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A Study on the Expression of Voltage-gated L-type Calcium Channels in Adult Rat Odontoblast

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ABSTRACT

A Study on the Expression of Voltage-gated L-type Calcium Channels in Adult Rat Odontoblast

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The primary role of odontoblasts is dentin formation that produces collagen-based mineralized tissue by the involvement of intracellular calcium signaling. There are many potential sources that increase intracellular calcium. Among these sources, strong candidate molecules involved in dentin formation are L-type calcium channels. It is a high-voltage activated family of voltage-dependent calcium channel, which is comprised of Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, and Ca_V 1.4 isoforms. However, it has not been demonstrated how calcium signaling is mediated by L-type calcium channels in acutely isolated adult rat odontoblasts.

In the present study, I initially hypothesized that the expression of L-type calcium channels mediates the calcium response by depolarization in acutely isolated rat odontoblasts. The molecular and functional expression of L-type calcium channels were examined through RT-PCR, single cell RT-PCR, and Fura-2-based ratiometric calcium imaging in odontoblasts.

The intracellular calcium responses of the odontoblasts were observed by calcium imaging with combination of extracellular 50 mM KCl solution and the L-type calcium channels agonist, Bay K 8644, application. In contrast to TG

neurons which showed significantly enhanced calcium transients by Bay K 8644 treatment, depolarization-induced calcium transients were unaltered by Bay K 8644 in odontoblasts.

These results revealed that L-type calcium channels may not be functionally expressed in odontoblasts to mediate intracellular calcium signals in response to membrane depolarization in acutely isolated adult rat odontoblasts. Moreover, scRT-PCR further validated the results of the calcium imaging experiment, as mRNA of L-type calcium channel isoforms were not detected in odontoblasts. Thus, it is unlikely that L-type calcium channels mediate calcium signaling in adult rat odontoblasts.

Key Words: Odontoblast, L-type Calcium Channels, Calcium imaging, Bay K 8644

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ABBREVIATIONS

Ca_V 1.1	L-type calcium channel isoform 1.1
Ca_V 1.2	L-type calcium channel isoform 1.2
Ca_V 1.3	L-type calcium channel isoform 1.3
Ca_V 1.4	L-type calcium channel isoform 1.4
Ca_V 3.1	T-type calcium channel isoform 3.1
E_K	Equilibrium potential for the potassium ion
DSPP	Dentin sialophosphoprotein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
L-type calcium channel	Long lasting-type calcium channel
OD	Odontoblast
scRT-PCR	Single cell reverse transcriptase polymerase chain reaction
TG	Trigeminal ganglia

1. INTRODUCTION

The primary role of odontoblast is formation of dentin in teeth. This biological function contributes to not only physiological dentin formation but also mediates pathological dentin formation in order to protect the dental pulp. When the dentin is damaged by external stimuli, the odontoblast starts to produce injury-induced dentin formation called tertiary dentin in order to protect dental pulp (Sangwan et al., 2013).

In odontoblast, the transportation and accumulation of Ca^{2+} ions are important for dentin formation. It has been demonstrated that concentration of Ca^{2+} ions are utilized by odontoblast as main source in dentinogenesis. Odontoblasts can uptake Ca^{2+} ions and secrete hydroxyapatite to mineralize extracellular matrix proteins (Linde, 1995). As Ca^{2+} ion plays the important role in mineralization of dentin formation, various calcium entry routes for mineralization have been investigated in odontoblasts. Among them, L-type calcium channels has been suggested as a main candidate for Ca^{2+} entry to mediate mineralization in odontoblast (Linde & Lundgren, 1995).

The L-type calcium channels are long lasting, high-threshold, and slowly inactivating channels, comprised of Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, and Ca_V 1.4 isoforms (Lipscombe et al., 2004). In recent studies, the possible existence of L-type calcium channels in odontoblasts have been demonstrated by Ca^{2+} ion uptake studies in dental pulp cells, using the L-type calcium channels inhibitor nifedipine and the agonist Bay K 8644 in combination with Ca^{2+} imaging (Lundgren & Linde, 1997). The localization of L-type calcium channels has also

been detected in the neonate rat odontoblast cell membrane by using monoclonal antibodies (Seux et al., 1994). However, the functional expression of L-type calcium channels and the contributing isoforms have not been demonstrated yet in adult rat odontoblasts.

In this study, I hypothesized that L-type calcium channels are molecularly and functionally expressed in acutely isolated adult rat odontoblasts. To verify this hypothesis, I performed calcium imaging to observe L-type calcium channels mediated intracellular calcium signaling in response to membrane depolarization. In addition, single cell RT-PCR was used to detect molecular expression of L-type calcium channel isoforms in odontoblasts. However, L-type calcium channels are not likely to involved in intracellular calcium signaling in acutely isolated adult odontoblast.

2. MATERIALS AND METHODS

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. Male Sprague Dawley rats (Orient Bio Inc.) of 6~8 weeks old (n = 26) were used for the experiments. The animals were housed 2 per cage at a temperature-controlled room (23 ± 1°C, 12 h/12 h light/dark cycle) and maintained with pellet diet and tap water *ad libitum*. The study conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for preclinical studies.

