Activation of G Proteins by Aluminum Fluoride Enhances RANKL-Mediated Osteoclastogenesis

Boryung Park¹, Yu-Mi Yang¹, Byung-Jai Choi³, Min Seuk Kim³, and Dong Min Shin¹

Departments of ¹Oral Biology, BK21 PLUS Project, ²Pediatric Dentistry, Yonsei University College of Dentistry, Seoul 120-752, ³Department of Oral Physiology, College of Dentistry, Wonkwang University, Iksan 570-749, Korea

Receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis is accompanied by intracellular Ca²⁺ mobilization in a form of oscillations, which plays essential roles by activating sequentially Ca²⁺/calmodulin-dependent protein kinase, calcineurin and NFATc1, necessary in the osteoclast differentiation. However, it is not known whether Ca²⁺ mobilization which is evoked in RANKL-independent way induces to differentiate into osteoclasts. In present study, we investigated Ca²⁺ mobilization induced by aluminum fluoride (AlF₄⁻), a G-protein-activator, with or without RANKL and the effects of AlF₄⁻ on the osteoclastogenesis in primary cultured mouse bone marrow-derived macrophages (BMMs). We show here that AlF₄⁻ induces intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) oscillations, which is dependent on extracellular Ca²⁺ influx. Notably, co-stimulation of AlF₄⁻ with RANKL resulted in enhanced NFATc1 expression and formation of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells. Additionally, we confirmed that mitogen-activated protein kinase (MAPK) is also activated by AlF₄⁻. Taken together, these results demonstrate that G-protein would be a novel modulator responsible for [Ca²⁺]ᵢ oscillations and MAPK activation which lead to enhancement of RANKL-mediated osteoclastogenesis.

Key Words: AlF₄⁻, Ca²⁺ signaling, G protein, MAPK activation, Osteoclastogenesis

INTRODUCTION

Receptor activator of NF-κB ligand (RANKL) expressed from osteoblasts binds to its own receptor, RANK, in osteoclast precursor cells, bone marrow-derived macrophage (BMMs), and initiates osteoclastogenesis by activating various intracellular signal pathways including mitogen-activated protein kinases (MAPKs), NF-κB, AP-1, c-fos, and NFATc1 [1-5]. Among them, NFATc1, which is regarded as a key factor to determines the late-stage of differentiation to osteoclast, is well defined to be modulated by intracellular Ca²⁺ mobilization [5]. According to previous reports including ours, RANKL generates intracellular Ca²⁺ mobilization via co-stimulatory signals mediated through immunoreceptor tyrosine-based activation motif (ITAM)-harbouring adaptors, such as Fc receptor common γ subunit (FcRγ) and DNAx-activating protein (DAP) 12 and reactive oxygen species (ROS) generation. RANKL-mediated intracellular Ca²⁺ mobilization is presented in a form of oscillations which needs Ca²⁺ flux into cytoplasm from both extracellular Ca²⁺ stores to form and sustain oscillation frequencies that is essential for sequential activation of Ca²⁺/calmodulin-dependent kinase, calcineurin, and NFATc1 [6,7]. In contrast, it has not been reported that Ca²⁺ mobilization generated by RANKL-independent way affects on differentiation into osteoclast.

Diverse signal pathways mediated by G-protein coupled receptors (GPCR) is reported to be involved in various osteogenic activities including cell survival, tumorigenesis, and differentiation of osteoclast [8-12]. For example, ovarian cancer G protein-coupled receptor 1 (OGGR1) activated by protons or lysolipids modulates not only osteoclast survival through NFAT-independent but also osteoclastogenesis through an OGR1/NFAT pathway [8,11]. Notably, regulator of G-protein signaling (RGS) 18, which is known to act as a GTPase activating protein (GAP), negatively regulates Ca²⁺ influx.

