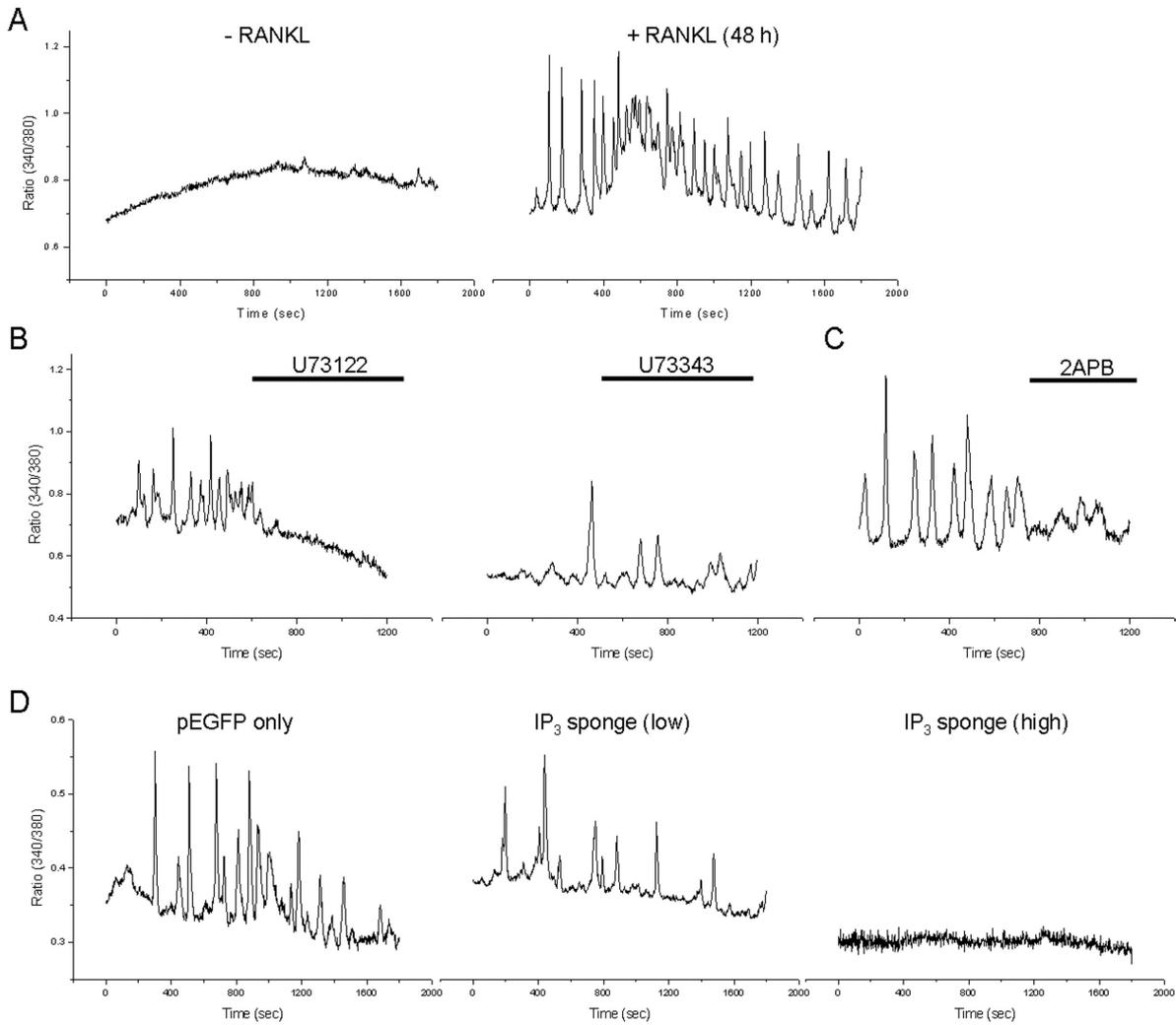### Effect of PLC and IP$_3$R on RANKL-mediated osteoclast differentiation and osteoclast activity

Based on our finding that RANKL induced an increase in intracellular IP$_3$ levels, we further investigated the role of PLC and IP$_3$R activation on RANKL-mediated osteoclast differentiation and osteoclast activity using a TRAP staining assay and pit assay. The blockage 10 μM U73122, and 75 μM 2APB inhibited the generation of RANKL-induced multinucleated cells to 60% and 84.3% of the controls, respectively (Fig. 4A, n=3). In addition, we measured osteoclast activity in RAW264.7 cells and BMMs using dentin slices following U73122 and 2APB treatment. Pit areas in RANKL-induced osteoclastogenesis were decreased to 48.4% and 93.6% of the controls, respectively (Fig. 4B, n=3).