Preparation of Odontoblast

Odontoblast cells were cultured as previously described (Guo et al, 2000; Won et al., 2018; Yeon et al., 2009). Rats were anesthetized with isoflurane and sacrificed by cervical dislocation. The upper and lower incisors were extracted within 5 min after cervical dislocation and kept in cold extracellular solution (ECS) containing (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES; and 10 glucose; 290 to 310 mOsm, adjusted pH 7.3 to 7.4 with NaOH. The incisors were sectioned transversely into 2-mm pieces and supplied cold ECS to prevent drying or overheating of the tissue. Teeth sections were incubated for 25min at 37 °C treated with 3 mL of an enzyme cocktail containing collagenase IA (3 mg/mL) and dispase (0.22 mg/mL) in Ca^{2+} and Mg^{2+} free ECS. The sections were

triturated with series of Pasteur pipettes to dissociate the pulp tissue and odontoblasts from dentin. The supernatant was collected in a separate tube with fresh ECS, and centrifuged at centrifuged at 1,000 rpm for 5 min. Cells were plated on a glass coverslip pre-coated with 0.5 mg/mL of poly-D-lysine (Sigma) and Cell-tak (Corning). Coverslips were kept at 3 to 5 °C until used.

Whole tissue Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from rat pulp tissue by using Trizol reagent (Life Technology). Then, the first-strand cDNA synthesis was obtained from 1 μg of total RNA by M-MLV reverse transcriptase (Invitrogen) followed by protocols. The primers (Cosmogenetech) used are listed in the **Table 1**. Isoforms of Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, and Ca_V 1.4 in smooth muscle, heart, lung, and retina were used for positive control respectively.

Single-cell reverse transcription-polymerase chain reaction (RT-PCR)

The dissociated odontoblasts were collected with patch pipette with a tip diameter of about 10 μm. Single-cell reverse transcription polymerase chain reaction (scRT-PCR) was performed as previously described (Won et al., 2018). The dissociated odontoblasts were collected with patch pipette with a tip diameter of about 10 μm. The collected cells were put into a polymerase chain reaction (PCR) tube containing 6 μl collection buffer containing: 0.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 1 μl of 100 ng/μl BSA, 1 μl of 50 μM $Oligo(dT)_{20}$, 1 μl of Random hexamer, 0.7 μl of RNaseOUT. After single cell collection, the tubes were added with 5 μl reverse transcription reagents containing: 1 μl of 10x RT

buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 0.1 M DTT, 0.5 μl of RNaseOUT, 1 μl of Superscript III. For cDNA synthesis, tubes were incubated for 10 min at 25˚C, 90 min at 50˚C, and 5 min at 85˚C. All PCR amplifications were performed with nested primers listed in **Table 1**. The first round of PCR was performed in reaction buffer total 25 μl containing: 2.5 μl of 10x PCR buffer, 0.75 μl of 50 mM $MgCl_2$, 0.5 μl of 10 mM dNTP, 0.1 μM of outer primers, 1 μl of cDNA, 0.2 μl of 5 U/μl Platinum Taq. For the second round, the first round reaction buffer total 25 μl was used except outer primers and cDNA were substituted with 0.1 μM inner primers and 1 μl of first round product. All scRT-PCR agents were purchased from Invitrogen unless stated otherwise.

Fura-2-based Ratiometric Calcium Imaging

Fura-2-based ratiometric calcium imaging was performed to measure intracellular calcium level change in odontoblast as previously described (Won et al., 2018). Before the experiment, coverslip was loaded with 4 μM Fura-2 AM (Invitrogen) in ECS for 40 min at room temperature and washed for 30 min. The coverslips were mounted onto the inverted microscope (Nikon Eclipse Ti). The morphology of odontoblast was confirmed by the microscope while perfusing with ECS containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, and 10 glucose; 290 to 310 mOsm, pH 7.3 to 7.4 adjusted with NaOH. Cells were illuminated with a 175-W xenon arc lamp, and excitation wavelengths (340/380 nm) were selected by a monochromator filter changer (Lambda DG-4; Shutter Instrument). Intracellular free calcium concentration was measured by digital video microfluorometry with an intensified charge-coupled device camera (Zyla; Andor) coupled to a microscope and software (Metafluor 7.8; Molecular

Devices). Equilibrium potential for the potassium ion (E_k) that activates voltage-gated calcium channels can be theoretically calculated by using the Nernst equation; $E_{ion} = (RT/zF) \ln([ion]_e/[ion]_i)$, where R is the constant of an ideal gas (8.314 VCK^{-1} mol^{-1}); T is the absolute temperature in kelvin (273.16 + temperature in °C; 298.16); F is the Faraday constant (96500 C mol^{-1}); *z* is the valence of the ion (+1); and $[ion]_e$ is the concentration of K^+ in the extracellular fluid (application of 50, 80, and 120 mM). $[ion]_i$ is the concentration of K^+ in the intracellular fluid (140 mM).

Drugs

(±)-Bay K 8644 and ionomycin were purchased from Tocris. They were dissolved in DMSO to make stock solution, and kept in -20ºC. The drug was diluted to their final concentration using the extracellular solution, and then applied by gravity through bath perfusion system.

Statistical Analysis

All data are presented as mean ± SEM unless stated otherwise. Statistical analyses were performed with Prism software (version 5.01, Graphpad Software Inc.). Statistical significance was assessed by paired or unpaired Student's 2-tailed *t* test, or repeated measures 1-way analysis of variance (ANOVA) followed by post hoc Bonferroni test. The criteria for significance was $P < 0.05$.