ABBREVIATIONS: RANKL, receptor activator of NF-κB ligand; NFATc1, nuclear factor of activated T cells cytoplasmic 1; AlF₄⁻, aluminum fluoride; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; MAPK, mitogen-activated protein kinase; FcRγ, Fc receptor common γ subunit; ROS, reactive oxygen species; OGR1, ovarian cancer G protein-coupled receptor 1; GAP, GTPase activating protein; MNCs, multinucleated cells; RGS, regulator of G-protein signaling; CREB, cAMP response element binding protein.
ulates osteoclastogenesis by modulating the activity of Gα subunit [11]. Here an important question has arisen whether modulating the activity of Gα subunit alone affects on the RANKL-induced Ca2+ oscillations and osteoclastogenesis.

Aluminum-fluoride complex (AlF4–) act as an analog of a phosphate group and stimulates cellular heteromeric G-proteins because of its structural similarity with phosphate group. AlF4– is tetrahedral and its Al-F bond length is very similar to P-O bond length of phosphate [13]. AlF4– can be used as useful tools investigating signal pathways following G-proteins. AlF4– stimulates G-protein and mimics the action of many neurotransmitters, hormones, and immune system [13,14]. AlF4–-induced Ca2+ oscillations were showed in smooth muscle cell [15] and pancreatic acinar cells [16]. AlF4– is also known to transmit signals modulating activities of bone cells, such as cell proliferation, differentiation [17] and protein phosphorylation [18]. It was reported that the effects of fluoride and aluminum on levels of the second messenger molecules are dependent on the type of cells and tissues [13]. Along with these reports, we postulated that induced Ca2+ signaling by aluminum-fluoride complexes may affect osteoclast differentiation, and the investigation was undertaken to study the effects of AlF4– on Ca2+ signaling and osteoclasts differentiation in primary cultured mouse bone marrow-derived macrophages (BMMs). In this study, we demonstrate that co-stimulation of AlF4– with RANKL has synergistic effects enhancing RANKL-induced Ca2+ oscillations, NFATc1 expression, and forming multinucleated cells (MNCs).

METHODS

Chemicals and antibodies

Recombinant mouse soluble RANK ligand and recombinant mouse M-CSF were purchased from KOMA Biotech (Seoul, Korea). AlCl3 and NaF were purchased from Sigma Aldrich (St. Louis, MO, USA) and Fluka (Buchs, Switzerland) respectively. Fura-2/AM was purchased from Teflabs (Austin, TX, USA). Pluronic F-127 was obtained from Invitrogen (Eugene, Oregon, USA). Monoclonal antibodies against ERK, phospho-ERK, JNK and phospho-JNK were purchased from Cell Signaling (Danvers, MA, USA); polyclonal antibody for NFATc1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Non-adherent bone marrow-derived macrophages (BMMs) were isolated from tibia and femur of 4-week-old male ICR mice (weight 21–25 g) as described previously [19]. Briefly, isolated BMMs were cultured in α-minimum essential medium (α-MEM; Gibco:BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco:BRL, Grand Island, NY, USA) in the presence of M-CSF (20 ng/ml).

Intracellular Ca2+ imaging

The cells were seeded on cover glass in a 35-mm dish (1×10^5 per dish) and treated with RANKL (50 ng/ml) and/or aluminum-fluoride complex (1.25 mM NaF+25 nM AlCl3). Cells were loaded with 3 μM fura-2/AM, 0.05% Pluronic F-127 in an extracellular physiological salt solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, and 10 mM glucose, titrated to pH 7.4 with NaOH. The osmolarity of the PSS was 310 mOsm) at room temperature and washed out with standard solution. [Ca2+]i was measured using wave lengths of 340 and 380 nm, and the emitted light was passed through a 510 nm cut off filter and was collected with a CCD camera and analyzed with MetaFluor software (Universal Imaging).

