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Role of Voltage-gated Potassium Channel, Kv7.3 in Osteoblast Maturation and Mineralization

2016년 2월

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Role of Voltage-gated Potassium Channel, $K_v7.3$ in Osteoblast Maturation and Mineralization

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February 2016
ABSTRACT

Role of Voltage-gated Potassium Channel, Kv7.3 in Osteoblast Maturation and Mineralization

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KCNQ channel is one of the voltage-gated potassium (Kv) channels. The functions of Kv7 channels in muscles, neurons and sensory cells are well characterized. However, the function in osteoblast cells has not been investigated.

The present study demonstrated that Kv7.3 channel could regulate osteoblast differentiation. First, expression of Kv7 channels was examined in MG-63 and Saos-2 osteoblast cells. All subtypes of Kv7 channels, including Kv7.1, Kv7.2, Kv7.3, Kv7.4 and Kv7.5, were expressed in MG-63 cells, and Kv7.3 and Kv7.5 channels existed in Saos-2 cells. The results illustrated that the expression of
K\textsubscript{v}7.3 channel, which was highly expressed in MG-63 and Saos-2 cells, changed during osteoblastic differentiation at the mRNA and protein levels. Inhibition of K\textsubscript{v}7.3 by linopirdine or XE991 increased the matrix mineralization during osteoblast differentiation. This was confirmed by the increase in mRNA of alkaline phosphatase, osteocalcin, transcription factor, osterix, and by the deposition of type I collagen proteins in MG-63 cells. Furthermore, the extracellular glutamate secreted by osteoblasts was measured to investigate its effect on the matrix mineralization in MG-63 osteoblast cells. Blockade of K\textsubscript{v}7.3 promoted the release of glutamate via the phosphorylation of extracellular signal-regulated kinase 1/2-mediated up-regulation of synapsin. On the other hand, activation of K\textsubscript{v}7.3 using flupirtine did not produce notable changes in matrix mineralization during osteoblast differentiation.

In conclusion, these results suggest that blockade of K\textsubscript{v}7.3 channels could enhance the matrix mineralization during osteoblast differentiation. Although further studies are required to clarify the underlying mechanisms, the present study implicates that K\textsubscript{v}7.3 channel can be a possible target of bone-loss-related diseases.

**Keywords**: Voltage-gated potassium channels; KCNQ3; MG-63 cells; Osteoblast differentiation; Extracellular matrix mineralization

**Student number**: 2013-21538
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INTRODUCTION

Voltage-gated potassium channels

Voltage-gated K⁺ (Kᵥ) channel is one of the largest gene families among the K⁺ channel groups. Kᵥ channels are known for regulating cellular electrophysiological properties in excitable cells such as neurons (Coleman et al., 1999; Nashmi and Fehlings, 2001; Misonou et al., 2005) and muscle cells (Bijlenga et al., 1998; Grunnet et al., 2008; Hancox et al., 2008). In neurons or cardiac muscle cells, Kᵥ channels repolarize the cell membrane after the action potential. In association with this action, Kᵥ channels modulate the firing rate of the action potential, membrane stabilization, and neurotransmission (Martire et al., 2004; Martire et al., 2007). Moreover, Kᵥ channels also serve as regulators in non-excitable cells. Specific Kᵥ channels are expressed in almost all cancer cells including leukemia (Pillozzi et al., 2002), breast cancer (Ouadid-Ahidouch et al., 2000; Abdul et al., 2003; Roy et al., 2008; Jang et al., 2009), and colonic (Lastraioli et al., 2004; Jiraporn et al., 2007; Spitzner et al., 2007; Shimizu et al., 2014) or gastric cancers (Mei et al., 2005), and they regulate cell proliferation (Ouadid-Ahidouch et al., 2000; Conforti et al., 2003; Wulff et al., 2003; Spitzner et al., 2007; Jang et al., 2009; Iannotti et al., 2010; Jang et al., 2011; Lee et al., 2014; Shimizu et al., 2014), migration (Levite et al., 2000; Lastraioli et al., 2004; Cherubini et al., 2004).
2005), and differentiation (Iannotti et al., 2010; Zhou et al., 2011; You et al., 2013). K_v channels could also affect cell volume (Jespersen et al., 2005; Roy et al., 2008) and cell signaling (Leung et al., 2011), which leads to diverse cellular activities.

**KCNQ channel families**

KCNQ channel, also known as K_v7 channel, is one of the K_v channel members, which comprises K_v7.1 to K_v7.5. K_v7 channels are widely distributed in various tissues (Soldovieri et al., 2011). K_v7.1 was first found in the heart and was well-characterized in cardiac muscle cells (Jesper sen et al., 2005; Soldovieri et al., 2011). K_v7.1 is also present in the inner ear epithelium (Wang et al., 2015), lung (Evans et al., 1996) and gastrointestinal tract (Heitzmann and Warth, 2007). Furthermore, K_v7.2 and K_v7.3 are mainly expressed in the central nervous system (Pan et al., 2006; Zhou et al., 2011; Miceli et al., 2013) and usually form a K_v7.2/7.3 heterotetramer, which contributes M-current (Robbins, 2001). Lastly, K_v7.4 is present in skeletal muscle cells (Iannotti et al., 2010; Iannotti et al., 2013) and the membrane of outer hair cells (Soldovieri et al., 2011), and K_v7.5 is widely distributed in the brain (Schroeder et al., 2000). Previous studies have determined the physiological role of KCNQ channels in cell proliferation, differentiation (Iannotti et al., 2010; Iannotti et al., 2013; Lee et al., 2014; Shimizu et al., 2014), and survival (Iannotti et al., 2010).
Bone cell differentiation

Bone is a complicated organ that continuously goes through the formative and resorptive activities of osteoblasts and osteoclasts (Long, 2012; Iniguez-Ariza and Clarke, 2015). Bone development depends on various extracellular signals and transcription factors to maintain bone structure and homeostasis, so its microenvironment is significant for bone physiology. Sequential expressions of regulatory signals are necessary for bone cell differentiation. First, cytokines such as bone morphogenic protein, TGF-β (Matsubara et al., 2008; Chen et al., 2012; Rahman et al., 2015), and transcription factors such as Runx2 (Stein et al., 2004; Komori, 2006; Matsubara et al., 2008) are required to commit a pluripotent stem cell into an osteoprogenitor cell and then become a pre-osteoblast. This committed pre-osteoblast then undergoes the matrix mineralization stage, a distinctive step in osteoblast differentiation. At this stage, osteoblast-derived factors, such as osteocalcin, alkaline phosphatase, collagens, and bone sialoproteins (Stein et al., 2004; Ellis E. Golub and Kathleen Boesze-Battaglia, 2007), mediate the initiation and formation of extracellular matrix mineralization by vesicle-mediated exocytosis (Rohde and Mayer, 2007).
Purpose of the present study

