High pH-Sensitive Store-Operated Ca\textsuperscript{2+} Entry Mediated by Ca\textsuperscript{2+} Release-Activated Ca\textsuperscript{2+} Channels in Rat Odontoblasts

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Odontoblasts play a crucial role in dentin formation and sensory transduction following the application of stimuli to the dentin surface. Various exogenous and endogenous stimuli elicit an increase in the intracellular free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in odontoblasts, which is mediated by Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores and/or Ca\textsuperscript{2+} influx from the extracellular medium. In a previous study, we demonstrated that the depletion of Ca\textsuperscript{2+} stores in odontoblasts activated store-operated Ca\textsuperscript{2+} entry (SOCE), a Ca\textsuperscript{2+} influx pathway. However, the precise biophysical and pharmacological properties of SOCE in odontoblasts have remained unclear. In the present study, we examined the functional expression and pharmacological properties of Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels that mediate SOCE and evaluated the alkali sensitivity of SOCE in rat odontoblasts. In the absence of extracellular Ca\textsuperscript{2+}, treatment with thapsigargin (TG), a sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor, induced an increase in [Ca\textsuperscript{2+}]\textsubscript{i}. After [Ca\textsuperscript{2+}]\textsubscript{i} returned to near-resting levels, the subsequent application of 2.5 mM extracellular Ca\textsuperscript{2+} resulted in an increase in [Ca\textsuperscript{2+}]\textsubscript{i} which is a typical of SOCE activation. Additionally, application of 2-methylthioadenosine diphosphate trisodium salt (2-MeSADP), a P2Y\textsubscript{1,12,13} receptor agonist, or carbachol (CCh), a muscarinic cholinergic receptor agonist, in the absence of extracellular Ca\textsuperscript{2+}, treatment with thapsigargin (TG), a sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor, induced an increase in [Ca\textsuperscript{2+}]\textsubscript{i}. After [Ca\textsuperscript{2+}]\textsubscript{i} returned to near-resting levels, the subsequent application of 2.5 mM extracellular Ca\textsuperscript{2+} resulted in an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, which is a typical of SOCE activation. Additionally, application of 2-methylthioadenosine diphosphate trisodium salt (2-MeSADP), a P2Y\textsubscript{1,12,13} receptor agonist, or carbachol (CCh), a muscarinic cholinergic receptor agonist, in the absence of extracellular Ca\textsuperscript{2+}, induced a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}. The subsequent addition of extracellular Ca\textsuperscript{2+} resulted in significantly higher [Ca\textsuperscript{2+}]\textsubscript{i} in 2-MeSADP- or CCh-treated odontoblasts than in untreated cells. SOCE, that is activated by addition of extracellular Ca\textsuperscript{2+} in the TG pretreated odontoblasts was then suppressed by Synta66, BTP2, or lanthanum, which are CRAC channel inhibitors. Treatment with an alkaline solution enhanced SOCE, while treatment with HC030031, a TRPA1 channel antagonist, inhibited it. The amplitude of SOCE at pH 9 in the presence of HC030031 was higher than that at pH 7.4 in the absence of HC030031. These findings indicate that CRAC channel-mediated alkali-sensitive SOCE occurs in odontoblasts. SOCE is mediated by P2Y and muscarinic-cholinergic receptors, which are activated by endogenous ligands in odontoblasts.

Keywords: odontoblast, store-operated Ca\textsuperscript{2+} entry, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel, alkaline stimulation, dentinogenesis
INTRODUCTION