In vitro osteoclastogenesis and TRAP staining

RANKL (50 ng/ml) and/or AlF4– (1.25 mM NaF+25 nM AlCl3) were added to BMMs (2×10^5 per well) cultured in 24-well plate with α-MEM containing M-CSF (20 ng/ml). Six days later osteoclastogenesis was confirmed by TRAP (tartrate-resistant acid phosphatase) staining to evaluate TRAP-positive multinucleated osteoclast formation. Naphthol AS-MX (Amresco, Cleveland, OH, USA) was used as the substrate and fast red violet LB salt (Sigma Aldrich, St. Louis, MO, USA) as the diazonium salt. TRAP+ multinucleated cells (>3 nuclei) were counted.

Immunocytochemistry

Cells seeded on 12-mm cover glasses were treated with RANKL (50 ng/ml) and/or AlF4– (1.25 mM NaF+25 nM AlCl3). After fixation with 10% methanol at –20°C for 10 min, cells were washed with cold phosphate buffered saline (PBS) and neutralized with 50 mM Glycine for 10 min at 4°C. Cells were sequentially blocked with 5% goat serum in incubation buffer (0.1% gelatin, 1% BSA, 0.01% sodium azide in PBS) and stained with 1 drop of 200 U/ml phalloidin in the dark for 1 hr. And then, cells were washed with cold PBS.

Western blot

Whole cell extracts of BMMs were prepared by washing the cells with cold PBS and lysed in RIPA 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, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO3) for 30 min. 20 μg/well proteins were loaded on 10% SDS-PAGE respectively, and then were separated by size. Separated proteins were transferred onto nitrocellulose membranes, blocked with 5% BSA in TBS, and probed with Abs against phospho-ERK (1:1,000), total-ERK (1:1,000), phospho-JNK (1:1,000), total-JNK (1:1,000), NFATc1 (1:1,000), and β-actin (1:1,000) for overnight. After washing out, the blots were incubated with HRP-conjugated secondary antibodies for 1 hr, and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, IL, USA) using Agfa CP-BU new film (Mortsel, Belgium). The level of protein expression was digitized film images and analyzed with MetaMorph software (Universal Imaging).

Statistical analysis

Results are expressed as means±S.E. from at least 3 independent experiments. The statistical significances of differences between groups were determined using the Stu-
dent t-test. The difference is significant if the value is <0.05.

RESULTS

Treatment of AlF₄⁻ induces Ca²⁺ mobilization similar to RANKL-induced [Ca²⁺]i oscillation

We have previously reported that RANKL stimulation on BMMs induces [Ca²⁺]i oscillations which is dependent on Ca²⁺ flux from both external and internal store [6]. To determine whether direct activation of G-protein by AlF₄⁻ would affect on the intracellular Ca²⁺ mobilizations, we analyzed AlF₄⁻ -mediated Ca²⁺ responses in the presence or absence of RANKL. As shown in Fig. 1, [Ca²⁺]i oscillations, which is dependent on extracellular Ca²⁺ influx, were observed from 24 to 48 hrs after RANKL treatment in BMMs (Fig. 1, upper panel). Notably, co-stimulation with AlF₄⁻ and RANKL (Fig. 1, middle panel) and AlF₄⁻ treatment alone (Fig. 1, lower panel) led to increase of oscillations frequency in comparison to RANKL alone, suggesting that G-protein activation would amplify the RANKL-mediated signals and be enough to induce [Ca²⁺]i oscillations regardless of the presence of RANKL. Furthermore, these all [Ca²⁺]i oscillations presented dependency on extracellular Ca²⁺ influx. Along with these results, we could assumed that [Ca²⁺]i oscillations caused by AlF₄⁻ has similar characteristics with the one caused by RANKL. Moreover, AlF₄⁻ is capable to not only induce [Ca²⁺]i oscillations which has similar characteristics with RANKL-induced [Ca²⁺]i oscillations but also enhance it.