$K_{\text{V}7}$ channels have been reported to regulate cell differentiation. For example, $K_{\text{V}7.4}$ has a role in skeletal muscle cell development (Iannotti et al., 2010; Iannotti et al., 2013). Another study indicated that $K_{\text{V}7.2/7.3}$ was involved in neuronal differentiation through synaptic vesicle protein-mediated endo/exocytosis of neurotransmitters (Zhou et al., 2011). M-current by $K_{\text{V}7}$ channels is controlled by multiple factors, one of which is intracellular $\text{Ca}^{2+}$ (Marrion et al., 1991; Marrion, 1997; Haitin and Attali, 2008; Hernandez et al., 2008; Soldovieri et al., 2011; Kosenko and Hoshi, 2013). Considering the fact that $\text{Ca}^{2+}$ is pivotal for bone homeostasis and that KCNQ channels modulate vesicular exocytosis, the potential role of KCNQ channels in bone differentiation was explored in the present study, focusing on biological mineralization of bone matrix. $K_{\text{V}7}$ channel expression was confirmed in osteoblast cells, and the present study determined the tentative theory that $K_{\text{V}7}$ channels, at least $K_{\text{V}7.3}$, may play potential roles in osteoblast differentiation, especially for matrix maturation and mineralization.
MATERIALS AND METHODS

Materials

Flupirtine maleate, linopirdine dihydrochloride, and XE991 dihydrochloride were purchased from Tocris Bioscience (MN, USA). Riluzole, glutamate release inhibitor, was obtained from Sigma-Aldrich (MO, USA).

Cell culture and osteoblast induction

MG-63 cells and Saos-2 cells were purchased from Korean Cell Line Bank (Seoul, South Korea). MG-63 cells were cultured in growth medium (GM) consisting of high-glucose DMEM containing 10% FBS and 1% antibiotic antymycotic solution in an incubator at 37°C with 95% air and 5% CO₂. Saos-2 cells were maintained in RPMI 1640 medium with 25 mM HEPES containing 10% FBS and 1% antibiotic antymycotic solution in the same conditions. MG-63 cells at passages 121 to 130 and Saos-2 cells at passages 52 to 61 were used in all experiments.

To induce osteoblast differentiation, 50 mg/ml of ascorbic acid (Sigma-Aldrich), 10 mM of beta-glycerol-phosphate (Sigma-Aldrich) and 10 nM of dexamethasone (Sigma-Aldrich) were supplemented to the maintaining GM. For osteoblast differentiation, cells were harvested using trypsin/EDTA. The detached
cells were then plated onto 6-well plates at a density of $10^5$ cells/well for 24 hours to allow cell attachment. On the following day, the cells were pre-incubated with flupirtine (30 μM), linopirdine (30 μM), XE991 (10 μM), and riluzole (30 μM) for 2 hours and then transferred to an osteoblast-induction medium (OM) containing these drugs and incubated for 14 days. The drugs-containing medium was replaced twice a week. Cells in the control groups were pre-incubated with GM for 2 hours and then transferred to OM without drugs for 14 days.

**Cell viability assay**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability. Through mitochondria-dependent enzymatic activity, MTT is reduced to insoluble formazan, which indicates the extent of viable cells. MG-63 cells were plated onto 96-well plates at a concentration of $10^4$ cells/well in GM or OM. The cells were treated with flupirtine (30 μM), linopirdine (30, 50, 100, and 200 μM) and XE991 (10 μM) for 24, 48, and 72 hours, respectively. The concentration of these drugs was based on the EC$_{50}$ or IC$_{50}$ values for each drug. After washing the cells with DPBS, the cells were incubated with 200 μL of DPBS containing 0.5 mg/ml of MTT for 4 hour. The formazans created by the viable cells were solubilized with 200 μL dimethylsulfoxide. The absorbance of each well was measured at 570 nm.
RNA extraction, RT-PCR and quantitative RT-PCR

Total cellular RNA was extracted with RiboEx™ (GeneAll, Seoul, South Korea) and treated with DNase I (TaKaRa, CA, USA) according to the manufacturer’s protocol. M-MLV reverse transcriptase (Invitrogen, CA, USA) was used to synthesize cDNA. Specific primers were employed for RT-PCR (Table 1). The PCR products were then electrophoresed in a 1.6% agarose gel and the expression levels of the target genes were confirmed quantitatively by real-time PCR (StepOne Plus, Applied Biosystems, MA, USA) using SYBR® Premix Ex Taq (TaKaRa). Gene expression was quantified using the comparative threshold cycles and the relative gene expressions were compared to the ratios of the reference gene (GAPDH) threshold cycles.
<table>
<thead>
<tr>
<th>Gene (Accession number)</th>
<th>Primer</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_7.1 (NM_000218)</td>
<td>Forward</td>
<td>CCCAAGAAAGTCTGTGGTGGT</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTCAAGCCGACGACAGAG</td>
<td></td>
</tr>
<tr>
<td>K_7.2 (NM_004518)</td>
<td>Forward</td>
<td>GCAAGCTGAGAACATTCCCTC</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGTACGGAAGCCCAACACC</td>
<td></td>
</tr>
<tr>
<td>K_7.3 (NM_001204824)</td>
<td>Forward</td>
<td>GTGGCAGGTCAGGAGATATT</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGCTGACTTTGTCAATGGT</td>
<td></td>
</tr>
<tr>
<td>K_7.4 (AH007377)</td>
<td>Forward</td>
<td>CTGGGCATCTCTTCTTTTC</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GACCAGGGTGCTGTCAGGT</td>
<td></td>
</tr>
<tr>
<td>K_7.5 (NM_001160134)</td>
<td>Forward</td>
<td>CGCTTTCGTTTTTTCTCTTG</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGAGCAAACCTCTAGTCTCC</td>
<td></td>
</tr>
<tr>
<td>ALP (NM_000478)</td>
<td>Forward</td>
<td>CCTCCTCGGAAGACACTCTG</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAGTGAAAGGGCTTCTTGTC</td>
<td></td>
</tr>
<tr>
<td>OSC (NM_001199662)</td>
<td>Forward</td>
<td>GACTGTGACGAGCTGTCAGGT</td>
<td>119</td>
</tr>
<tr>
<td>Runx2 (NM_001015051)</td>
<td>Forward</td>
<td>CACCAGGAAAGCAGGATGCA</td>
<td>95</td>
</tr>
<tr>
<td>Osterix (AF477981)</td>
<td>Forward</td>
<td>TGATGCCATAGCCCTCCCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAGTGAAAGCTCTATTCCAAACC</td>
<td></td>
</tr>
<tr>
<td>GAPDH (NM_002046)</td>
<td>Forward</td>
<td>CTCTGCTTCTCTGTTCGAC</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACGACCAAATCCGGTAACTC</td>
<td></td>
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</table>
**Alizarin Red S staining and quantification**

Alizarin Red S staining was used to determine the extent of calcium deposits, an indicator of mineralization. MG-63 and Saos-2 cells were cultivated in OM with or without drugs for 14 days. They were then fixed with 70% ice-cold ethanol for 1 hour and stained with 2% Alizarin Red S solution, pH 4.1–4.3 (Sigma-Aldrich). After Alizarin Red S staining, cells were dissolved in 10% cetylpyridinium chloride and the OD values at 570 nm were analyzed for the quantification of calcium deposits.