Along with their role in physiological dentin formation and mineralization (dentinogenesis), odontoblasts are important players in sensory transduction following various stimuli to the dentin surface (Linde, 1995; Linde and Lundgren, 1995; Tsumura et al., 2012, 2013; Sato et al., 2013, 2015; Shibukawa et al., 2015; Kimura et al., 2016; Nishiyama et al., 2016). The stimuli to the dentin surface induce Ca\(^{2+}\) signaling, resulting in increased intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in odontoblasts, which triggers the release of ATP from pannexin channels, and glutamate from the volume-sensitive outwardly rectifying anion channels as neuro-/intercellular-transmitters (Sato et al., 2015; Shibukawa et al., 2015; Nishiyama et al., 2016). The released ATP and glutamate play a role in the sensory signal transduction sequence for dentinal pain by transmitting sensory signals to neurons, and may promote dentinogenesis by a cluster of odontoblasts via odontoblast-odontoblast and odontoblast-trigeminal ganglion (TG) neuron signal communication (Sato et al., 2015; Shibukawa et al., 2015; Nishiyama et al., 2016). In addition, we also reported that high pH-sensitive transient anion channels as neuro-/intercellular-transmitters (Sato et al., 2015; Shibukawa et al., 2015; Nishiyama et al., 2016). The depletion of Ca\(^{2+}\) stores is sensed by Ca\(^{2+}\) store-localized stromal interaction molecule 1 (STIM1), a Ca\(^{2+}\) store calcium-sensor, and causes translocation of STIM1 to the plasma membrane. In the plasma membrane, STIM1 interacts directly with Orai1, resulting in the activation of CRAC channels (Frischauf et al., 2008, 2016; Desai et al., 2015). Recent studies have shown the importance of Orai1 in bone formation by osteoblasts. Both odontoblasts and osteoblasts evoke the secretion of the extracellular matrix and formation of mineralized hydroxyapatite (Hwang et al., 2012). In addition, in ameloblasts, SOCE contributes to enamel formation and regulation of the expression of enamel matrix proteins. CRAC

Store-operated Ca\(^{2+}\) entry (SOCE) has been well described as a Ca\(^{2+}\) entry pathway in the plasma membrane that is activated by the depletion of IP\(_3\)- and/or ryanodine-sensitive Ca\(^{2+}\) stores (Putney, 1986, 2010; Parekh and Putney, 2005). SOCE is a ubiquitous and important Ca\(^{2+}\) influx mechanism in excitable and non-excitable cells. SOCE participates not only in the replenishment of Ca\(^{2+}\) stores but also in the modulation of many physiological functions such as secretion, cell proliferation, endothelial cell migration, T cell activation, mast cell degranulation, thrombus formation, and tumor cell metastasis (Cheng et al., 2011). SOCE is mediated via store-operated Ca\(^{2+}\) (SOC) channels. The best-characterized SOC channels are Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels composed of the pore-forming subunit Orai1, Orai2, or Orai3 (Desai et al., 2015). The depletion of Ca\(^{2+}\) stores is sensed by Ca\(^{2+}\) store-localized stromal interaction molecule 1 (STIM1), a Ca\(^{2+}\) store calcium-sensor, and causes translocation of STIM1 to the plasma membrane. In the plasma membrane, STIM1 interacts directly with Orai1, resulting in the activation of CRAC channels (Frischauf et al., 2008, 2016; Desai et al., 2015). Recent studies have shown the importance of Orai1 in bone formation by osteoblasts. Both odontoblasts and osteoblasts evoke the secretion of the extracellular matrix and formation of mineralized hydroxyapatite (Hwang et al., 2012). In addition, in ameloblasts, SOCE contributes to enamel formation and regulation of the expression of enamel matrix proteins. CRAC