Table 1. List of Primers Used

Target gene (bp)	Outer primers (F, R)	Inner primers (F, R)	GenBank No.
GAPDH (118)	AGTGGGGAAGTTCTATGCCACA CTGGTGACTCCAGCTCTTCCAT		NM_017008
DSPP (453, 409)	GCTGAGGTGACACCAAGCATT ACTTTTGTTGCCCGTGCTG	GGAAGGTGCTGGTTTGGATAAT ACCTTCGGTTTCTAATCCCTGA	NM_012790
Cav 1.1 (368, 200)	AGTGGGGAAGTTCTATGCCACA CTGGTGACTCCAGCTCTTCCAT	GACCCAAAAAGGACACAGTTC CCAGGAAGTTGTCCACTTGACC	NM_053873.1
Cav 1.2 (497, 232)	GGTGGTCCTGAACTCCATCATC AGTCTCCTCGAGCTTTGGCTTT	TAGATGTTCCAGCGGAAGAGGA CAGGGCAACTCATAGCCCATAG	NM_012517.2
Cav 1.3 (323, 202)	ATGAGGTAACCGTGGGGAAGTT GGTGGTATTGGTCTGCTGAAGG	AAATTCAAGAAGCGGAAAGAGCAAG GCAGGGCACCATTTCTTTTGAATAC	NM_017298.1
Cav 1.4 (366, 152)	AAGCACATCCTCTGTCCTCCAG GTGAACATGAAAACCCCAGAGC	TGAGACCACCCCAGAAACATACA AGTTGGGAGCCACTCCATCAT	NM_053701.1
Cav 2.1 (356, 141)	ACAAGCAGAGCTTCCAGTATCG AAGGTAGGGCCTTGAAAGACTG	CCCACCCTTTGAGTACACCAT TCGAGAGAGAAGAGGGAGGTG	NM_012918.3
Cav 2.2 (356, 102)	CCCCACAAACCTGACGAGATG CCCCATCCGGTCCTCTCAAT	CAGAGGACCCAGGACGTACTTT GACATCTACAGCCAGTGCTCCT	NM_147141.1
Cav 2.3 (356, 159)	ACCTGGAACTGGCTGTACTTCA AGGTGTGCCCACTGAAGAGATA	GTCGGAGTGGATACCCTTCAAT AGATGCCATGGTTAGAGGACTG	NM_019294.2
Cav 3.1 (356, 137)	CTGGGTCCTGGCTAGATGAAC GTCCATGTCTGTTGGGTCAGA	GGAGACACTCCATTGCTGTCA GATACTGGGTGGGCTGAGTTT	NM_031601.4
Cav 3.2 (356, 230)	ATGTCCATGGAGCACTACAACC AAGACCAAGGTTCCCTACCTGA	CGTCTTTACCATCGTCTTCGTC TCTTCAGTAGCTTCAGCACACG	NM_153814.2
Cav 3.3 (356, 197)	TCACACTGGAGGAGATCGAGA CACAAACTGCAGGCTGCTTAG	CTGGTCTGCAATGACGAGAAC TAGAGCGGTGACACAAACTGC	NM_020084.3

3. RESULTS

Adult rat odontoblasts respond to extracellular high K^+ solutions

The morphology of acutely isolated odontoblasts was verified under microscope before Fura-2-based ratiometric intracellular calcium measurement (**Fig. 1A**). The various concentration of extracellular K^+ of 50 mM, 80 mM, 120 mM was used to depolarize the membrane potential of odontoblasts in order to activate voltage-gated calcium channels. The theoretical equilibrium potential for K^+ when using extracellular high K^+ solutions containing 50 mM, 80 mM, 120 mM KCl was -26 mV, -14 mV, -4 mV when calculated by using the Nernst Equation (presuming that internal K^+ in odontoblast was 140 mM). When membrane potential of odontoblast was depolarized by applying increasing concentration of K^+ in the bath, intracellular calcium transient was enhanced as K^+ concentration increased (n = 8, repeated measures 1-way ANOVA, $^{**}p$ = 0.0004, **Fig. 1B, C**). Ionomycin, a calcium ionophore, was used to confirm cellular viability at the end of each experiment (Lipscombe et al., 2004).

Effect of Bay K 8644 on 50 mM KCl-induced calcium transient in odontoblast

To determine whether the calcium response by high K^+ was mediated by L-type calcium channels, I monitored the potentiating effect of L-type calcium channels agonist Bay K 8644 on calcium transient induced by 50 mM KCl (20 sec). Successive 50 mM KCl treatment induced intracellular calcium transients in odontoblasts. In trigeminal ganglia (TG) neurons which was used as positive control for L-type calcium channels expression, Bay K 8644 treatment resulted in increased calcium transient when compared to the original response (**Fig. 2A**). However, pretreatment of Bay K 8644 failed to potentiate the amplitude of intracellular calcium response in acutely isolated adult rat odontoblast (**Fig. 2B**). There was no calcium response when the drug alone was administered. There was no effect of treatment with only drugs. The 50 mM KCl-induced calcium transient was significantly potentiated by Bay K 8644 treatment in TG neurons (251.1 ± 92.56 vs 465.4 ± 173.0, n = 10, paired Student's *t*-test, **p* = 0.0330; **Fig. 2C**) but not in odontoblast (0.038 ± 0.0069 vs 0.043 ± 0.007, n = 13, paired Student's *t*-test, *p* = 0.0729; **Fig. 2D**). Only TG neuron showed substantial enhancement of calcium transient area when odontoblast and TG neuron were compared. In TG neurons, the area under curve of the calcium response increased more than twice. (1.141 ± 0.0983 vs 1.944 ± 0.2039, n = 13, Student's *t*-test, ***p* = 0.0010; **Fig. 2E**).

The mRNA expression of L-type calcium channels in acutely isolated adult rat odontoblasts

As calcium imaging results indicated that odontoblasts may not express L-type calcium channels, I used scRT-PCR to further verify the expression of this isoforms. L-type calcium channel isoforms were successfully detected in positive control tissues by the designed primers (Ca_V 1.1; smooth muscle, Ca_V 1.2; heart, Ca_V 1.3; lung, and Ca_V 1.4; retina, **Fig. 3A**). Although these four isoforms were detected in pulp tissue (**Fig. 3B**), scRT-PCR revealed that the mRNA of L-type calcium channel isoforms were not detected from individual odontoblasts (n = 22; **Fig. 4**).