DISCUSSION

Osteoclastogenesis requires costimulatory receptor signaling mediated through adaptor proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs), such as the Fc receptor γ (FcR γ) and the 12-kDa DNAX-activating protein [5]. The active signal transmitted through adaptor proteins then induces Ca^{2+} signaling, which sequentially activates NFATc1 and induces osteoclast differentiation [5,10]. In addition, the signal via TRAF6 activates transcription factors such as c-fos and NF-κB [4] and the Ca^{2+} signal and activation of NFATc1 are transduction signals occurring after the middle stage of osteoclast differentiation in most cells. It is likely that activation of IP$_3$Rs in the ER may induce Ca^{2+} oscillations. Because there was no way to directly measure the intracellular concentration of IP$_3$, we transfected cells with a PLC δ-PH domain [16]. The transfected PLC δ-PH domain binds to PIP$_2$ with high specificity and affinity during the resting state. However, it is translocated to the cytoplasm and binds to IP$_3$ when IP$_3$ accumulates via an external signal because the PLC δ-PH domain has a stronger affinity for IP$_3$ than for PIP$_2$ [16]. We demonstrated that RANKL induces an increase in IP$_3$ levels at 24 h after RANKL treatment, after which
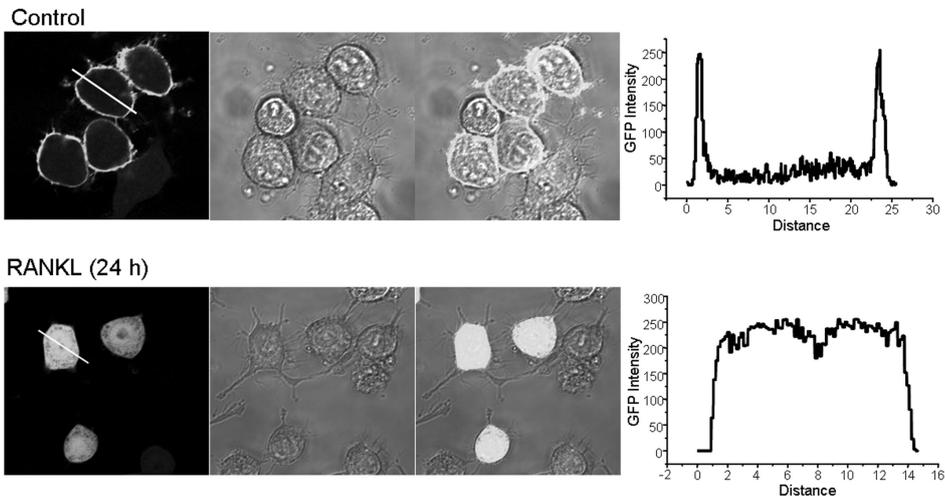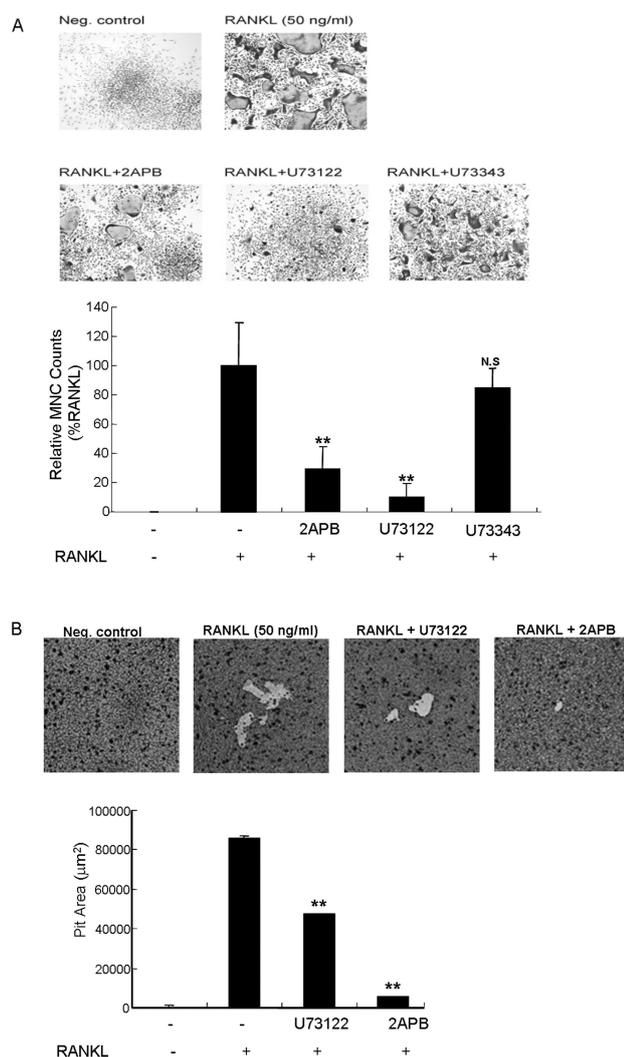
Fig. 2. Effect of PLC and IP_3 inhibition on RANKL-induced $[Ca^{2+}]_i$ oscillations in BMMs. (A) Generation of Ca^{2+} oscillations after 48 h with RANKL treatment. (B, C) Inhibitory effect of RANKL-induced $[Ca^{2+}]_i$ oscillations by the PLC inhibitor, U73122 and IP_3R inhibitor, 2APB. (D) Inhibition of RANKL-induced $[Ca^{2+}]_i$ oscillations by elimination of IP_3.