FIGURE 1 | Addition of extracellular Ca\(^{2+}\) increases [Ca\(^{2+}\)]\(_i\), following TG-induced [Ca\(^{2+}\)]\(_i\) increase. (A) Representative trace of [Ca\(^{2+}\)]\(_i\), increase in response to application of 10 µM TG and subsequent application of 2.5 mM extracellular Ca\(^{2+}\) (white box at bottom) after 10 µM TG application. Black box at the top indicates the application of 10 µM TG. (B) Summary bar graph shows [Ca\(^{2+}\)]\(_i\), increases by application of 10 µM TG (gray column) and 2.5 mM extracellular Ca\(^{2+}\) (open column). Each column indicates the mean ± SE of 7–9 independent experiments.
FIGURE 2 | Effects of PLC-coupled receptor agonists on the Ca\(^{2+}\) influx. [A,C,E] Representative traces of \([\text{Ca}^{2+}]_i\) increase in response to application of PLC-coupled receptor agonists, 50 nM 2-MeSADP (A), 100 \(\mu\)M CCh (C), or 100 \(\mu\)M DHPG (E), and subsequent application of 2.5 mM extracellular Ca\(^{2+}\) (white box at bottom) with (red line), or without (black line) agonists (A,C,E). Black boxes at the top indicate the application time period of 50 nM 2-MeSADP (A), 100 \(\mu\)M CCh (C), or 100 \(\mu\)M DHPG (E). (B,D,F) Summary bar graphs show \([\text{Ca}^{2+}]_i\) increase by application of 50 nM 2-MeSADP (B), 100 \(\mu\)M CCh (D), or 100 \(\mu\)M DHPG (F) (upper column) and subsequent application of 2.5 mM extracellular Ca\(^{2+}\) in the presence (gray column) or absence (middle column) of these agonists. Each column indicates the mean ± SE of 5–11 independent experiments. Statistically significant differences between columns (shown by solid lines) are denoted by asterisks, *\(P < 0.05\).

channels are also involved in enamel development (Nurbaeva et al., 2015b).

It has been reported that Orai1 is expressed in mouse odontoblasts (Zheng et al., 2015). In a previous study, we had also demonstrated that SOCE and CRAC currents were activated in response to depletion of Ca\(^{2+}\) stores in acutely dissociated odontoblasts (Shibukawa and Suzuki, 2003). However, the detailed biophysical as well as pharmacological properties of SOCE in odontoblasts remain unclear. In the present study, to elucidate pharmacological properties of SOCE, we investigated the expression, and pharmacological properties of CRAC channels in odontoblasts. In addition, we examined extracellular pH-sensitivity of the SOCE in odontoblasts.

MATERIALS AND METHODS

Ethical Approval

All animals were treated in accordance with the Guiding Principles for the Care and Use of Animals in the field of physiological sciences approved by the Council of the Physiological Society of Japan and the American Physiological
Society. All animal experiments in this study followed the guidelines established by the National Institutes of Health, United States regarding the care and use of animals for experimental procedures, as well as the United Kingdom Animals (Scientific Procedures) Act, 1986. This study was approved by the Ethics Committee of our institute (Nos. 270302, 280302, and 290301).

**Dental Pulp Slice Preparation**

Dental pulp slice preparations were obtained from newborn Wistar rats (6- to 8-day-old) using a previously described method (Son et al., 2009; Shibukawa et al., 2015; Tokuda et al., 2015). Briefly, under isoflurane (3%) and pentobarbital sodium anesthesia (25 mg/kg), the mandible was dissected. The hemimandible embedded in alginate impression material was sliced transversely through the incisor at 500-µm thickness with a standard vibrating tissue slicer (Dosaka EM, Kyoto, Japan). A section of mandible was sliced to the required level, so that the dentin and enamel were directly visible between the bone tissue and the dental pulp. The surrounding impression material, bone tissue, enamel, and dentin were removed from the mandible section under a stereoscopic microscope, and the remaining dental pulp slice was used in further experiments. We selected mandible sections in which the dentin layer was thin and the enamel, and dentin were clearly distinguishable under the microscope, to avoid cellular damage to odontoblasts. Pulp slices were treated with a standard Krebs solution containing 0.03% trypsin and 0.17% collagenase (30 min at 37°C). For [Ca^{2+}]i measurement, enzymatically treated and isolated odontoblasts from the dental pulp slice were plated onto a culture dish, immersed in alpha-minimum essential medium (Life Technologies, Carlsbad, CA, United States) including 10% fetal bovine serum and 5% horse serum, and maintained at 37°C in a 5% CO2 incubator. The primary cultured odontoblasts from the dental pulp slice were used for [Ca^{2+}]i measurements; we stably measured [Ca^{2+}]i increases within 24 h of isolation. Cells were confirmed to be odontoblasts in a previous study with the odontoblast markers dentin matrix protein-1, dentin sialoprotein, and nestin within 24 h of isolation (Tsumura et al., 2012).