The mRNA expression of P/Q, N, R, T-type calcium channels in acutely isolated adult rat odontoblasts

Since calcium imaging and scRT-PCR results indicated that odontoblasts do not express L-type calcium channels, the mRNA expression of P/Q (Ca_V 2.1), N (Ca_V 2.2), R (Ca_V 2.3), T (Ca_V 3.1, Ca_V 3.2, and Ca_V 3.3)-type calcium channels were screened by scRT-PCR. All isoforms of voltage-gated calcium channels were successfully detected in the positive control tissues by the designed primers (Ca_V 2.1, Ca_V 2.2, Ca_V 2.3, Ca_V 3.1, and Ca_V 3.3; cerebral cortex, Ca_V 3.2; colon**).** Among these voltage-gated calcium channels, only T-Type calcium channels, especially Ca_V 3.1, was detected in 46% (n = 6/13) of odontoblasts (**Fig. 5A**).

Figure 1.

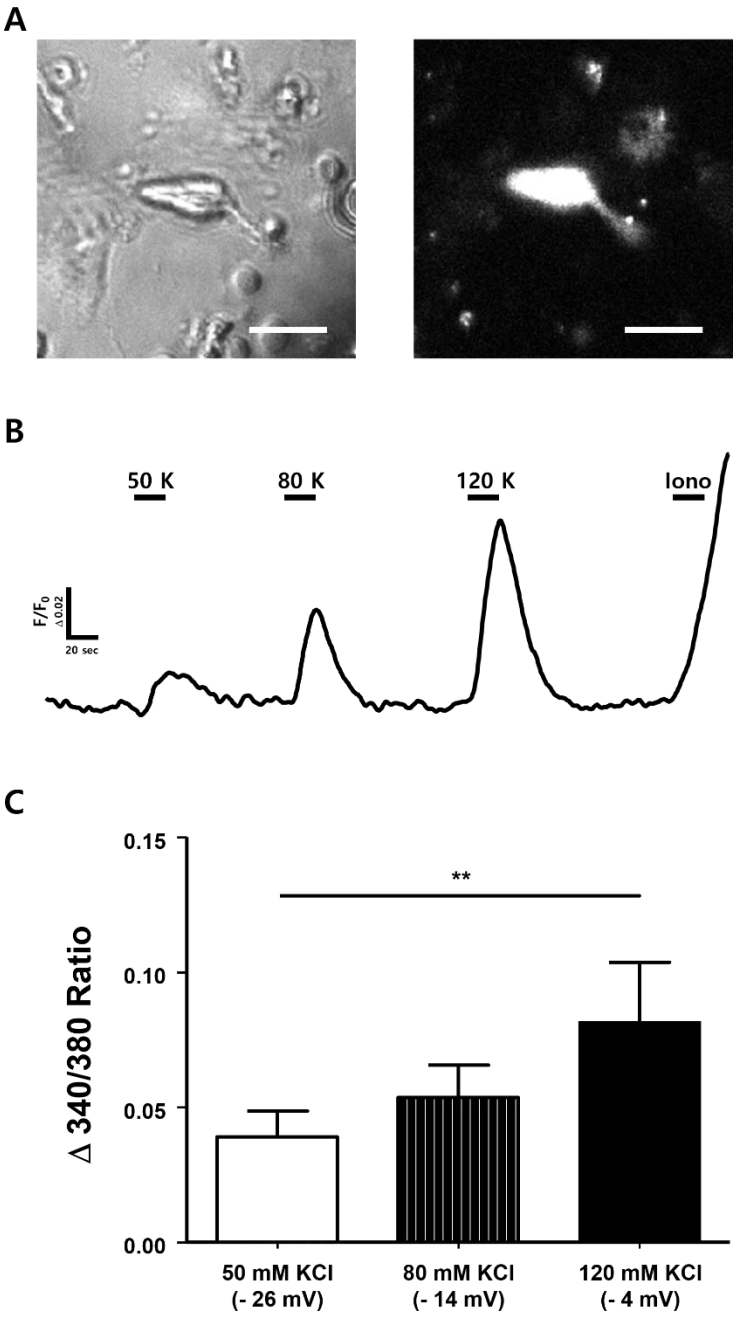

A
B
50 K
80 K
120 K
Iono
F/F0
Δ0.02
20 sec
C
0.15
0.10
0.05
0.00
**
Δ 340/380 Ratio
50 mM KCl
(- 26 mV)
80 mM KCl
(- 14 mV)
120 mM KCl
(- 4 mV)

Figure 1. Adult rat odontoblasts responded to extracellular high K^+ solutions

(A) A representative figure of acutely isolated adult rat odontoblast shown by bright field image (left) and Fura-2–based fluorescent image (right). Scale bar: 10 μm. **(B)** A representative trace of extracellular high K^+-induced calcium transient by Fura-2-based ratiometric intracellular calcium measurement as K^+ concentration increased. **(C)** Comparison of high K^+-induced calcium transient peak amplitudes (n = 8, repeated-measures 1-way analysis of variance followed by Bonferroni's multiple comparison test, ***p = 0.0004*). Equilibrium membrane potentials of the extracellular high K^+ (E_K) was calculated by Nernst Equation.

Figure 2.