• PLC δ-PH-GFP transfected RAW264.7 cells

Fig. 3. Localization of phospholipase C (PLC) δ-PH-GFP in RAW264.7 cells after RANKL stimulation. RAW264.7 cells were treated with RANKL following their transfection with the PLC δ-PH-GFP domain. After 24 h with RANKL treatment, GFP intensity was measured using confocal laser microscopy. The graph shows the fluorescence intensity along the lines indicated in the images.

Fig. 4. Effects of IP$_3$R inhibitor and PLC inhibitor on RANKL-induced multinucleated cell formation and pit formation in BMMs. (A) BMMs were seeded in 48-well plates and cultured for 6 days with RANKL and M-CSF. RANKL was simultaneously added to 2-aminoethoxydiphenyl borate (2APB)-, U73122-, and U73343-treated cells. Multinucleated cells (MNCs; ≥ 3 nuclei) in a well are presented as MNCs per well. Data from three independent experiments are represented as mean±SD (**p<0.005 compared with RANKL-induced MNC counts). (B) BMMs were seeded on OAAS plates, the bottom of which was covered with dentin slices, and cultured for 15 days with RANKL and M-CSF. RANKL was simultaneously added to 2APB- and U73122-treated cells. Data show the relative pit area compared to RANKL-treated BMMs and mean±SD obtained from three independent experiments (**p< 0.005 compared with RANKL-induced pit area).

IP$_3$ binds to the PH-domain and translocates from the membrane to the cytosol. On the other hand, during osteoclastogenesis, PLCγ activation is important to generate Ca^{2+} signaling, and this has been confirmed by using the PLC inhibitor U73122 [15]. In our study, treatment of cells with U73122 inhibited the generation of RANKL-induced Ca^{2+} oscillations, multinucleated cells, and reduced the bone-resorption rate. In addition, we observed that treatment with

2APB, an IP$_3$R inhibitor, inhibited the generation of RANKL-induced Ca^{2+} oscillations, multinucleated cells, and reduced bone-resorption rate. This result is consistent with a previous finding that, Ca^{2+} oscillations were not generated and *in vitro* osteoclastogenesis was impaired in IP$_3$R2/3-deficient mice [17]. Therefore, IP$_3$-mediated Ca^{2+} signaling is essential for osteoclastogenesis.

Previous studies showed that Ca^{2+} influx channels located on the plasma membrane play an important role in osteoclastogenesis [18,19]. Transient receptor potential cation channel subfamily V member 4 (TRPV4)-deficient mice led to an increase in bone mass; Ca^{2+} oscillations were normal in these cells at the early stage of osteoclastogenesis; however, they gradually disappeared at the later stages. These results suggest that Ca^{2+} influx via TRPV4 is necessary for Ca^{2+} signaling [19]. TRPV5-deficient mice also showed an increase in bone mass, although increased number and size of osteoclasts *in vivo*, suggesting the importance of Ca^{2+} influx for resorption activity in mature osteoclasts [18]. Therefore, it remains to be determined how Ca^{2+} oscillations and Ca^{2+} channel-mediated Ca^{2+} influx relate to osteoclastogenesis.

In summary, these results suggest that RANKL induces increases in intracellular IP$_3$ and IP$_3$ plays an important role in osteoclstogenesis. Therefore, manipulation of this signaling pathway may provide a target for treatment of bone-related disease.

ACKNOWLEDGEMENTS

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