**Measurement of Ca^{2+}-Sensitive Dye Fluorescence**

Cells in dental pulp slices were loaded with 10 µM fura-2-acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan) (Tsien et al., 1985) and 0.1% (w/v) pluronic acid F-127 (Life Technologies) in standard Krebs solution for 30 min at 37°C. They were then washed with fresh Krebs solution. A dish including fura-2-loaded odontoblasts was mounted on the stage.
of a microscope (IX73, Olympus, Tokyo, Japan) with HCImage software, an excitation wavelength selector, and an intensified charge-coupled device camera system (Hamamatsu Photonics, Shizuoka, Japan). Fura-2 fluorescence emission was recorded at 510 nm in response to alternating excitation wavelengths of 380 nm (F380) and 340 nm (F340). The \([\text{Ca}^{2+}]_{i}\) was defined using the fluorescence ratio (\(R_{F340/F380}\)) of F340 to F380 (F340/F380), and is described as \(F_{i}/F_{0}\) units; the \(R_{F340/F380}\) value (\(F\)) was normalized to the resting value (\(F_{0}\)). The \(F_{i}/F_{0}\) baseline was configured at 1.0. All experiments were performed at room temperature (30 ± 1.0°C).

**Solutions and Reagents**

Krebs solution containing (in mM) 136 NaCl, 5 KCl, 0 or 2.5 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, 10 glucose, and 12 NaHCO\(_3\) (pH 7.4 Tris) was used as the standard extracellular solution. To prepare high pH (pH 9) extracellular solutions, 12 mM NaHCO\(_3\) in Krebs solution was replaced by 10 mM (for pH 9) NaOH. This replacement did not affect extracellular free \([\text{Ca}^{2+}]_{i}\) concentrations in the test solution. Synta66 was obtained from ABOIOUS INC. (Gloucester, MA, United States). BTP2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). HC030031, 2-Methylthioadenosine diphosphate, and DHPG were obtained from Tocris Bioscience (Bristol, United Kingdom). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, United States). Stock solutions of lanthanum chloride, carbachol, and DHPG were prepared in ultra-pure water (Millipore, MA, United States). All other stock solutions were prepared in dimethyl sulfoxide. Stock solutions were diluted to the appropriate concentration with Krebs solution (pH 7.4 or 9) before use.

**Statistics and Offline Analysis**

Data are represented as the mean ± standard error (SE) of the mean of \(N\) observations, where \(N\) shows the number of independent experiments. The Wilcoxon test or Mann–Whitney test were used to evaluate the non-parametric statistical significance. A \(P\)-value < 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, United States).

**RESULTS**

**Addition of Extracellular \([\text{Ca}^{2+}]_{i}\)**

In the absence of extracellular \([\text{Ca}^{2+}]_{i}\), application of 10 \(\mu\)M thapsigargin (TG), an inhibitor of sarco/endoplasmic reticulum \([\text{Ca}^{2+}]_{i}\)-ATPase (Thastrup et al., 1990; Shibukawa and Suzuki, 2003), induced transient \([\text{Ca}^{2+}]_{i}\) increases to a peak value of 1.04 ± 0.006 \(F_{i}/F_{0}\) units (\(N = 7\)) (Figures 1A,B). The transient increases in \([\text{Ca}^{2+}]_{i}\) are caused by the release of \([\text{Ca}^{2+}]_{i}\) from intracellular \([\text{Ca}^{2+}]_{i}\) stores. After \([\text{Ca}^{2+}]_{i}\) returned to the nearesting levels, subsequent application of 2.5 mM extracellular \([\text{Ca}^{2+}]_{i}\) increased \([\text{Ca}^{2+}]_{i}\) (Figure 1A) to a peak value of 1.32 ± 0.04 \(F_{i}/F_{0}\) units (\(N = 9\)) (Figures 1A,B).