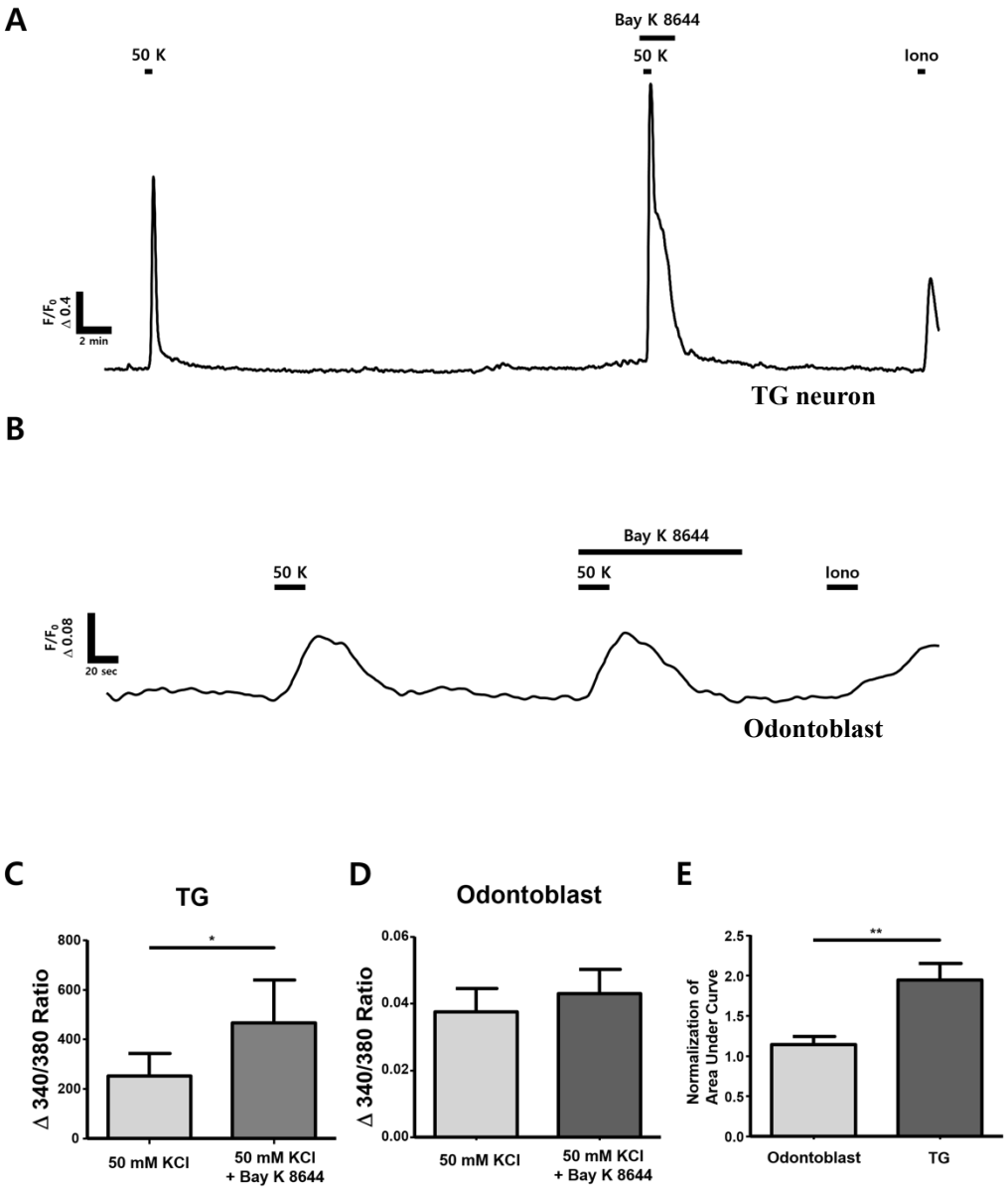
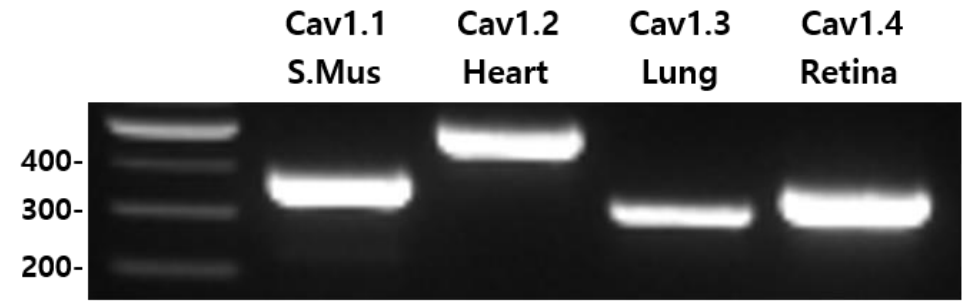

A
Bay K 8644
50 K
50 K
Iono
F/F0
Δ 0.4
2 min
TG neuron
B
Bay K 8644
50 K
50 K
Iono
F/F0
Δ 0.08
20 sec
Odontoblast
C
TG
Δ 340/380 Ratio
800
600
400
200
0
*
50 mM KCl
50 mM KCl
+ Bay K 8644
D
Odontoblast
Δ 340/380 Ratio
0.06
0.04
0.02
0.00
50 mM KCl
50 mM KCl
+ Bay K 8644
E
Normalization of
Area Under Curve
2.5
2.0
1.5
1.0
0.5
0.0
**
Odontoblast
TG

Figure 2. Effect of Bay K 8644 on 50 mM KCl-induced calcium transient in odontoblast

(A) The representative trace in TG neuron shown by combination of Bay K 8644 and 50 mM KCl-induced calcium transient. **(B)** The representative trace in acutely isolated adult rat odontoblast shown by combination of Bay K 8644 and 50 mM KCl-induced calcium transient. **(C)** Statistical analysis shown by area under curves of first (Application of 50 mM KCl) and second transient (Pretreatment of Bay K 8644 and application of 50 mM KCl) in TG neurons (n= 10, Student's 2-tailed paired *t* test, **p* = 0.0330). **(D)** Statistical analysis shown by area under curves of first and second transient in odontoblast (n= 13, Student's 2-tailed paired *t* test, *p* = 0.0729). **(E)** Area under curves evoked by 50 mM KCl and Bay K 8644 were normalized by those evoked by 50 mM KCl. When compared in odontoblast and TG neuron, only TG neuron showed substantial enhancement of calcium transient area by Bay K 8644 treatment. (n = 13, Student's 2-tailed unpaired *t* test, ***p* = 0.0010).

