Spontaneous calcium responses of SF2 rat dental epithelial cells stably expressing the calcium sensor G-GECO

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ABSTRACT

Genetically-encoded calcium indicators such as G-GECO are useful for studying Ca^{2+} responses during long-term processes. In this study, we employed a lentiviral vector and established a rat dental epithelial cell line that stably expressed G-GECO (SF2-G-GECO). Ca^{2+} imaging analysis under cell culture conditions revealed that SF2-G-GECO cells exhibited spontaneous Ca^{2+} responses, which could be classified into the following three major patterns depending on the cell density: localized Ca^{2+} responses at cell protrusions at a low density, a cell-wide spread of Ca^{2+} responses at a medium density, and Ca^{2+} responses in clusters of 3–20 cells at a high density. The P2Y receptor inhibitor suramin (10 μM), the ATP-degrading enzyme apyrase (5 units/mL), and the fibroblast growth factor (FGF) receptor inhibitor FIIN-2 (1 μM) decreased the frequency of spontaneous Ca^{2+} responses. These results indicate that ATP and FGF are involved in the spontaneous Ca^{2+} responses. SF2 cells differentiate into ameloblasts via interactions with mesenchymal cells. Therefore, SF2-G-GECO cells are expected to be a useful tool for studying the functions of Ca^{2+} responses in regulating gene expression during tooth development.

INTRODUCTION

Ca^{2+} is an intracellular messenger involved in the regulation of a wide range of biological processes, including contraction, secretion, gene expression, proliferation, and differentiation (Berridge 1993; Ghosh and Greenberg 1995; Barritt 1999; Kreideweiss *et al.* 1999). The major mechanism of Ca^{2+} signaling in non-excitable cells is release of Ca^{2+} from intracellular stores by inositol trisphosphate (IP_3), which is produced intracellularly via stimulation of G protein-coupled receptors and receptor tyrosine kinases

(Clapham 2007; Berridge 2009). In addition, Ca^{2+} signaling is sustained by influx of extracellular Ca^{2+} by store-operated Ca^{2+} entry (SOCE), which is activated by a decrease in the Ca^{2+} concentration in intracellular stores (Putney 2007).

Stim1 and Orai1, two molecules responsible for severe combined immunodeficiency, regulate SOCE by functioning as an endoplasmic reticulum Ca^{2+} sensor and store-operated Ca^{2+} channel, respectively (Feske *et al.* 2005; Roos *et al.* 2005; Prakriya *et al.* 2006). Deficiency in these molecules is known to be associated with a unique clinical phenotype that is characterized by defective dental enamel calcification, a defining feature of amelogenesis imperfecta type III (Feske 2011). In addition, knockout of these molecules impairs bone calcification and enamel formation, highlighting the importance of SOCE and Ca^{2+} signaling in tooth development (Picard *et al.* 2009; Robinson *et al.* 2012).

The dental cups, the basis of teeth, are formed

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from dental epithelial cells derived from the oral epithelium and mesenchymal cells derived from the neural crest, and tooth development proceeds through epithelial–mesenchymal interactions between these cells (Du *et al.* 2018). During this process, dental epithelial cells differentiate into ameloblasts, which form tooth enamel, and mesenchymal cells differentiate into odontoblasts and pulp stem cells, which form dentin and pulp (Arakaki *et al.* 2012). Ca^{2+} signaling may be involved in the regulation of cell migration, cell proliferation, and gene expression during such epithelial–mesenchymal interactions.

To visualize Ca^{2+} responses associated with such a slow cellular process over time, genetically encoded calcium indicators (GECIs), such as G-GECO, are particularly useful. Unlike chemically synthesized fluorescent Ca^{2+} indicators, GECIs do not leak out of cells or accumulate in organelles, meaning they are suitable for long-term imaging studies under cell culture conditions (Tanimura 2015). We previously reported vitamin D3-induced spontaneous Ca^{2+} responses of SF2 cells by performing long-term Ca^{2+} imaging of SF2 cells that expressed G-GECO using adenovirus vectors (Murata *et al.* 2016). Despite the high efficiency of gene transfer, adenovirus vectors are relatively cytotoxic and the delivered genes are transiently expressed (Russell 2000). Lentiviruses have relatively low cytotoxicity, and are used to integrate genetic material into the host genome to allow long term, stable expression of transgenes (Shai *et al.* 2002; Zheng *et al.* 2013). This property has been used for *in vivo* gene transfer and the establishment of cells with stable transgene expression (Shai *et al.* 2002; Cockrell and Kafri 2007; Zheng *et al.* 2018).