**Effects of 2-MeSADP, Carbachol and DHPG Pre-application on the \([\text{Ca}^{2+}]_{i}\) Influx**

PLC-coupled receptors, P2Y (Sato et al., 2015; Shibukawa et al., 2015; Wang et al., 2016), muscarinic-cholinergic (Shibukawa and Suzuki, 2003), and group I metabotropic glutamate receptors (Kim et al., 2009; Nishiyama et al., 2016), are expressed in odontoblasts. We, thus, examined the participation of these PLC-coupled receptors in the activation of \([\text{Ca}^{2+}]_{i}\) influx by store depletion. In the absence of extracellular \([\text{Ca}^{2+}]_{i}\), application of 50 nM 2-methylthioadenosine diphosphate (2-MeSADP), a P2Y\(_{1,12,13}\) receptor agonist (Abbracchio et al., 2006; Kawaguchi et al., 2015), increased \([\text{Ca}^{2+}]_{i}\) transiently to a peak value of 1.08 ± 0.02 \(F_{i}/F_{0}\) units (\(N = 6\)) (Figures 2A,B). Carbachol (CCh) (100 \(\mu\)M), a muscarinic-cholinergic receptor agonist (He et al., 2005; Piergentili et al., 2007), evoked transient \([\text{Ca}^{2+}]_{i}\) increases

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**FIGURE 5** | \(\text{La}^{3+}\) inhibits SOCE. (A) Representative trace of the effect of \(\text{La}^{3+}\) on SOCE induced by subsequent application of 2.5 mM extracellular \([\text{Ca}^{2+}]_{i}\) (white box at bottom) after pre-incubation of 10 \(\mu\)M TG (white box at the top). Black box shows the time period of 100 \(\mu\)M \(\text{La}^{3+}\) addition to the extracellular solution. (B) Summary bar graph shows the effect of \(\text{La}^{3+}\) on SOCE (gray column) or the peak \(F_{i}/F_{0}\) values in SOCE activation (open column). Each column indicates the mean ± SE of seven independent experiments. Statistically significant differences between columns (shown by solid lines) are denoted by asterisks, \(*P < 0.05\).
to the value of 1.04 ± 0.01 F/F0 units (N = 6) (Figures 2C,D), while application of 100 μM DHPG, an agonist of group I metabotropic glutamate receptors (Ito et al., 1992; Lin et al., 1997; Schoepf et al., 1999), induced transient [Ca\(^{2+}\)]\(_i\) increases to the value of 1.02 ± 0.002 F/F0 units (N = 11) (Figures 2E,F). These transient [Ca\(^{2+}\)]\(_i\) increases are elicited by the Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. After [Ca\(^{2+}\)]\(_i\) returned to near-resting levels following each application of 50 nM 2-MeSADP, 100 μM CCh, and 100 μM DHPG, subsequent addition of 2.5 mM extracellular Ca\(^{2+}\) increased [Ca\(^{2+}\)]\(_i\), (Figures 2A,C,E). The peak values following application of 2.5 mM extracellular Ca\(^{2+}\) with 50 nM 2-MeSADP were 1.72 ± 0.04 F/F0 units (N = 6) (Figure 2B), while those with 100 μM CCh were 1.38 ± 0.05 F/F0 units (N = 5) (Figure 2D). After pretreatment of 2-MeSADP, and CCh, the Ca\(^{2+}\) influx induced by subsequent application of 2.5 mM extracellular Ca\(^{2+}\) was significantly larger than that without pretreatment; the values of Ca\(^{2+}\) influx without any pretreatment were 1.23 ± 0.01 F/F0 units (N = 5) (Figures 2B,D). However, there were no significant differences in the Ca\(^{2+}\) increases (that was elicited by subsequent application of 2.5 mM extracellular Ca\(^{2+}\)) between with DHPG pretreatment (1.24 ± 0.007 F/F0 units; N = 8) and without any pretreatment (Figure 2F).