Fig. 1. Effects of the [Ca²⁺]i increases mediated by RANKL and AlF₄⁻ in BMMs. BMMs were treated with RANKL (50 ng/ml) and/or AlF₄⁻ (1.25 mM NaF+25 nM AlCl₃). After 48 h of the stimulations, [Ca²⁺]i oscillations in BMMs were measured using Fura-2 fluorescence dye. Compared to RANKL alone, application of AlF₄⁻ similarly increased frequency of the [Ca²⁺]i oscillations in the presence or absence of RANKL (left panels). These induction of [Ca²⁺]i oscillations presented dependency on extracellular Ca²⁺ influx. (right panel).

Fig. 2. Increased expression of NFATc1 in BMMs induced by RANKL and AlF₄⁻. Whole cells lysates were collected from cells stimulated with RANKL and/or AlF₄⁻ for indicated time. NFATc1 and actin were blotted with its antibody (upper panels) and then showed means of protein expression levels between RANKL and AlF₄⁻ treatments (n=3). Data were expressed as the means±SEM. **p<0.01, ***p<0.001 compared with RANKL treated group.
Co-stimulation with AlF<sub>4</sub>− and RANKL enhances NFATc1 expression, but not AlF<sub>4</sub>− alone

NFATc1 is known to be up-regulated by RANKL stimulation and modulates cell fusion and maturation [3]. As described previously, we found that AlF<sub>4</sub>− solely induces intracellular Ca<sup>2+</sup> mobilization in a form of oscillations which has similar characteristics with RANKL-induced [Ca<sup>2+</sup>]i oscillations. However, it is not clear that AlF<sub>4</sub>−-mediated [Ca<sup>2+</sup>]i oscillations is sufficient to transmit signals related on the differentiation to osteoclasts. To answer this question, we employed a standard in vitro osteoclast culture system and prepared whole cell lysates from cells maintained with M-CSF and RANKL in the presence of absence of AlF<sub>4</sub>−. As shown in Fig. 2, similar tendency of the NFATc1 induction was observed in both samples treated with RANKL alone and both of RANKL and AlF<sub>4</sub>−, which starts to be increased from 24 hrs and maximized around 48 hrs after stimulation. Whereas, in terms of quantity of expression, co-stimulation with RANKL and AlF<sub>4</sub>− more than 48 hrs resulted in 1.7-fold increase compared to RANKL alone. Unlike our expectation, sole treatment of AlF<sub>4</sub>− did not induce NFATc1 expression.

Co-stimulation with AlF<sub>4</sub>− and RANKL enhances the formation of TRAP-positive multinucleated cells, but not AlF<sub>4</sub>− alone

To further investigate the effects of AlF<sub>4</sub>− on RANKL-induced osteoclastogenesis, we examined the effects of enhanced [Ca<sup>2+</sup>]i oscillations by AlF<sub>4</sub>− on the formation of MNCs and actin ring. BMMs were stimulated with AlF<sub>4</sub>− (1.25 mM NaF + 25 nM AlCl<sub>3</sub>) in the presence or absence of RANKL (50 ng/ml) for 6 days, and TRAP and phalloidin staining were conducted. Interestingly, TRAP<sup>+</sup> MNCs were only confirmed in RANKL treated cells, but not in the cells with only AlF<sub>4</sub>− (Fig. 3A). However, BMMs treated with AlF<sub>4</sub>− and RANKL simultaneously showed remarkably enhanced MNCs formation (Fig. 3A). Consistent with TRAP staining results, we also confirmed that co-stimulation with AlF<sub>4</sub>− and RANKL increased actin ring formation compared to RANKL alone (Fig. 3B), but not in AlF<sub>4</sub>− alone. These results suggest that AlF<sub>4</sub>−-mediated signals are not sufficient for cell fusion and attachment but it improves osteoclastogenesis when it coexists with RANKL.