**Western blot analysis**

Total cell lysates were extracted by treating cells with lysis buffer containing 50 mM of Tris-HCl, pH 8.0, with 150 mM of sodium chloride, 1% igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (Sigma-Aldrich) and adding 1% protease inhibitors (Sigma-Aldrich) and 10% phosphatase inhibitors (Roche, Basel, Switzerland). Cell lysates were incubated on ice for 10 mins and centrifuged at 10,000 g for 10 mins at 4°C and the supernatant was used for whole-cell lysates. The BCA assay was used to determine protein concentrations.

SDS-PAGE electrophoresis was conducted on 8%–12% acrylamide gels according to the size of target proteins, and the separated proteins were transferred
onto the nitrocellulose membranes. Then, the membranes were blocked with 0.1% tween-20 TBS containing 5% skim milk for 1 hour at room temperature. Specific primary antibodies (Table 2) were added to 0.1% tween-20 TBS containing 5% skim milk and the membranes were incubated at 4°C overnight. After washing with 0.1% tween-20 TBS three times, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat antibodies for 1 hour at room temperature. After washing with 0.1% tween-20 TBS three times, proteins were identified using ECL solutions (Advansta, CA, USA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse secondary antibodies were purchased from GenDEPOT (TX, USA) and anti-goat secondary antibody came from Santa Cruz Biotechnology, Inc. (TX, USA).

**Table 2. Primary antibodies used for western blot analysis**

<table>
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<tr>
<th>Primary Antibody</th>
<th>Size (kDa)</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_v7.2</td>
<td>100</td>
<td>Alomone Labs (Jerusalem, Israel)</td>
<td>1:500</td>
</tr>
<tr>
<td>K_v7.3</td>
<td>55/100</td>
<td>Alomone Labs (Jerusalem, Israel)</td>
<td>1:500</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>42/44</td>
<td>Cell Signaling Technology (MA, USA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>42/44</td>
<td>Cell Signaling Technology (MA, USA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Synapsin Ia/b</td>
<td>80/86</td>
<td>Santa Cruz Biotechnology (TX, USA)</td>
<td>1:200</td>
</tr>
<tr>
<td>Type 1 collagen</td>
<td>70-90</td>
<td>Santa Cruz Biotechnology (TX, USA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>43</td>
<td>Santa Cruz Biotechnology (TX, USA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Vinculin</td>
<td>118</td>
<td>Santa Cruz Biotechnology (TX, USA)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Extracellular glutamate assay

The release of glutamate into culture medium by MG-63 cells was measured by using glutamate colorimetric assay kit (BioVision Inc., CA, USA) according to the manufacturer’s procedure. To determine the release of glutamate into the extracellular medium, the cells were plated onto 6-well plates at a density of $10^5$ cells/well and followed by the same protocol of inducing osteoblast differentiation described above. On days 2 and 4, the cultured medium was collected for the assay of glutamate, and the remained cells were used for the quantification of proteins. The medium used in the glutamate assay was phenol red-free DMEM.

Statistical analysis

The values were presented as mean ± standard error of the mean. Student’s $t$-test was used when comparing two different groups. P-values of less than 0.05 were considered to be statistically significant.
RESULTS

Expression of Kv7 channels in osteoblast-like cell lines, MG-63 and Saos-2 cells

To identify the effect of Kv channels on osteogenic properties, the mRNA expressions of KCNQ gene subfamilies, including Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 in MG-63 and Saos-2 cells, were analyzed using RT-PCR. While all subtypes of Kv7 channel were present in MG-63 cells (Fig. 1A), two subtypes of Kv7 channel, Kv7.3 and Kv7.5 were expressed in Saos-2 cells (Fig. 1B).
Figure 1. RT-PCR analysis of the $K_V7$ channels in osteoblast-like cells.

The PCR products using cDNA from the MG-63 and Saos-2 cells were electrophoresed in a 1.6% agarose gel. (A) $K_V7.1$, $K_V7.2$, $K_V7.3$, $K_V7.4$, and $K_V7.5$ were expressed in MG-63 cells (n=3). (B) $K_V7.3$ and $K_V7.5$ were expressed in Saos-2 cells (n=3).
Regulation of Kv7.3 transcripts and proteins during osteoblast differentiation in MG-63 and Saos-2 cells

To investigate the mRNA and protein expression level of Kv7.3 during osteoblastic differentiation, quantitative RT-PCR (qRT-PCR) and western blot analysis were performed. The qRT-PCR demonstrated that in MG-63 cells, the mRNA expression level of Kv7.3 significantly decreased 8 hours after osteoblast induction; however, at day 14, the mRNA level of Kv7.3 showed a substantial increase (Fig. 2A). Similarly in Saos-2 cells, while the Kv7.3 transcripts decreased 1 hour after the osteoblast induction, the level of Kv7.3 transcripts considerably increased at day 14 of osteoblast differentiation (Fig. 2B). Changes in Kv7.3 protein expression were investigated, and western blot analysis illustrated that Kv7.3 proteins increased at days 4 and 14 after osteoblast induction; however, Kv7.2 expression showed similar level during differentiation in MG-63 and Saos-2 cells (Figs. 2C and D).
Figure 2. Changes in Kv7.3 expression levels during osteoblastic differentiation.

The relative expression level of Kv7.3 during osteoblast differentiation was analyzed by quantitative RT-PCR. (A) In MG-63 cells, Kv7.3 transcripts decreased for 4 days after osteoblast induction. However, at day 14, Kv7.3 mRNA expression was considerably increased (n=4). (B) In Saos-2 cells, the mRNA expression of Kv7.3 was reduced 1 hour after osteoblast induction, whereas at day 14, Kv7.3 expression was significantly augmented (n=4). (C, D) Western blot analysis shows that Kv7.2 channels remained similar expression level during osteoblast differentiation in MG-63 and Saos-2 cells. The expression level of Kv7.3 protein was increased at days 4 and 14 after osteoblast induction (n=3). Data are presented as mean ± SEM. *P<0.05, **P<0.01, and ***P<0.005.
**Effect of the Kv7 opener and Kv7.3 blockers on MG-63 cell viability**

To confirm the effect of Kv7.3 modulators on cell viability, the MTT assay was performed on MG-63 cells. A Kv7 opener, flupirtine (30 μM), and Kv7.3 blockers, linopirdine (30 μM) and XE991 (10 μM), were used. There was no significant difference in the groups treated with flupirtine, linopirdine, and XE991 compared to the controls until 48 hours after treatment. However, at 72 hours, all of the groups treated by flupirtine, linopirdine, and XE991 showed significant decreases in cell viability (Fig. 3A). To further understand the effect on cell viability during osteoblast differentiation, MG-63 cells were cultured in osteoblast-induction medium (OM) with flupirtine (30 μM), linopirdine (30 μM), and XE991 (10 μM), and then MTT assay was performed. The results indicated that although linopirdine reduced cell proliferation at 24 hours, there was no significant change in overall cell viability when treated with OM containing flupirtine, linopirdine, and XE991 (Fig. 3B).