**Synta66 and BTP2 Inhibited Store-Operated Ca\(^{2+}\) Entry (SOCE)**

To identify the pathway of Ca\(^{2+}\) influx activated by subsequent application of 2.5 mM extracellular Ca\(^{2+}\) after store depletion, we investigated the effects of CRAC channel inhibitors, synta66 (Beech, 2012; Kruchten et al., 2012; Derler et al., 2013; Molnár et al., 2016) and BTP2 (Ishikawa et al., 2003; Zitt et al., 2004; Zeng et al., 2017), on the Ca\(^{2+}\) influx. After store depletion by pretreatment of 10 μM TG in the absence of extracellular Ca\(^{2+}\), application of 2.5 mM extracellular Ca\(^{2+}\) increased [Ca\(^{2+}\)]\(_i\), to a peak value of 1.32 ± 0.04 F/F0 units (N = 9). The increases in [Ca\(^{2+}\)]\(_i\) were significantly suppressed in the presence of 10 μM synta66 to 1.20 ± 0.03 F/F0 units (N = 6) (Figures 3A,B). In addition, when the cells were subjected to preincubation with 1 μM BTP2 for 60 min at 37°C, the [Ca\(^{2+}\)]\(_i\) increases following Ca\(^{2+}\) store depletion by TG pretreatment were inhibited to 1.08 ± 0.01 F/F0 units (N = 10) (Figures 4A,B) compared to those without BTP2 (1.32 ± 0.04 F/F0 units (N = 9)).

**Lanthanum Inhibited SOCE**

After store depletion by pretreatment with 10 μM TG in the absence of extracellular Ca\(^{2+}\), application of 2.5 mM extracellular Ca\(^{2+}\) increased [Ca\(^{2+}\)]\(_i\), to a peak value of 1.32 ± 0.02 F/F0 units (N = 7), and an application of 100 μM lanthanum (La\(^{3+}\)), a non-specific CRAC channel inhibitor (Ross and Cahalan, 1995; Derler et al., 2013; Guido et al., 2015; Prakriya and Lewis, 2015), caused a decrease in [Ca\(^{2+}\)]\(_i\), to a peak value of 1.16 ± 0.01 F/F0 units (N = 7) (Figures 5A,B). After La\(^{3+}\)-induced suppression of SOCE, removal of La\(^{3+}\) resulted in a slow return of SOCE activity over several minutes (Figure 5A).

**Alkaline Stimuli Enhanced SOCE**

We examined the effects of extracellular alkalization on SOCE in odontoblasts. After store depletion by continuous treatment with 10 μM TG in the absence of extracellular Ca\(^{2+}\), subsequent application of alkaline solution (pH 9) with 2.5 mM extracellular Ca\(^{2+}\) enhanced SOCE to a peak value of 2.17 ± 0.1 F/F0 units (N = 5) (blue; Figures 6A,B), while the peak value of [Ca\(^{2+}\)]\(_i\) increase by application of standard (pH 7.4) extracellular solution with 2.5 mM Ca\(^{2+}\) was 1.49 ± 0.13 F/F0 units (N = 14) (black in Figure 6A). Odontoblasts express alkalinsensitive TRPA1 channels (Tsumura et al., 2013; Kimura et al., 2016). To remove the Ca\(^{2+}\) influx component via TRPA1 channel activation from SOCE by the subsequent application of alkaline solution with extracellular Ca\(^{2+}\), we applied HC030031, a TRPA1 channel antagonist (McNamara et al., 2007; Tsumura et al., 2013). HC030031 (100 μM) suppressed SOCE by the subsequent application of alkaline solution with 2.5 mM extracellular Ca\(^{2+}\) to
1.78 ± 0.04 F/F₀ units (N = 15) (red; Figures 6A,B). The SOCE evoked by subsequent application of 2.5 mM extracellular Ca²⁺ with 100 µM HC030031 at pH 9 were larger than those without HC030031 at pH 7.4 (N = 14) (Figures 6A,B).