Figure 3.

A


Cav1.1
S.Mus
Cav1.2
Heart
Cav1.3
Lung
Cav1.4
Retina
400-
300-
200-

B

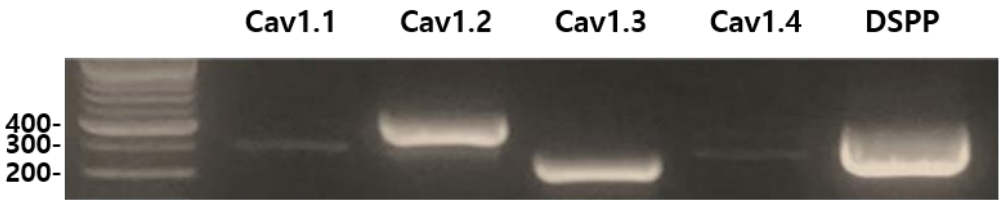

Cav1.1
Cav1.2
Cav1.3
Cav1.4
DSPP
400-
300-
200-

Figure 3. The mRNA expression of L-type calcium channels in rat pulp tissue determined by RT-PCR

(A) The mRNA detections of L-type calcium channel isoforms in positive control tissues (Ca_V 1.1: smooth muscle, Ca_V 1.2: heart, Ca_V 1.3: lung, Ca_V 1.4: retina). Predicted sizes for selected markers are Ca_V 1.1, (368 bp) Ca_V 1.2 (497 bp), Ca_V 1.3 (323 bp), and Ca_V 1.4 (366 bp). **(B)** The mRNA detections of L-type calcium channel isoforms in rat whole pulp tissues. DSPP (453 bp) was used as positive control for odontoblasts.

Figure 4.

A.

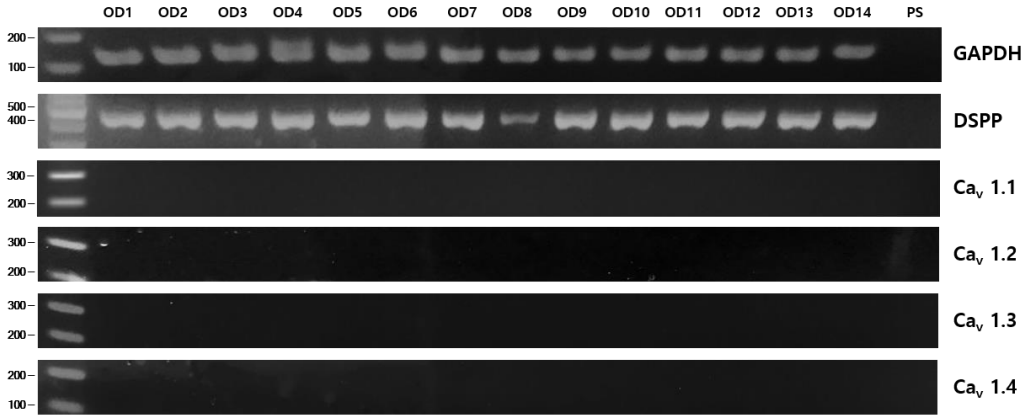
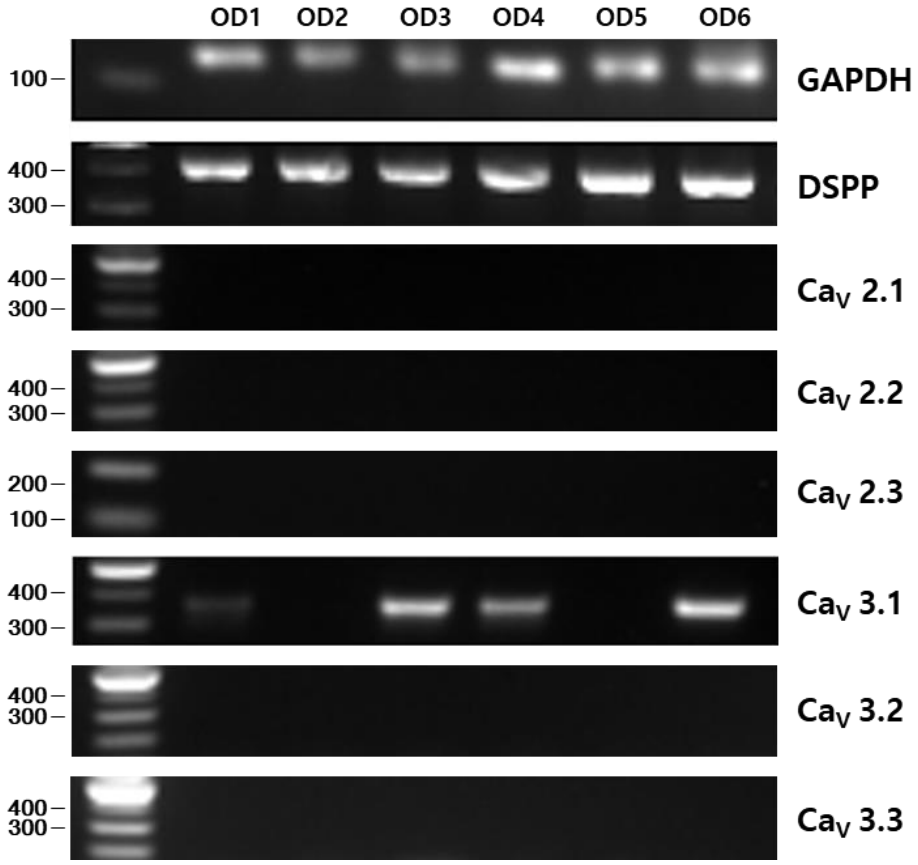