![Fig. 3. RANKL and AlF<sub>4</sub>−-induced osteoclast differentiation in BMMs.](image)
**Induction of MAPK signal pathway in response to AlF$_4^-$**

RANKL is also known to transmit signals through activation of MAPK pathway including ERK, JNK, and AP-1 crucial for gene expression by clustering with CREB, MITF, PU.1, and NFATC1 [20-23]. To gain insight of G-protein activation into MAPK pathway, we measured phosphorylation of ERK and JNK in response of RANKL and both of RANKL and AlF$_4^-$ (Fig. 4). Interestingly, phosphorylation of ERK and JNK was also detected in only AlF$_4^-$ treated sample, suggesting that activation of G-protein is somehow directly related with MAPK. Taken together, these results clearly indicate that activation of G-protein synergistically improve RANKL-mediated osteoclastogenesis through enhancing Ca$^{2+}$ oscillations and MAPK pathway.

**DISCUSSION**

Diverse Ca$^{2+}$ signals in osteoclast are essential for cellular functions including motility, differentiation, and bone resorption [6,8-10]. Several reports including ours clearly presents that RANKL-induced [Ca$^{2+}$]i oscillation occurred by activation of phospholipase C (PLC) and IP$_3$ production, which evokes Ca$^{2+}$ release from the ER, resulting in induction of Ca$^{2+}$ oscillations. There are numerous hormones and neurotransmitters which lead to intracellular Ca$^{2+}$ mobilization relied on sequential activation of GPCR, G-protein, and PLC [24,25]. Recent studies showed specific GPCRs are involved in osteoclastogenesis and deficiency of RGS impairs to differentiate into osteoclast due to the absence of Ca$^{2+}$ signaling [26]. Inspired by these, we assumed that activation of G-protein by itself may be sufficient or necessary for the differentiation into osteoclast regardless of RANKL stimulation. Under this hypothesis, we first confirmed that sole activation of G-protein generates [Ca$^{2+}$]i oscillations using AlCl$_3$ and NaF because traces of aluminum can form aluminum-fluoride complex (most likely AlF$_4^-$) easily in aqueous solutions [13] and makes a synergic action of fluoride to activate G-proteins [27]. AlF$_4^-$ is well known to directly activate a GTP-binding protein coupled to PLC [28]. Interestingly, we observed that not only co-stimulation with RANKL and AlF$_4^-$ but also AlF$_4^-$ alone induce [Ca$^{2+}$]i oscillations in both BMMs. However, we could not see any Ca$^{2+}$ responses when the same concentration of AlF$_4^-$ was treated acutely. Moreover, as our previous report, we also confirmed that extracellular Ca$^{2+}$ influx is crucial for sustained [Ca$^{2+}$]i oscillations in response to AlF$_4^-$ alone and both of RANKL and AlF$_4^-$, clearly presenting that AlF$_4^-$-induced [Ca$^{2+}$]i oscillations is generated through similar mechanism with RANKL's. The characteristic of intracellular Ca$^{2+}$ signaling varies as following signal molecules, which gives diversity and versatility on the cell functions [29]. Based on our results, it is assumed that activation of G-protein by AlF$_4^-$ possibly determines cell fate of BMMs alike with RANKL.