**DISCUSSION**

We elucidated the expression and pharmacological properties of CRAC channels in odontoblasts. After store depletion, application of extracellular Ca²⁺ induced Ca²⁺ influx (SOCE). The Ca²⁺ influx was suppressed by CRAC channel inhibitors, synta66, BTP2 and lanthanum. The activation of P2Y and muscarinic-cholinergic receptors triggered SOCE. However, the activation of group I metabotropic glutamate receptors did not elicit SOCE. After store depletion, alkaline solution containing Ca²⁺ enhanced SOCE under TRPA1 channel inhibition, compared to that using the extracellular solution with Ca²⁺ (pH 7.4) without TRPA1 inhibition. These results indicate that, in odontoblasts, store depletion activates CRAC channel-mediated SOCE, which is promoted in an alkaline environment. The results are in line with previous results showing the expression of Orai1, subunits of CRAC channels, by immunohistochemical analysis in odontoblasts (Zheng et al., 2015). CRAC channels, which mediate SOCE, are involved in various functions in diverse cells. In ameloblasts, CRAC channel-mediated SOCE contributes to the mechanism for Ca²⁺ uptake in enamel formation (Nurbaeva et al., 2015a,b). It has been also reported that dental pulp stem cells (DPSCs) express Orai1, and Orai1-knocked down shRNA suppress mineralization by DPSCs (Sohn et al., 2015). These results suggest that CRAC channel-mediated SOCE in odontoblasts has a potential role in dentinogenesis.

TRP canonical subfamily (TRPC) channels have been also proposed as possible candidates for the channel proteins mediating SOCE (Cheng et al., 2011). Among the TRPC channels, TRPC1 channels have been well-characterized and reported to participate in endogenous SOCE in several cell types (Cheng et al., 2011). Following store depletion, STIM1 translocates to the endoplasmic reticulum-plasma membrane junction and interacts with Orai1, resulting in CRAC channel activation. Ca²⁺ entry via Orai1 initiates the recruitment of TRPC1 channels into the plasma membrane, where the channels interact with STIM1 and are activated. According to this mechanism (Hogan and Rao, 2015; Ong et al., 2016; Ambudkar et al., 2017), TRPC1 channel function crucially depends on Orai1-mediated Ca²⁺ entry, and SOCE is generated by both Orai1 and STIM1, and TRPC1 channels. Odontoblasts in rats and humans have been shown to express TRPC1 channels (Kwon et al., 2014; Song et al., 2017). It has been reported that BTP2 (CRAC channel inhibitor) also inhibit SOCE via TRPC3 and TRPC5 channels (He et al., 2005). If odontoblasts express TRPC3 or/and TRPC5 channels, these channels may also contribute to SOCE in odontoblasts. Thus, TG-induced SOCE arises not only via CRAC channels but also via TRPC channel family in odontoblasts.

In the absence of extracellular Ca²⁺, P2Y, muscarinic-cholinergic, and group I metabotropic glutamate receptor
agonists increased \([Ca^{2+}]_i\) in odontoblasts. The results demonstrate the expression of these receptors in odontoblasts, and are in line with previous reports (Shibukawa and Suzuki, 2003; Kim et al., 2009; Sato et al., 2015; Shibukawa et al., 2015; Nishiyama et al., 2016). These PLC-coupled receptors facilitate the activation of SOCE by store depletion. In odontoblasts, P2Y and muscarinic-cholinergic receptor activation elicited store depletion, resulting in the activation of SOCE. Non-excitable cells, such as microglia, cells in the adrenal cortex, and salivary gland cells, also express SOCE activated by P2Y and/or muscarinic-cholinergic receptor activations to mediate cellular functions (Nishi et al., 2013; Ambudkar, 2014; Michaelis et al., 2015). On the other hand, we could find a few reports describing SOCE activated by group I metabotropic glutamate receptor activation in hippocampal neuron and astrocytes (Ng et al., 2011; Ronco et al., 2014). The present study could not find group I metabotropic glutamate receptor-induced SOCE. Further study will be needed to clarify SOCE induced by the activation of glutamate receptors.