Additionally, high concentration of linopirdine (50, 100, and 200 μM) was used to inhibit Kv7.5 channel. MG-63 cells were not significantly influenced by GM containing linopirdine with all concentrations (Fig. 4A). However, under OM conditions, 100 μM and 200 μM of linopirdine attenuated cell viability at 48 hours, and 200 μM of linopirdine reduced cell proliferation at 72 hours (Fig. 4B).
Figure 3. Effect of flupirtine, linopirdine and XE991 on MG-63 cell viability.

The MTT assay was performed on MG-63 cells. (A) MG-63 cells were incubated in growth medium (GM) with 30 μM flupirtine, 30 μM linopirdine, and 10 μM XE991. At 72 hours, linopirdine, XE991, and flupirtine caused notable decreases in cell viability (n=12). (B) MG-63 cells were cultured in osteoblast-induction medium (OM) with 30 μM flupirtine, 30 μM linopirdine, and 10 μM XE991. The cell viability was not significantly influenced by OM containing flupirtine, linopirdine, and XE991 for 72 hour treatment (n=12). The values are presented as mean ± SEM. *P<0.05 and **P<0.01. GM, growth medium; OM, osteoblast-induction medium; CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991
Figure 4. Effect of K$_V$7.5 channel inhibition by high concentration linopirdine on MG-63 cell viability.

The MTT assay was performed on MG-63 cells to investigate the effect of high dose linopirdine (50, 100, and 200 μM). (A) MG-63 cells were incubated in growth medium (GM) with 50, 100, and 200 μM of linopirdine. The cells were not significantly affected by all concentration of linopirdine (n=8). (B) MG-63 cells were cultured in osteoblast-induction medium (OM) under same condition. Treatments with 100 μM of linopirdine decreased cell viability at 48 hours. Treatment with 200 μM linopirdine also attenuated cell proliferation at 48 and 72 hours in MG-63 cells (n=8). The values are presented as mean ± SEM. *$P$<0.05 and ***$P$<0.005. GM, growth medium; OM, osteoblast-induction medium.
Regulation of mineralization by a K\textsubscript{V}7 opener or K\textsubscript{V}7.3 blockers in MG-63 and Saos-2 cells

Effect of K\textsubscript{V}7.3 channel on calcium deposition in the extracellular matrix using was confirmed using Alizarin Red S staining. The K\textsubscript{V}7.3 blockade by linopirdine (30 μM) or XE991 (10 μM) noticeably increased mineralization in MG-63 cells (Figs. 5A and B). On the other hand, K\textsubscript{V}7 activation by flupirtine (30 μM) did not show an effect on mineralization (Figs. 5A and B). These experiments were conducted with another osteoblast-like cell line, Saos-2, to investigate the effect of blocking K\textsubscript{V}7 channels. The results showed that linopirdine (30 μM) and XE991 (10 μM) significantly promoted mineralization (Figs. 6A and B), whereas flupirtine (30 μM) reduced the mineralization of Saos-2 cells (Figs. 6A and B). Therefore, the inhibition of K\textsubscript{V}7.3 channels augmented mineralization, facilitating osteoblast differentiation.
Figure 5. Regulation of mineralization by K<sub>v</sub>7 channels in MG-63 cells.

(A) Alizarin Red S staining data shows that 30 μM linopirdine and 10 μM XE991 augmented mineralization in the extracellular matrix in MG-63 cells (n=10). On the other hand, there was no significant change in mineralization when treated by 30 μM flupirtine (n=3). (B) The OD values presented the quantified Alizarin Red S staining, and demonstrated that linopirdine or XE991 increased the amount of calcium deposits. Data are presented as mean ± SEM. ***P<0.005. Scale bar represents 100 μm. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991; OD, optical density
Figure 6. Regulation of mineralization by K<sub>v</sub>7 channels in Saos-2 cells.

(A) Alizarin Red S staining data illustrates that 30 μM linopirdine and 10 μM XE991 augmented mineralization in the extracellular matrix in Saos-2 cells (n=8). On the other hand, 30 μM flupirtine reduced mineralized calcium deposits (n=7).

(B) The OD values are shown parallel to Alizarin Red S staining results. Data are presented as mean ± SEM. ***P<0.005. Scale bar represents 100 μm. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991; OD, optical density.
Regulation of osteoblast differentiation markers by $K_v7$ channels in MG-63 cells

Expressions of alkaline phosphatase (ALP), osteocalcin (OSC), Runx2, and osterix were examined by qRT-PCR. The data illustrated that $K_v7.3$ blockade by linopirdine (30 μM) and XE991 (10 μM) significantly increased ALP gene expression at days 7 and 10 of osteoblast induction, whereas the $K_v7$ activation by flupirtine (30 μM) lowered the expression of ALP (Fig. 7A). Also, mRNA expression of OSC was increased by linopirdine and XE991 at days 7 and 10, respectively, whereas flupirtine showed no significant effect on OSC expression (Fig. 7B). Furthermore, the expressions of Runx2 and osterix were confirmed, which are known as essential transcription factors that promote hMSCs to differentiate into osteogenic lineages. The mRNA expression of Runx2 was significantly decreased by flupirtine at day 4. Although the level of Runx2 was decreased by linopirdine at day 10, linopirdine and XE991 did not produce significant changes in Runx2 levels through overall osteoblast differentiation (Fig. 7C). However, at day 10, linopirdine and XE991 augmented the mRNA expression of osterix, and flupirtine did not produce changes in osterix expression level (Fig. 7D).
Figure 7. mRNA expression of osteoblastic differentiation markers in MG-63 cells.