To further investigate physiological phenomenon induced by AlF$_4^-$ or co-stimulation with RANKL and AlF$_4^-$, we decided to check NFATC1 activity and the formation of TRAP$^+$ MNCs and actin ring, which are widely used as a criterion to confirm the osteoclastogenesis. Intriguingly, results strongly suggest that activation of G-protein by AlF$_4^-$ acts as a synergic factor that somehow interacts with RANKL-mediated other signals responsible for transmitting differentiation-related signals to downstream. Our results raise an important question of which G$\alpha$ subunit is activated and causes the enhancement of osteoclastogenesis. There are the major four families, G$\alpha_{q/11}$, G$\alpha_s$, G$\alpha_{12/13}$ whose main effector molecules are thought to be PLC, adenylyl cyclase, and small GTPase families [30]. Following studies support the involvement of G$\alpha$ subunits including G$\alpha_{q11}$, G$\alpha_s$, and G$\alpha_1$ in osteoclastogenesis. OGR1, which is one of GPCR coupled to G$\alpha_{q11}$ and triggers [Ca$^{2+}$]i mobilization, is reported to be involved in RANKL-induced osteoclastogenesis by modulating NFATC1 signaling pathway [11,12,31]. Continuous production of cAMP mediated by dopamine D1-like receptor, which is coupled to G$\alpha_s$ subunit and inhibits adenylyl cyclase, suppresses RANKL-induced osteoclastogenesis [32]. Furthermore, critical role of CREB (cAMP response element binding protein) in RANKL-mediated osteoclastogenesis strongly supports that finely tuned cAMP production by G$\alpha_s$ and G$\alpha_1$, and G$\alpha_q$/PLC/IP$_3$/Ca$^{2+}$ signaling pathway are a key modulator in osteoclastogenesis [25,33,34]. Taken together, it is convinced that modulating the activities of G$\alpha_{q11}$, G$\alpha$, and
and Gaq affects on RANKL-induced osteoclastogenesis. Since our current results clearly show that treatment of AlF4- induces [Ca2+]i mobilization in a form of oscillations and enhances RANKL-mediated osteoclastogenesis, Gaq11 seems to dominantly be involved in RANKL-mediated osteoclastogenesis. However, due to the property of AlF4- as a universal activator of Ga subunit, it should be another important study to determine which subtype of Ga subunit governs signaling pathway related to differentiation.

Before the finding of co-stimulatory signal pathway including Ca2+ increase via DAP12 and FcγR7, it is generally accepted that TRAF6/MAPK/AP-1/NFATc1 pathway mainly dominates gene induction responsible for late-stage of osteoclastogenesis [2,35,36]. As shown in Fig. 4, RANKL is known to activate MAPK pathway, such as ERK and JNK, at early moment. Consistent with previous results, activation of ERK and JNK is appeared to be activated in response to not only co-stimulation with RANKL and AlF4-, but also AlF4- alone. Although this result bring puzzling question that is subject to different interpretations with respect to the cause-effect relationship between [Ca2+]i oscillations and MAPK, we could assure that AlF4- is sufficient to activate MAPK necessary for osteoclastogenesis. One of important finding in this result is that function of G-protein responsible for differentiation into osteoclast is associated with either pathway of Ca2+ mobilization and MAPK by which enhance cell fusion and attachment of cell on bone surface. The role of GPCR in osteoclastogenesis is still uncertain though there are several reports showing involvement of signal molecules following GPCR activation in osteoclastogenesis. The other is the application of AlF4- for inducing various cell functions by activating G-protein. AlF4- acts as a messenger of false information [13] and has been often used in many laboratories to investigate their effects on various cells and tissues. Further studies are needed to clarify the potential risks for human health of long-term exposure to AlF4-.

Notably, our study indicates the possibility that Ga subunits can be regarded as a target molecule necessary on modulating bone resorption. As mentioned previously, OGR1 (GPR68), which is coupled to Gaq11 subunit and transmit signals to PLC, is one of few GPCRs that is demonstrated the roles in osteoclastogenesis. Despite of the absence of direct evidence, following studies presented the answer for the question. 1) Deficiency of OGR1 caused abnormalities in osteoclastogenesis [9], 2) RGS18, which accelerates intrinsic GTP hydrolysis on heterotrimeric G-protein alpha subunits, inhibits G alpha subunits may not block the osteoclastogenesis. However, assuming Ga subunits is inhibited in vivo, it can cause the reduction of osteoclastogenesis and bone resorption.

In summary, present study demonstrates that AlF4- induces [Ca2+]i oscillations and MAPK, which lead to improve RANKL-mediated osteoclastogenesis by enhancing NFATc1 expression. Although not described in detail here, it is needed that further verification for signal molecules related with G-protein, such as GPCR and RGS protein, during osteoclastogenesis. With this finding, it should be a novel therapeutic target for bone-related disorders including osteoporosis and osteopetrosis.

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