In this study, we utilized a lentiviral vector to create a SF2 cell line that stably expressed G-GECO as a tool to investigate the relationship between Ca^{2+} responses and development and differentiation, which occur over a long period of time. These cells exhibited spontaneous Ca^{2+} responses under cell culture conditions in the presence of serum, and ATP and fibroblast growth factor (FGF) were involved in these responses.

MATERIALS AND METHODS

Materials. The SF2 rat dental epithelial cell line was a gift from Dr. Satoshi Fukumoto (Department of Oral Health and Development Sciences, Tohoku University Graduate School of Dentistry). CMV-G-GECO1.1 (Addgene plasmid #32445) was a gift from Dr. Robert Campbell (University of Alberta,

Canada). ATP was purchased from Boehringer Mannheim (Mannheim, Germany). Gefitinib and FIIN-2 were obtained from Aobious Inc (Gloucester, MA, USA). Suramin was from Calbiochem (La Jolla, CA, USA). All other reagents were of analytical grade.

Construction of lentiviruses for G-GECO expression. Lentiviruses were constructed and prepared using the ViraPower Lentivital Expression System (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Briefly, the ccdB region of the pENTR-1A entry plasmid (Thermo Fisher Scientific) was removed by digestion with EcoRI, and the 2.3 kbp fragment was self-ligated (pENTR- Δ ccdB). Next, the endogenous NheI site of pENTR-1A was removed and a new NheI site was added upstream of the EcoRI site in the multiple cloning site of pENTR-1A using an AMAP Multi Site-directed Mutagenesis Kit (Amalgaam, Nagoya, Japan) and the following primers: 5'-ATA ACC CTA TTA CCG ATA CGCA TGG ATC TCG G-3' and 5'-GGA ACC AAT TCA GCT AGC GCT GTC GAC TGG ATC CGG-3' (the introduced NheI site is underlined), according to the manufacturer's instructions. The resulting mutated pENTR-1A was referred to as pENTR- Δ Nhe+Nhe.

The pG-GECO1.1 plasmid was digested with BamHI and EcoRI, and the resulting DNA fragment containing G-GECO was inserted into the BamHI/EcoRI sites of pENTR- Δ Nhe+Nhe. The resulting construct was referred to as pENTR-G-GECO. The G-GECO gene of the entry clone (pENTR-G-GECO) was transferred into pLenti6.3/V5-DEST (Thermo Fisher Scientific) by the LR recombination reaction to generate the pLenti-G-GECO vector. To produce recombinant lentiviruses, 293FT cells were co-transfected with pLenti-G-GECO and ViraPower Packaging Mix using Lipofectamine 2000. After 3 days of culture, the supernatant containing the lentivirus particles was collected and used in subsequent experiments. The titer of G-GECO lentiviruses (LV-G-GECO) was approximately 1×10^9 plaque-forming units (pfu)/mL.

Cell culture and transfection. SF2 cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture with Ham's F12 (DMEM/F12; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 . Cells were infected with LV-G-GECO at a concentration of 10^8 pfu/mL, incubated for 15–20 h, and then cultured in DMEM/F12. Selection was not performed because

Fig. 1 ATP- and ionomycin-induced changes in fluorescence in SF2-G-GECO cells. Fluorescence images before (A) and after addition of 100 μ M ATP (B) and 10 μ M ionomycin (C), and changes in the fluorescence intensity of G-GECO (D). The presence of ATP and ionomycin is indicated by the horizontal bars at the top of the panel D. Images in A–C were captured at the time points indicated by the corresponding letters in panel D. Scale bar, 10 μ m.

fluorescence was observed in almost all of these cells. Fluorescence was maintained in almost all cells even after ten passages.

Ca^{2+} imaging. G-GECO-expressing SF2 cells (SF2-G-GECO cells) were cultured in DMEM/F12 lacking phenol red (Wako Pure Chemical Industries, Osaka, Japan) in fibronectin-coated chambers consisting of plastic cylinders (7 mm in diameter) attached to cover glasses. These chambers were placed on a Nikon ECLIPSE Ti2-E inverted fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Nikon Fluor $\times 40$ oil immersion objective lens (NA = 1.30) under cell culture conditions (37°C, 5% CO_2) during imaging using a stage chamber and a stage-and-objective lens heating system (INU-NI-F1; Tokai Hit, Shizuoka, Japan). Fluorescence images of G-GECO were captured at 30 sec intervals using an excitation wavelength of 480 nm and an emission wavelength of 535 nm with an imaging system consisting of a DL-604M-#VP EM-CCD camera (Andor Technology, Belfast, UK) and NIS-Elements software (Nikon).