OD1
OD2
OD3
OD4
OD5
OD6
OD7
OD8
OD9
OD10
OD11
OD12
OD13
OD14
PS
200
100
GAPDH
500
400
DSPP
300
200
Cav 1.1
300
200
Cav 1.2
300
200
Cav 1.3
200
100
Cav 1.4

Figure 4. The mRNA expression of L-type calcium channels determined by scRT-PCR in acutely isolated rat odontoblasts

(A) The representative gels showing scRT-PCR products from fourteen single odontoblast cells. Results indicating mRNA expression of GAPDH, DSPP, Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, and Ca_V 1.4. Predicted sizes for selected markers are GAPDH (118 bp), DSPP (409 bp), Ca_V 1.1, (200 bp) Ca_V 1.2 (232 bp), Ca_V 1.3 (202 bp), Ca_V 1.4 (152 bp). DSPP was used as odontoblast-specific marker and GAPDH was used as house keeping gene marker. Pipette solution (PS) which contained only extracellular solution without cell contents was used as negative control.

Figure 5.

A.


OD1
OD2
OD3
OD4
OD5
OD6
100
GAPDH
400
300
DSPP
400
300
Ca_V 2.1
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300
Ca_V 2.2
200
100
Ca_V 2.3
400
300
Ca_V 3.1
400
300
Ca_V 3.2
400
300
Ca_V 3.3

Figure 5. The mRNA expression of voltage-gated calcium channels determined by scRT-PCR in acutely isolated rat odontoblasts

(A) The representative gels showing scRT-PCR products from six odontoblast cells. Results indicating mRNA expression of GAPDH, DSPP, Ca_V 2.1, Ca_V 2.2, Ca_V 2.3, Ca_V 3.1, Ca_V 3.2 and Ca_V 3.3. Predicted sizes for selected markers are GAPDH (118 bp), DSPP (409 bp), Ca_V 2.1 (497 bp), Ca_V 2.2 (356 bp), Ca_V 2.3 (159 bp), Ca_V 3.1 (356 bp), Ca_V 3.2 (356 bp) and Ca_V 3.3 (356 bp).

4. DISCUSSION

This study aimed to elucidate whether L-type calcium channels are molecularly and functionally expressed in acutely isolated adult rat odontoblast. Although the intracellular calcium signaling was induced by membrane depolarization, positive modulator of L-type calcium channels, Bay K 8644, failed to potentiate intracellular calcium signal in odontoblast. ScRT-PCR further verified that odontoblasts lack L-type calcium channel isoforms. These results indicate that L-type calcium channels may not be functionally expressed in odontoblasts from the mature teeth, thus minimally contributing to the depolarization-induced calcium transient.

First of all, Fura-2-based ratiometric calcium imaging was used to observe the L-type calcium channels by Bay K 8644-induced potentiation. TG neurons were used to compare with odontoblast as positive control for L-type calcium channels. Calcium transients were potentiated by Bay K 8644 in TG neurons but not in odontoblasts. To verify that the calcium signals originated from the activation of L-type calcium channels, odontoblasts were pretreated with Bay K 8644 and the calcium responses to 50 mM KCl solution were observed. The concentration of Bay K 8644 was set to 1 μM with reference to the previous paper (Greenberg et al., 1984). The activation range of the L-type calcium channels was determined to be -20 to 0 mV according to the IV curve (Bechem & Hoffmann, 1993). 50 mM KCl solution was used to prevent the membrane potential from being fully potentiated in comparison to the use of 80 or 120 mM KCl in order to optimally observe the potentiating effect of Bay K 8644 on L-type calcium channels.

scRT-PCR was used to detect L-type calcium channel isoforms on single cell level and obtained consistent result. The designed primers Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, and Ca_V 1.4 successfully detected the mRNA of the respective isoforms in positive control tissues such as smooth muscle, heart, lung, and retina, respectively (**Fig. 3**). Therefore, it seems unlikely that L-type calcium channels mRNAs not being detected in odontoblasts is due to experimental errors.

In contrast to our results, other groups have reported L-type calcium channels expression in odontoblasts. This discrepancy might be due to the developmental stage of the odontoblasts under study as odontoblasts from neonatal rats or differentiated human pulp cells were shown to express L-type calcium channels. Odontoblasts from the injured pulp were found to express of L-type calcium channels by immunohistochemical analysis. (Seux et al., 1994; Westenbroek et al., 2004). A previous study demonstrated the expression of L-type calcium channels in dissociated dental pulp cells by performing calcium imaging with Bay K 8644 application with high extracellular K^+. However, the cells under study was only presumed as odontoblasts as the cells were not identified with odontoblastic markers such as DSPP, and morphological features describing odontoblastic characteristics such as long process and polarized nuclei were also not provided in the study (Lundgren & Linde, 1997).

As acutely isolated adult rat odontoblasts show high K^+-induced calcium transient, it can be inferred from this study that odontoblasts express voltage-gated calcium channels which can mediate calcium transients by membrane depolarization, although the contribution of L-type calcium channels seems to be less likely. Because of this results, L-type calcium channels may not be expressed in odontoblast from the mature teeth. During the formation of dentin, intracellular

calcium signaling via L-type calcium channels may be involved, but in mature teeth, the expression of L-type calcium channels may be down-regulated in odontoblasts as dentin formation is minimal. Therefore, the possibility of other channels to be functionally expressed in odontoblasts cannot be excluded.