The relative mRNA expression levels of osteoblastic differentiation markers, alkaline phosphatase (ALP), osteocalcin (OSC), Runx2, and osterix, were measured by qRT-PCR and normalized against GAPDH expression (n=3-7). (A) While linopirdine (30 μM) or XE991 (10 μM) increased ALP mRNA expression at days 7 and 10 of osteoblastic differentiation, flupirtine (30 μM) decreased ALP mRNA expression at days 7 and 10. (B) The mRNA expression level of OSC was increased by linopirdine or XE991 at days 7 and 10, respectively. (C) mRNA expression of Runx2 was decreased by flupirtine at day 4, and also declined by linopirdine at day 10. (D) Osterix mRNA expression was increased by linopirdine or XE991 at day 10 of osteoblastic differentiation. The values are presented as mean ± SEM. *P<0.05, **P<0.01, and ***P<0.005. ALP, alkaline phosphatase; OSC, osteocalcin; CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991
Effect of $K_v7$ channel modulations on synaptic vesicle-related protein, synapsin and the MAPK signaling pathway

In previous experiments, $K_v7.3$ inhibition using linopirdine or XE991 increased mineralization during osteoblast differentiation. Focusing on these results, extracellular signal-regulated kinase 1/2 (ERK1/2) and synapsin expression were examined to verify whether $K_v7$ channels were involved in vesicular exocytosis during osteoblast mineralization. First, the expression of synaptic vesicle-related protein, synapsin, was increased by linopirdine (30 μM) and XE991 (10 μM), whereas flupirtine (30 μM) had no effect on the expression of synapsin (Fig. 8). In the same manner, while inhibition of $K_v7.3$ by linopirdine or XE991 increased the expression of ERK1/2 phosphorylation (pERK1/2), $K_v7$ activation by flupirtine showed no significant change of pERK1/2 level. On the other hand, at day 14, the blockade by linopirdine or XE991 produced no increase in pERK1/2 and flupirtine, but rather, increased the expression of pERK1/2 (Fig. 9).
Figure 8. Regulation of synaptic vesicle-related protein, synapsin, by Kv7 channels in MG-63 cells.

Western blot analysis shows that linopirdine (30 μM) or XE991 (10 μM) increased synapsin expression during osteoblast differentiation. However, flupirtine (30 μM) had no significant effect on the protein expression levels of synapsin (n=3). Vinculin is used as a loading control for western blot analysis. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991
Figure 9. Alteration of ERK1/2 phosphorylation by the Kv7 opener or Kv7.3 blockers in MG-63 cells.

Western blot analysis shows that while linopirdine (30 μM) or XE991 (10 μM) increased the expression level of ERK1/2 phosphorylation at days 4 and 7 of osteoblast differentiation, flupirtine (30 μM) had no significant effect on the level of ERK1/2 phosphorylation. However, treatment with linopirdine or XE991 showed no increase of EKR1/2 phosphorylation at day 14 of osteoblast differentiation. Flupirtine augmented the expression of ERK1/2 phosphorylation at day 14 (n=3). Vinculin is used as a loading control for western blot analysis. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991; ERK1/2, extracellular signal-regulated kinase 1/2; pERK1/2, ERK1/2 phosphorylation.
**Induction of glutamate release and type 1 collagen by Kv7.3 channels during MG-63 osteoblast differentiation**

To investigate the effect of Kv7 channels on glutamate release during osteoblastic differentiation, the amount of extracellular glutamate secreted by osteoblasts was measured. Fig. 10 demonstrated the difference of total glutamate amounts between the flupirtine (30 μM), linopirdine (30 μM), or XE991 (10 μM) treated groups and the control groups. The Kv7 activation by flupirtine significantly reduced the amount of glutamate release on days 2 and 4 after osteoblast induction (Figs. 10A and B). In contrast, Kv7.3 blockade by linopirdine or XE991 increased the amount of extracellular glutamate (Figs. 10A and B).

Furthermore, riluzole, glutamate release inhibitor, was used to confirm that the augmentation of glutamate release by Kv7.3 blockers can directly affect osteoblast differentiation. Riluzole (30 μM) co-treatment with flupirtine (30 μM), linopirdine (30 μM), or XE991 (10 μM) produced no significant changes in the extracellular mineralization (Figs. 11A and B). The mRNA expressions of ALP and OSC were examined. The mRNA expression of ALP was increased by riluzole treatment with Kv7.3 blockers, including linopirdine or XE991, at day 4 (Fig. 11C). Also, riluzole treatment co-applied with linopirdine or XE991 increased the OSC level at day 7 (Fig. 11D). However, co-treatment with riluzole and Kv7 opener, flupirtine, made no significant changes in the expressions of ALP and OSC compared to the controls (Figs. 11C and D).
The expression of intracellular type 1 collagen was investigated during osteoblastic differentiation to further understand the role of $K_V7.3$ channels in the extracellular matrix maturation. Linopirdine (30 μM) or XE991 (10 μM) augmented the level of type 1 collagen at day 7 of osteoblastic differentiation. The $K_V7$ activation by flupirtine (30 μM) decreased the expression of type 1 collagen (Fig. 12).
Figure 10. Effect of K\textsubscript{V}7 channels on glutamate release during osteoblastic differentiation in MG-63 cells.

(A) On day 2, flupirtine (30 μM) significantly decreased glutamate release after inducing osteoblast induction (n=6). On the other hand, linopirdine (30 μM) or XE991 (10 μM) caused a significant increase of glutamate release (n=5). (B) On day 4, flupirtine also caused decreased glutamate release (n=5), whereas XE991 significantly increased the extracellular glutamate (n=7). Linopirdine augmented the amount of glutamate, but not statistically significant (n=8). The values are presented as mean ± SEM. *P<0.05, **P<0.01, and ***P<0.005. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991
Figure 11. Counter effect of riluzole, glutamate release inhibitor, on mineralization augmented by K$_{V}$7.3 blockers in MG-63 cells.

(A) Alizarin Red S staining data shows that riluzole (30 μM) treatment co-applied with flupirtine (30 μM), linopirdine (30 μM), or XE991 (10 μM) produced similar level of mineralization compared with the controls (n=3). (B) The OD values are shown parallel to Alizarin Red S staining results. The relative mRNA expression levels of osteoblastic differentiation markers, including alkaline phosphatase (ALP) and osteocalcin (OSC), were measured by qRT-PCR and normalized against GAPDH expression (n=3). (C) At day 4, riluzole treatment with linopirdine or XE991, reduced the mRNA expression of ALP. At day 7, there was no significant change in the ALP level. (D) At day 7, riluzole treatment with linopirdine or XE991 reduced the OSC mRNA level. Data are presented as mean ± SEM. *P<0.05 and **P<0.01. Scale bar represents 100 μm. CONT, non-treated controls; RLZ, riluzole; FLU, flupirtine; LINO, linopirdine; XE, XE991; ALP, alkaline phosphatase; OSC, osteocalcin; OD, optical density
Figure 12. Induction of intracellular type 1 collagen by K\textsubscript{V7} channels during osteoblast differentiation in MG-63 cells.

Western blot analysis demonstrates that 30 μM linopirdine or 10 μM XE991 considerably increased the expression of type 1 collagen on day 7 of osteoblast differentiation (n=6). On the other hand, 30 μM flupirtine attenuated the expression of type 1 collagen (n=6). Actin is used as a loading control for western blot analysis. CONT, non-treated controls; FLU, flupirtine; LINO, linopirdine; XE, XE991
DISCUSSION

In the present study, the results demonstrated that Kv7.3 channels have a potential effect on osteoblast differentiation in MG-63 osteoblast-like cells. The present study showed that blockade of Kv7.3 channels by linopirdine or XE991 remarkably increased extracellular mineralization during osteoblast differentiation. In contrast, activation of Kv7.3 channels by flupirtine did not produce significant changes. ALP, OSC, osterix, ERK1/2 phosphorylation, synaptic-vesicle protein synapsin, glutamate signals, and type 1 collagens were involved in the process of osteoblast differentiation.