Data analyses. Time-dependent changes in G-GECO fluorescence were analyzed using NIS-Elements imaging software (Nikon). Changes in fluorescence intensity were visualized using a ratio image, which is an image from a time point (image T) divided by an image from the previous time point (image T-1) and overlaid with the fluorescent image using NIS-Elements imaging software (Nikon) and Aquacosmos imaging software (Hamamatsu Photonics, Shizuoka, Japan). Results are presented as the mean $\pm$ standard error of the mean (SEM) of least three independent experiments. Statistical analysis was performed using Bartlett's test for homogeneity of variance followed by Fisher's least significant difference post-hoc test for comparison of individual means.

RESULTS

Fig. 1 shows changes in fluorescence of SF2-G-GECO cells associated with ATP- and ionomycin-induced increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Stimulation with ATP, which increases $[Ca^{2+}]_i$ by activating P2Y receptors, increased the

Fig. 2 Spontaneous Ca^{2+} responses of SF2 cells at a low density. **(A)** Differential interference contrast image (a) and fluorescence ratio images (b–f) at the time points indicated in panel B. **(B)** Time-dependent changes in fluorescence ratios of single SF2-G-GECO cells. Traces 1–6 in panel B are the changes in fluorescence ratios of the cells indicated by the corresponding numbers in panel Aa. Scale bar, 10 μm . Fluorescence of SF2-G-GECO cells was monitored at 37°C under cell culture conditions. Ca^{2+} responses were mostly observed at cell protrusions (images Ac, Ae, and Af).

fluorescence intensity in most cells, and subsequent removal of ATP returned the fluorescence intensity to the resting level. A further large increase in fluorescence intensity was observed upon application of Ca^{2+} ionophore ionomycin. These results showed that most SF2-G-GECO cells generated in this study expressed functional G-GECO.

Ca^{2+} imaging analysis under culture conditions revealed that SF2-G-GECO cells exhibited spontaneous Ca^{2+} responses, including Ca^{2+} oscillations with repeated increases in $[\text{Ca}^{2+}]_i$ and single spike-like increases in $[\text{Ca}^{2+}]_i$. The spatial characteristics of these Ca^{2+} responses can be classified into three major patterns depending on the cell density. At a low density (<50 cells/frame), Ca^{2+} responses were observed at cell protrusions (Fig. 2). At a medium density (50–100 cells/frame), a cell-wide spread of Ca^{2+} responses was observed in addition to localized responses at cell protrusions (Fig. 3). At a high density (100 < cells/frame), Ca^{2+} responses were ob-

served in clusters of synchronized $[\text{Ca}^{2+}]_i$ elevations in about 3–20 cells (Fig. 4c and 4f). Concordant increases in $[\text{Ca}^{2+}]_i$ in distant cells were also observed (Fig. 4d). Most of the Ca^{2+} responses in these clusters showed a large increases in $[\text{Ca}^{2+}]_i$. These responses in cell clusters and distant cells were considered to be caused by the diffusion of one or more bioactive substances from the cells. It was also possible that intracellular Ca^{2+} propagation through gap junctions may be involved in the Ca^{2+} response in cell clusters.

To investigate the mechanism of the spontaneous Ca^{2+} responses, the frequency of the increases in $[\text{Ca}^{2+}]_i$ was quantitatively analyzed under medium density conditions. It is known that ATP functions as an extracellular messenger after its release from cells in response to mechanical stimulation or an increase in $[\text{Ca}^{2+}]_i$ (Nezu *et al.* 2010). To investigate the involvement of ATP in the generation of these spontaneous Ca^{2+} responses, we examined the ef-

Fig. 3 Spontaneous Ca^{2+} responses of SF2 cells at a medium density. **(A)** Differential interference contrast image (a) and fluorescence ratio images (b–f) at the time points indicated in panel B. **(B)** Time-dependent changes in fluorescence ratios of single SF2-G-GECO cells. Traces 1–8 in panel B are the changes in fluorescence ratios of the cells indicated by the corresponding numbers in panel Aa. Scale bar, 10 μm . Fluorescence of SF2-G-GECO cells was monitored at 37°C under cell culture conditions. Ca^{2+} responses were observed in whole cells (images Ac, Ae, and Af) and cell protrusions (image Ad).