There are other possible channels expressed in odontoblasts which may be involved in calcium signaling during dentin formation. Calcium signaling can be mediated by plasma membrane calcium channels and pumps such as Na^{+}-Ca^{2+} exchangers, TRPC, N-methyl-D-aspartate receptors (NMDAR), plasma membrane Ca^{2+}-ATPases (PMCA), and intracellular Ca^{2+} channels including inositol triphosphate receptors (IP3R) and sarcoplasmic reticulum Ca^{2+}-ATPases (Duan, 2013; Kawashima & Okiji, 2016; Krebs et al., 2015; Nurbaeva et al., 2017). These membrane proteins serve as part of Ca^{2+} transporter involved in mineralization in odontoblasts (Tsumura et al., 2010).

Voltage-gated calcium channels expressed in osteoblasts can be possible candidates as calcium signaling. It has been well documented to mediate bone formation. Osteoblasts, which involved in bone formation, are comparable to odontoblasts because they play similar role in ability of calcified tissue formation (Song et al., 2017). Osteoblast significantly expressed various voltage-gated calcium channels, especially L-type, T-Type, N-type, P/Q type calcium channels (Barry, 2000; Shao et al., 2005).

Due to these investigations, the possibility of these channels to be expressed in odontoblasts cannot be ruled out. Since calcium imaging result reveal the extracellular high K^{+}-induced intracellular calcium responses (**Fig. 1**), other voltage-gated calcium channels may express in acutely isolated adult rat odontoblast. Therefore, scRT-PCR was conducted to screen other calcium

channel (**Fig. 5A**). Surprisingly, only T-Type calcium channel isoform, Ca_V 3.1, was detected in a subpopulation of acutely isolated adult rat odontoblast.

In conclusion, it is unlikely that adult rat odontoblasts express L-type calcium channels. The extracellular high K^+-induced intracellular calcium responses were detected in odontoblasts, but the depolarization-induced calcium transients were not enhanced by L-type calcium channels agonist, Bay K 8644, in odontoblasts. In odontoblasts from the mature teeth, T-type calcium channels, rather than L-type calcium channels, may mediate intracellular calcium responses following membrane depolarization. Identification of the physiological function of T-type calcium channels, especially Ca_V 3.1 in odontoblasts, needs to be investigated in the further study.

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국문초록

상아모세포에서 전압 의존성 L형 칼슘 통로의 발현에 대한 연구

상아모세포의 주요한 역할은 세포 내 칼슘 신호에 의해 형성되는 콜라겐 기반의 광화 조직인 상아질의 형성이다. 상아질 생성 과정 중 세포 내 칼슘 신호를 증가시키는 다양한 요인들이 보고되어 왔으며 그 중 주요한 요인 중 하나는 전압 의존성 L형 칼슘 통로의 활성이다. Cav 1.1, Cav 1.2, Cav 1.3, Cav 1.4 네 개의 동형 단백질로 구성되어 있는 전압 의존성 L형 칼슘 통로의 발현 및 기능은 현재까지 성체 쥐의 상아모세포에서 규명되지 않았다.

본 연구에서는 상아질 형성에서 세포 내 칼슘 신호 전달과 관련된 L형 칼슘 통로가 흰쥐 성체 쥐의 상아모세포에서 발현되어 있는지를 규명하고자 하였다. 그리고 이를 규명하기 위하여 RT-PCR, 단일 세포 RT-PCR 및 Fura-2를 기반으로 한 칼슘 이미징을 통해 상아모세포에 전압 의존성 L형 칼슘 통로의 발현 및 기능을 조사하였다.

흰쥐 성체 상아모세포의 세포 내 칼슘 반응을 조절하기 위하여 고농도의 칼륨 용액으로 세포막 전위를 탈분극 시키면서 전압 의존성 L형 칼슘 통로의 작용제인 Bay K 8644 약물을

처리하였을 때, 세포 내 칼슘 신호가 증가되지 않는 것을 관찰하였다. 또한, 이 결과를 검증하기 위해서 단일 상아모세포의 전압 의존성 L형 칼슘 통로 mRNA 발현을 단일 세포 RT-PCR 기법을 통해 확인 하였을 때 L형 칼슘 통로의 동형 단백질인 Ca_V 1.1, Ca_V 1.2, Ca_V 1.3, Ca_V 1.4가 발현 되어있지 않은 것을 확인하였다.

추가적으로, 상아모세포에서 탈분극으로 인한 세포내 칼슘 신호 증가를 관찰하였기 때문에, 단일 세포 RT-PCR 기법을 사용하여 다른 전압 의존성 칼슘 통로의 발현을 탐색하였고, 그 결과 T형 칼슘 통로 중 하나인 Ca_V 3.1의 mRNA 발현을 확인하였다.

요약하여, 본 실험을 통하여 흰쥐 성체 상아모세포에서 전압 의존성 L형 칼슘 통로가 기능적으로 발현되지 않는다는 결과를 얻었으며, 흰쥐 성체 상아모세포에서 세포막 탈분극에 반응하는 칼슘 신호는 L형 칼슘 통로를 매개로 하는 것이 아닌 T형 칼슘통로, 특히 Ca_V 3.1을 매개로 할 가능성이 높으며 상아모세포 내 T형 칼슘통로의 역할을 규명하기 위한 후속연구가 필요하다.

주요어: 상아모세포, L형 칼슘 통로, 칼슘 이미징, Bay K 8644.

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