Ion channels and osteoblastic differentiation

Several studies have reported that ion channels, including voltage-gated calcium channels (Barry, 2000) and ether-a-go-go 1 channels (Wu et al., 2013; Wu et al., 2013; Wu et al., 2014) were involved in cell proliferation of MG-63 or Saos-2 cells, and KCNQ5 channels were involved in the proliferation of CCL-184 canine osteosarcoma cells (Lee et al., 2014). However, ion channels are not widely known for regulating cellular differentiation, especially osteoblast differentiation. The overexpression of chloride channel-3, CIC-3, enhances osteogenic
differentiation through Runx2-mediated ALP, OSC, and bone sialoprotein (BSP) genes by regulating intracellular pH in MC3T3-E1 primary mouse osteoblasts (Wang et al., 2010). Chloride intracellular channel 1 also induces osteoblast marker genes but exclusive of Runx2, by hyperpolarization of the mitochondrial membrane in C3H10T1/2 mouse embryonic mesenchymal cells (Yang et al., 2009). The blockade of large-conductance potassium channels with low concentrations of TEA increased mineralization in human primary osteoblasts (Henney et al., 2009) and hSlo potassium channels might regulate bone remodeling by responding mechanical loads in MG-63 and CAL72 osteosarcoma cells (Rezzonico et al., 2003). Taken altogether, most studies concerning osteoblast differentiation focused on mesenchymal stem cells (Yang et al., 2009) or primary osteoblasts (Henney et al., 2009; Wang et al., 2010).

**Expression of Kv7 channels in osteoblast-like cells**

MG-63 and Saos-2 osteoblast-like cells, originally derived from osteosarcoma, have distinct characteristics compared to mesenchymal stem cells or primary osteoblasts (Pautke et al., 2004; Czekanska et al., 2012). MG-63 is the immature state of osteoblast, and the expressions of osteoblast markers OSC, BSP, and Runx2 are relatively lower than those of primary osteoblasts and there are inconsistencies in mineralization (Pautke et al., 2004; Czekanska et al., 2012). Unlike MG-63, Saos-2 mature osteoblast cells have high levels of ALP enzymatic
activity and have strong capability to make calcified matrix (Czekanska et al., 2012). Another study showed dissimilar patterns of osteoblast genes, such as Runx2 in the osteoblast differentiation of Saos-2 cells in comparison to primary osteoblasts (Prideaux et al., 2014). This is because mesenchymal stem cells can differentiate into pre-osteoblasts, immature osteoblasts, and mature osteoblasts sequentially (Stein et al., 2004), whereas Saos-2 cells are the mature state of the osteoblast. Subsequently, Saos-2 and MG-63 cells are undergoing osteoblastic differentiation from the immature or mature state of osteoblast (Czekanska et al., 2012), which make it possible to concentrate on osteoblast maturation and matrix mineralization (Komori, 2010).

To date, little is known about the role of $\text{K}_7$ channels in MG-63 and Saos-2 cells. Although several studies showed that TEA-sensitive (Yellowley et al., 1998) or $\text{K}_V2.1$-related (Li et al., 2013) outward $\text{K}^+$ currents were confirmed in MG-63 cells, the physiologic function of KCNQ channels are not widely known in MG-63 and Saos-2 cells. According to the results, all $\text{K}_7$ subtypes were detected in MG-63 cells, whereas $\text{K}_7.3$ and $\text{K}_7.5$ channels were expressed in Saos-2 cells. It is significant that the expression pattern of $\text{K}_7$ channels between two cells is different. Since MG-63 cells are immature state of osteoblast and Saos-2 cells reflect mature osteoblastic features (Pautke et al., 2004; Czekanska et al., 2012), $\text{K}_7.1$, $\text{K}_7.2$ and $\text{K}_7.4$ channels which exist only in MG-63 cells may have possibility of determining immature osteoblastic features or affecting differentiation potency. First, distinct from $\text{K}_7.3$ expression, $\text{K}_7.2$ channels showed similar level during osteoblast differentiation. In addition, $\text{K}_7.5$ channels
were not inhibited by 30 μM of linopirdine or 10 μM of XE991, though other Kv7 channels including Kv7.1, Kv7.2 and Kv7.4 channels were affected. Also, Kv7.5 channels were involved in cell proliferation of canine osteosarcoma CCL-185 cells (Lee et al., 2014). Hence, high concentration of linopirdine was applied to MG-63 cells to investigate the role of Kv7.5 channel in cell proliferation. Kv7.5 blockade with 100 and 200 μM of linopirdine reduced MG-63 cell viability only under OM conditions. This is because protein expression of Kv7.5 channels may increase as osteoblast differentiation. Since mRNA expressions do not directly mirror protein expression levels (Greenbaum et al., 2003), additional studies are required to clarify the role of Kv7.1, Kv7.4 and Kv7.5 channels in MG-63 cells.

The mRNA expression of Kv7.3 channels was confirmed commonly in MG-63 and Saos-2 cells. Moreover, during osteoblast differentiation, the Kv7.3 mRNA declined 1 hour after osteoblast induction and increased remarkably at day 14 in MG-63 and Saos-2 cells. Similar to these results, the alteration of gene expression during differentiation was also reported in studies of ion channels (Yamashita et al., 2003; Swayne and Wicki-Stordeur, 2012; You et al., 2013). The variation of the pannexin 2 gene was observed in an experiment on neurogenesis (Swayne and Wicki-Stordeur, 2012) and the Kv3.1 transcript level was oscillated during adipogenesis (You et al., 2013) and the cardiac Kv1.5 and Kv4.2 mRNA expression showed variation depending on the circadian rhythm (Yamashita et al., 2003). However, unlike mRNA expression of Kv7.3 channel, expression of Kv7.3 proteins remained at the same level and is augmented at days 4 and 14. Although discrepancies between mRNA and protein are commonly seen, the reason for this
disparity has not yet been clearly revealed. Some reports have demonstrated that mRNA and protein expression levels are relative but not causative (Greenbaum et al., 2003), and are affected by many factors, such as mRNA stabilization, translational modification, and protein degradation (Vogel and Marcotte, 2012).