In the present study, alkaline stimuli enhanced SOCE via CRAC channels in odontoblasts. In our previous study, we predicted that alkaline stimuli might also activate \(Ca^{2+}\)-permeable channels in addition to TRPA1 channels in odontoblasts (Kimura et al., 2016). It has been proposed that external pH modulates CRAC channel activation through its channel pore (Beck et al., 2014). In addition, external alkaline conditions augment the amplitudes of both CRAC current and \([Ca^{2+}]_i\) increases by SOCE (Iwasawa et al., 1997; Laskay et al., 2005; Beck et al., 2014). These evidences suggested directly regulated mechanisms for the activation of CRAC channels by alkaline stimuli. In our previous study, alkaline stimuli also elicited \(Ca^{2+}\) release from \(Ca^{2+}\) stores via metabotropic receptors in odontoblasts (Kimura et al., 2016). Although we speculated that the activation of alkali sensitive-metabotropic receptors may induce store depletion resulting in SOCE, further study will be needed to identify the molecular entity of these alkali sensitive-metabotropic receptors (Kimura et al., 2016). Odontoblasts also detect high pH produced by dental materials such as calcium hydroxide or mineral trioxide aggregate (MTA), and the alkaline stimuli increase the mineralization level in odontoblasts via TRPA1 channel activation (Kimura et al., 2016). Thus, we suggest that CRAC channel-mediated SOCE may also participate in dentinogenesis under high pH as well as physiologic conditions.

The stimuli to the dentin surface induce \([Ca^{2+}]_i\) increases via mechanosensitive TRP channels (Sato et al., 2015; Shibukawa et al., 2015), and Piezo channels (Sato et al., 2018) in odontoblasts. The \([Ca^{2+}]_i\) increases elicit the release of ATP from pannexin-1 channels (Sato et al., 2015; Shibukawa et al., 2015) in odontoblasts. The released ATP is also hydrolyzed by nucleoside triphosphate diphosphohydrolase-2 to produce ADP (Sato et al., 2015; Shibukawa et al., 2015). Therefore, ADP/ATP released from odontoblasts, as intercellular-/neuro-transmitters, also promotes \(Ca^{2+}\) signaling by the activation of ADP-induced SOCE, which involves P2Y receptor activation, in odontoblasts located in the periphery. The presence of cholinergic nerves, post-ganglionic parasympathetic fibers, in the dental pulp is controversial. If post-ganglionic parasympathetic nerves innervate the dental pulp, acetylcholine released by excitation of the neurons could activate SOCE in odontoblasts.

In conclusion (see Figure 7), we demonstrated SOCE mediated by CRAC channels in odontoblasts. SOCE is activated by PLC-coupled receptors in odontoblasts. Endogenous ADP, released from odontoblasts in the dental pulp in response to cellular deformation or cellular damage, as well as muscarinic-cholinergic agonist from intradental parasympathetic neurons, evoked SOCE in odontoblasts. SOCE was enhanced by an alkaline environment and may play important roles in accelerating cellular functions, such as high-pH sensitive tertiary/reactionary dentin formation, following alkaline stimuli applied to dentin. In addition, alkaline stimuli activate TRPA1 channels in odontoblasts and evoke \(Ca^{2+}\) influx via TRPA1 channels. \([Ca^{2+}]_i\) increases due to TRPA1 channel-mediated \(Ca^{2+}\) influx, which is closely involved in dentin formation under both physiological and high pH conditions (Kimura et al., 2016). Alkaline stimuli also activate alkali sensitive-metabotropic receptors (Kimura et al., 2016), and their activation induces \(Ca^{2+}\) release from intracellular \(Ca^{2+}\) stores via ryanodine and/or IP3 receptors. During dental treatments, the use of dental materials, such as calcium hydroxide or MTA, results in a high pH/\(Ca^{2+}\) extracellular environment. This external environment activates \(Ca^{2+}\) signaling mediated by SOCE, TRPA1 channels and alkali sensitive-metabotropic receptors, which can sense the increased pH in odontoblasts, resulting in the induction of dentinogenesis.

**AUTHOR CONTRIBUTIONS**

MK, KN, MT, and YS were responsible for the conception and design of the experiments. MK, KN, AH, SO, KS, MT, and YS were responsible for the acquisition, analysis, and interpretation of data. MK, KN, and YS were responsible for drafting and critically revising the intellectual content of the article. YS was responsible for final approval of the version to be submitted/published. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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