fects of suramin, a P2Y receptor (P2YR) inhibitor (Wildman *et al.* 2003). In this experiment, we first examined Ca^{2+} responses under normal culture conditions (first measurement) and subsequently examined Ca^{2+} responses in the same area in the absence and presence of suramin (second measurement). Comparison of the first and second measurements demonstrated that the frequency of Ca^{2+} responses (responses/100 cells/h) in the second measurement without the inhibitor was 86.9% of that in the first measurement. The frequency of spontaneous Ca^{2+} responses in the second measurement was reduced to 47.8% and 52.5% of that in the first measurement upon application of 10 and 30 μM suramin, respectively (Fig. 5). This result suggests that P2YR is involved in the spontaneous Ca^{2+} responses of SF2 cells. A further increase in the suramin concentration to 30 μM did not result in more inhibition, indicating that suramin exerted its maximum inhibitory

effect at 10 μM .

To further clarify the involvement of extracellular ATP in the spontaneous Ca^{2+} responses, we analyzed the effects of the ATP-degrading enzyme apyrase. Addition of apyrase (5 units/mL) reduced the frequency of the spontaneous Ca^{2+} responses to 27.9%. Addition of 1 μM ATP induced sustained increases in $[\text{Ca}^{2+}]_i$ or persistent Ca^{2+} oscillations in many SF2-G-GECO cells under cell culture conditions. These results indicate that extracellular ATP is involved in the spontaneous Ca^{2+} oscillations of SF2 cells. Consistent with the effect of suramin, apyrase also failed to completely inhibit Ca^{2+} responses, supporting the involvement of factors other than ATP.

It is known that serum contains a variety of growth factors, including epidermal growth factor (EGF) and FGF. We therefore examined the effects of the EGF receptor inhibitor Gefitinib (Wakeling *et al.* 2002) and the FGF receptor inhibitor FIIN-2 (Tan

Fig. 4 Spontaneous Ca^{2+} responses of SF2 cells at a high density. **(A)** Differential interference contrast image (a) and fluorescence ratio images (b–f) at the time points indicated in panel B. **(B)** Time-dependent changes in fluorescence ratios of single SF2-G-GECO cells. Traces 1–6 in panel B are the changes in fluorescence ratios of the cells indicated by the corresponding numbers in panel Aa. Scale bar, 10 μm . Fluorescence of SF2-G-GECO cells was monitored at 37°C under cell culture conditions. Ca^{2+} responses were observed in cell populations (images Ac and Af) and distant cells (image Ad).

et al. 2014) on the spontaneous Ca^{2+} responses (Fig. 5). Gefitinib did not elicit an inhibitory effect at 1 μM , but reduced the frequency of spontaneous Ca^{2+} responses to 16.6% at 10 μM . In addition, FIIN-2 (1 μM) reduced the frequency of spontaneous Ca^{2+} responses to 43.0%.

DISCUSSION

In the present study, we generated SF2 cells stably expressing G-GECO (SF2-G-GECO cells) as a tool to analyze the function of Ca^{2+} signaling during tooth development. $[\text{Ca}^{2+}]_i$ elevation upon application of ATP and ionomycin increased fluorescence in almost all cells. These cells expressed G-GECO at a sufficient intensity and exhibited changes in fluorescence and therefore Ca^{2+} responses could be analyzed in the presence of serum. Serum is necessary for many cellular responses associated with development, including cell proliferation, gene expression, and mi-

gration, but its weak fluorescence can interfere with the measurement cellular fluorescence. However, the fluorescence of SF2-G-GECO cells was sufficient to allow the analysis of Ca^{2+} responses even in the presence of serum. Thus, it was possible to detect changes in the fluorescence of SF2-G-GECO cells, and to measure Ca^{2+} responses in the presence of serum.

Observations of SF2-G-GECO cells under culture conditions revealed spontaneous increases in $[\text{Ca}^{2+}]_i$. The frequency of spontaneous Ca^{2+} responses in SF2-G-GECO cells varied greatly depending on the cell density. The frequency of spontaneous Ca^{2+} oscillations differed greatly depending on the area of observation and therefore we investigated the effects of inhibitors and other agents by sequentially monitoring Ca^{2+} responses in the same areas. The inhibitory effects of the P2YR inhibitor suramin and the ATP-degrading enzyme apyrase on the spontaneous Ca^{2+} responses suggest that extracellular ATP is in-