**Increased matrix mineralization during osteoblastic differentiation by Kv7.3 inhibition using linopirdine or XE991**

Mineralization occurs within the extracellular matrix at an end stage of osteoblast differentiation (Stein et al., 2004; Rohde and Mayer, 2007). Hence, extracellular matrix mineralization is used as an indicator to determine osteoblast differentiation or maturation (Stein et al., 2004). Due to the fact that the relationship between M-current of Kv7 channels and Ca$^{2+}$ (Marrion et al., 1991; Marrion, 1997; Haitin and Attali, 2008; Hernandez et al., 2008; Soldovieri et al., 2011; Kosenko and Hoshi, 2013), a key factor of bone homeostasis, was reported, the effect of Kv7 channels on osteoblast differentiation was examined by using osteoblast-like cells, MG-63 and Saos-2. According to the results, the inhibition of Kv7.3 by linopirdine or XE991 produced more mineralized deposits in the extracellular matrix. On the other hand, Kv7 activation by flupirtine did not increase the amount of calcium deposits.
Bone cell differentiation involves the distinct sequential expressions of different transcription factors and bone matrix proteins at each step (Stein et al., 2004; Komori, 2006). \( \text{K}_v 7.3 \) blockade increased osterix, but did not affect Runx2. Runx2 is a known transcription factor, that is a stringent regulator in differentiating pluripotent stem cells into immature osteoblasts, and it also induces essential osteoblast-derived matrix proteins (Stein et al., 2004). It is known that the level of Runx2 expression declines with bone cell differentiation (Maruyama et al., 2007). Rather, Runx2 suppresses osteoblast mineralization during bone development (Komori, 2010) and in vivo studies have demonstrated that overexpression of the Runx2 gene inhibits bone development during the maturation period (Liu et al., 2001; Kanatani et al., 2006). Furthermore, Runx2 is required to initially induce bone matrix proteins, whereas it is not essential for maintaining them (Komori, 2010). Since MG-63 cells are immature osteoblasts, the effect of the Runx2 transcription factor is not influential in MG-63 cell differentiation (Pautke et al., 2004; Czekanska et al., 2012). Osterix acts as a downstream signal of Runx2 and activates osteoblast marker genes, such as type 1 collagen and, BSP (Nakashima et al., 2002; Zhou et al., 2010). ALP and OSC mRNA levels were also increased by suppressing \( \text{K}_v 7.3 \) channels. ALP and OSC belong to bone matrix proteins induced by the transcription factors of bone formation (Stein et al., 1990). In this regard, \( \text{K}_v 7.3 \) inhibition stimulated not Runx2, but osterix, a downstream signal, to induce the expressions of ALP and OSC, which promoted osteoblast mineralization.
Glutamate, the most common excitatory neurotransmitter, is primarily found in the central nervous system (CNS). However, glutamate signaling is not confined to the CNS, but is also found in non-neuronal tissues, including lung (Said et al., 1996), megakaryocytes (Genever et al., 1999), pancreas (Inagaki N et al., 1995; Molnár et al., 1995; Weaver et al., 1996), and bone (Genever and Skerry, 2001; Chenu, 2002; Lin et al., 2008; Seidlitz et al., 2010; Brakspear and Mason, 2012). Specifically, glutamatergic innervation exists in bones, similar to that of the CNS (Chenu, 2002). Glutamate is released from bone cells, and in turn it acts as an autologous signal within the bone environment, facilitating osteoblast proliferation, differentiation, and maturation (Genever and Skerry, 2001; Lin et al., 2008; Seidlitz et al., 2010; Brakspear and Mason, 2012; Cowan et al., 2012). Other studies have reported that treatment with glutamate receptor agonists such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or N-methyl-D-aspartate (NMDA), increased OSC expression, ALP activity, and mineralization of calcium deposits in bone matrix in vitro (Genever and Skerry, 2001; Lin et al., 2008; Brakspear and Mason, 2012). Moreover, in vivo studies have indicated that local injection of AMPA or NMDA into bone augmented bone volume and mass (Lin et al., 2008).
MG-63 cells express various glutamate receptors, which consist of NMDA receptors including NR1, NR2A, NR2B, NR2D, and NR3A, and metabotropic receptors including mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, and mGluR8 at mRNA levels (Kalariti et al., 2004). The mechanism of glutamate regulation in bone is analogous to that of synapses in the CNS (Chenu, 2002). The exocytosis of glutamate involves in the activity of synapsin via adjusting the phosphorylation of the ERK signal (Bhangu et al., 2001; Blum and Konnerth, 2005; Jie-li et al., 2008). Additionally, Kv7.2/7.3 channel has been reported to be engaged in the ERK1/2 phosphorylation in hippocampal neurons (Zhou et al., 2011). Hence, to identify whether ERK1/2 and synapsin proteins are responsible for glutamate exocytosis, the protein expressions were examined. The results show that the ERK1/2 phosphorylation increased by Kv7.3 blockade on days 4 and 7, and the overall expression of synapsin by Kv7.3 inhibition was higher than that by Kv7 activation during differentiation. To further understand that the increase of glutamate induced by Kv7.3 blockers can promote extracellular mineralization, glutamate release inhibitor, riluzole, was co-applied with Kv7 modulators. Riluzole treatment with linopirdine or XE991 offset the extracellular mineralization which was enhanced by Kv7.3 blockers. Hence, in this pathway, the blockade of Kv7.3 enhanced the glutamate signals, which ultimately promoted mineralized deposits in the extracellular matrix.
Membrane potential and vesicular exocytosis

Depolarization of cell membrane is one of the important factors that can facilitate vesicular exocytosis. An action potential in excitable cells including neurons and muscle cells, is a sequential process of depolarization and repolarization, which involve in opening and closing of various types of voltage-gated channels including voltage-gated Na\(^+\), Ca\(^{2+}\), and K\(^+\) channels (Bean, 2007; Harvey Lodish et al., 2013). Pharmacologic modulation of membranous K\(_V\) channels has an effect on the membrane conductance, which can influence neurotransmitter release (Martire et al., 2004), vesicular exocytosis (MacDonald et al., 2002; Yang et al., 2014), or gastrointestinal mobility (Farrelly et al., 2003; Lee et al., 2015). In rat hippocampus neurons, inhibition of K\(_V\)7.2 channels prevented the retigabine-induced inhibition of norepinephrine, aspartate, and gamma-aminobutyric acid release by depolarizing membrane potential (Martire et al., 2004). MacDonald et al. (2002) illustrated that inhibition of K\(_V\)2.1 channels, which repolarizes cell membrane in the pancreas beta-cells, promoted insulin secretion. Similarly, blockade of K\(_V\)10.1 channels controls motility of human jejunum through their modulation of the electrical activity of smooth muscle cells (Farrelly et al., 2003). Since inhibition of K\(_V\)7.3 channels using linopirdine or XE991, makes cell membrane relatively depolarized, it may result in more favorable environment for vesicular exocytosis of bone matrix vesicle and glutamate.
**Deposition of type 1 collagens**