Fig. 5 Effects of inhibitors on spontaneous Ca^{2+} responses of SF2-G-GECO cells. Ca^{2+} responses were observed under cell culture conditions (first response) and subsequently in the presence or absence of inhibitors (second response). The frequencies of second Ca^{2+} responses relative to first Ca^{2+} responses are shown as percentages. The presence of suramin (10 and 30 μM), apyrase (5 units/mL), gefitinib (1 and 10 μM), and FIIN-2 (1 μM) and the absence of inhibitors (control) at the second observations are indicated. Data are presented as mean $\pm$ SEM ($n = 3-25$). Asterisks indicate significant differences from the control (* $P < 0.05$).

involved in the generation of these responses through P2YR. It is thought that apyrase reduced these responses by rapidly hydrolyzing the released ATP. The spontaneous Ca^{2+} responses of SF2-G-GECO cells were single spike-like responses with an irregular frequency. On the other hand, these cells showed sustained increases in $[\text{Ca}^{2+}]_i$ and sustained Ca^{2+} oscillations upon addition of extracellular ATP. Therefore, the localized and transient increase in $[\text{Ca}^{2+}]_i$ in the spontaneous responses are likely due to the hydrolysis and diffusion of ATP from SF2 cells.

The spontaneous Ca^{2+} responses were not completely attenuated by suramin or apyrase, suggesting that factors other than ATP are also involved. We speculate that serum growth factors such as EGF and FGF might be involved in these ATP-independent responses. The inhibitory effect of 1 μM FIIN-2, an FGF receptor inhibitor, suggests that FGF is involved in the spontaneous Ca^{2+} responses. Gefitinib had an inhibitory effect at 10 μM , but not at 1 μM . Gefitinib specifically inhibits the tyrosine kinase activity of EGF receptor at 1 μM , but acts non-specifically at concentrations higher than 10 μM , suggesting that receptor tyrosine kinases other than the

EGF receptor are involved in the spontaneous Ca^{2+} responses (Wakeling *et al.* 2002). The present study indicates that ATP, FGF, and unidentified serum factors are involved in spontaneous Ca^{2+} responses in SF2-G-GECO cells. Serum contains growth factors that activate receptor tyrosine kinases (Joh *et al.* 1986; Wang *et al.* 2016), which promote IP_3 production, and these are likely to be involved in the Ca^{2+} response.

The spontaneous Ca^{2+} responses of SF2 cells are likely involved in regulation of cell migration and gene expression. SF2-G-GECO cells are therefore expected to be a useful tool for direct analysis of the mechanisms that regulate these processes. Fukumoto *et al.* reported that co-culture of SF2 cells and dental pulp stem cells (DPSCs) leads to the differentiation of ameloblasts from SF2 cells and pulp cells from DPSCs (Eun-Jung *et al.* 2019). We also observed Ca^{2+} -dependent changes in gene expression of transforming growth factor β and Notch3 in these co-cultures (data not shown). We expect that the roles of Ca^{2+} signaling in the regulation of gene expression in SF2 cells and DPSCs can be explored by co-culture experiments using SF2-G-GECO cells.

Many different types of GECIs have been devel-

oped, and most comprise the Ca^{2+} -binding protein calmodulin (CaM), the CaM-binding domain of myosin light chain kinase (M13), and fluorescent proteins (FPs) (Miyawaki *et al.* 1997; Tanimura 2015). Binding of Ca^{2+} to these chimeric proteins causes the M13 and CaM domains to interact and leads to a change in the fluorescence ratio or intensity (Tanimura 2015). The ratiometric response is independent of the indicator concentration, which is an important advantage of FRET-based indicators (Nagai *et al.* 2004; Horikawa *et al.* 2010). On the other hand, single FP-based indicators are advantageous for multi-color imaging in combination with other fluorescent indicators because they only require measurement of a single wavelength (Zhao *et al.* 2011; Chen *et al.* 2013).

In addition, the difference in affinity for Ca^{2+} is important for selection of GECIs. The high-affinity ratiometric GECI YC-Nano50, which has a K_d value of ~ 50 nM for Ca^{2+} (Horikawa *et al.* 2010), has been used to calculate $[\text{Ca}^{2+}]_i$ at resting state (Nezu *et al.* 2019). By contrast, the K_d value of G-GECO1.1 for Ca^{2+} is relatively high at about 620 nM (Zhao *et al.* 2011), suggesting that the spontaneous Ca^{2+} responses of SF2 cells represent a relatively large increase in $[\text{Ca}^{2+}]_i$. In addition, G-GECO, a single FP-based indicator, facilitates simultaneous imaging with other fluorescent sensors, such as red FP-based GECIs R-GECO (Zhao *et al.* 2011). SF2-G-GECO cells are expected to be particularly useful when used in combination with DPSCs expressing R-GECO.

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