Collagens are involved in the matrix mineralization during bone cell differentiation (Mizuno and Kuboki, 2001; Salasznyk et al., 2004; Kihara et al., 2006; Viguet-Carrin et al., 2006; Rohde and Mayer, 2007; Mathews et al., 2011). Collagens are accumulated in the extracellular matrix, which promotes the formation of extracellular calcium deposits (Kihara et al., 2006; Viguet-Carrin et al., 2006; Rohde and Mayer, 2007; Mathews et al., 2011). Furthermore, the mutation in collagen genes, especially type 1 collagens, is one of the factors that cause osteogenesis imperfecta (Gajko-Galicka, 2002; Viguet-Carrin et al., 2006; Basel and Steiner, 2009). The results show that the level of intracellular type 1 collagens was enhanced by the blockade of Kv7.3 channels, whereas Kv7 activation reduced the expression of type 1 collagen.
Osteopenia and osteoporosis are common health problems around the world (Desiderio et al., 2014). Medications for osteoporosis generally are anti-resorptive reagents acting on osteoclasts (Komarova et al., 2001; Drake et al., 2008; Nardone et al., 2014; Iniguez-Ariza and Clarke, 2015; Maraka and Kennel, 2015) and the modulators of estrogen (Davies et al., 1999), calcitonin (Wimalawansa, 1993; Nardone et al., 2014), and parathyroid hormone (Nardone et al., 2014; Iniguez-Ariza and Clarke, 2015) to increase blood Ca$^{2+}$ concentrations. However, the side-effects have been reported that drugs that have inhibitory effects on osteoclasts, rather induced bone fracture (Watts, 2014; Acevedo et al., 2015), skeletal pain (Demonaco, 2009; Watts, 2014), and increased the possibility of cancers (Andrici et al., 2012; Oh et al., 2012). Hormone-modulating agents also have potential adverse effects (Wimalawansa, 1993; Davies et al., 1999). Therefore, advances have been recently explored such as targeting molecular pathways (Iniguez-Ariza and Clarke, 2015). In this respect, although further studies are necessary to elucidate the detailed mechanisms, $K_{V7.3}$ channel may serve as a potential therapeutic target for bone-loss-related diseases.
CONCLUSION

The present study demonstrates that $K_{V}$7.3 channels can affect osteoblast differentiation and help increase matrix maturation and mineralization. In the present study, $K_{V}$7.3 channel was confirmed in MG-63 and Saos-2 osteoblast cells at mRNA and protein levels. Fig. 13 depicts the summary of the results. Inhibition of $K_{V}$7.3 channel by linopirdine or XE991 led to the augmentation of osterix expression, and also increased the extracellular glutamate release that responds to the up-regulation of synapsin mediated by ERK1/2 phosphorylation. These pathways resulted in the increase of ALP and osteocalcin transcripts, and the deposition of type 1 collagen protein, which subsequently enhanced extracellular matrix maturation and mineralization during osteoblast differentiation. On the contrary, activation of $K_{V}$7.3 channel using flupirtine, did not produce significant changes in matrix mineralization.

Researches on $K_{V}$ channels related to osteoblast differentiation have not been widely investigated. Furthermore, developing medication for osteopenia or osteoporosis has been made steadily, since bone-loss-related diseases have become a widespread health problem. In this regards, the present study shows importance since it demonstrates that $K_{V}$7 channels are involved in osteoblast maturation and mineralization.
Taken together, the results of the present study suggest that Kv7.3 may be a novel regulator in osteoblast differentiation. Though further studies are required to elucidate the underlying mechanisms, the findings provide that Kv7.3 channel may be one of the potential therapeutic targets in bone loss-related diseases.
Figure 13. Summary of the effect of KCNQ3 channel on osteoblast differentiation.

$K_v7.3$ channel blockade induces up-regulation of synapsin via the ERK1/2 phosphorylation which promotes exocytosis of glutamate into extracellular matrix. Then, secreted glutamate acts on nearby osteoblasts or oneself, which enhances the expression of ALP and OSC. In another pathway, inhibition of $K_v7.3$ also increases osterix, inducing mRNA expression of ALP and OSC, and protein expression of type 1 collagen. In conclusion, blockade of $K_v7.3$ channels results in the augmentation of extracellular matrix mineralization during osteoblast differentiation. ERK, extracellular signal-regulated kinase; pERK, ERK phosphorylation; ALP, alkaline phosphatase; OSC, osteocalcin
REFERENCES


국문초록

뼈모세포의 분화에 있어서
전압의존성 K⁺ 채널, Kv7.3의 역할

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KCNQ 채널 또는 Kv7 채널은 전압 의존성 K⁺ 채널 (Kv 채널)의 하나이다. Kv7 채널의 기능은 근육, 신경, 그리고 간장 세포에서 널리 알려져 있으나, 뼈모세포에서의 기능에 대하여는 아직 알려진 바가 없다.

본 연구는 Kv7.3 채널이 뼈모세포의 분화를 조절할 수 있다는 결과를 보여주고 있다. 먼저, 뼈모세포주인 MG-63 및 Soas-2 세포에서 Kv7 채널의 발현을 확인하였다. MG-63 세포에서 Kv7.1, Kv7.2, Kv7.3, Kv7.4와 Kv7.5 채널의 발현을, Saos-2 세포에서는 Kv7.3 및
Kv7.5 채널의 발현을 mRNA 수준에서 확인하였다. 공통적으로 강하게 발현된 Kv7.3 채널이 뼈모세포의 분화에 따라 mRNA 및 단백질 수준에서 발현양이 변화함을 확인하였다. Kv7.3 채널 억제제인 linopirdine과 XE991을 이용하여 Kv7.3 채널을 억제했을 때, MG-63 및 Saos-2 뼈모세포 분화 과정에서 세포 외 기질의 골석화를 증가시켰다. Kv7.3 채널 억제에 의한 뼈모세포의 분화 증가는 뼈세포-기질 특이적 단백질인 alkaline phosphatase, osteocalcin, 그리고 전사인자 osterix의 mRNA 발현 증가와 type 1 collagen 단백질의 증가로 확인되었다. 또한, glutamate가 MG-63 뼈모세포의 세포 외 기질의 골석화 증가에 미치는 영향을 확인하기 위해 세포 밖으로 분비되는 glutamate의 양을 측정하였다. Kv7.3 채널의 억제는 세포 외 신호조절 인산화 효소 1/2 (extracellular signal-regulated kinase 1/2; ERK1/2)의 인산화를 통한 synapsin 단백질 발현 증가를 매개하여 glutamate의 분비를 촉진하였다. 이와 대조적으로 Kv7 채널의 활성작용제인 flupirtine 처리에 의한 Kv7.3 채널 활성화는 뼈모세포 분화 과정에서 세포 외 기질의 골석화에 유의적인 영향을 나타내지 않았다.

따라서, 본 연구 결과는 Kv7.3 채널 억제가 뼈모세포 기질의 골석화를 증가시킨다는 사실을 제시한다. 작용 메커니즘을 구체적으로 밝히기 위한 추가적인 실험이 필요하지만, 본 연구는 Kv7.3 채널이 뼈손실-관련질환의 치료 타겟로서 이용될 수 있는 가능성을 시사한다.

주요어: 전압의존성 포타슘 채널; KCNQ3; MG-63 cells; 뼈세포 분화; 세포외 기질의 골석화화

학번: 2013-21538