Difference in Calcium Mobilization between Two Strains of Mice That Have Different Abilities to Secrete Saliva

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Abstract

In salivary glands, fluid and ion secretion is mainly regulated by intracellular Ca2+. Muscarinic stimulation evokes rapid increase and sustained elevation of intracellular Ca2+, which are due to release of Ca2+ from intracellular pool and store-operated Ca2+ entry (SOCE), respectively. Two mouse strains, C57BL/6 (B6) and C3H, have been reported to have different sensitivities to caries. C3H, which has a lower sensitivity to caries, have a higher ability to secrete saliva compared with B6. In this study, we compared Ca2+ mobilization in submandibular gland cells of the two strains. When cells were stimulated by 1 μM carbachol, there is no significant difference between the peaks of Ca2+ response in the two strains. However, the sustained phase of Ca2+ elevation was higher in C3H than in B6. Following stimulation with 10 μM carbachol results in higher peak and sustained phase in C3H compared to in B6. Ca2+ elevation induced by the inhibitor of endoplasmic reticular Ca2+ pump thapsigargin in Ca2+-free medium was higher in C3H than in B6. In contrast, there was no difference in SOCE observed by addition of extracellular Ca2+ after depletion of Ca2+ by thapsigargin between the two strains. In addition, expression levels of genes that are involved in SOCE were not different between the two strains. These results suggest that the different response of Ca2+ elevation is due to the difference in capacity of intracellular Ca2+ pool.

Keywords:
salivary glands, Ca2+ response, Ca2+ pool

Introduction

Saliva is essential for maintenance of oral hygiene and hyposecretion of saliva causes severe caries, periodontitis and mucosal infections (1). Two mouse strains, C57BL/6 (B6) and C3H, have been reported to have different sensitivities to caries. Oral inoculation of Streptococcus mutans resulted in caries with a higher rate in B6 than in C3H (2). C3H, which has a lower sensitivity to caries, have a higher ability to secrete saliva compared with B6 (3). Although saliva flow rate may affect caries susceptibility, the reason that causes the difference in saliva flow rate has not been resolved.

In salivary glands, fluid and ion secretion is regulated by parasympathetic activation. Muscarinic agonists induce synthesis of inositol triphosphate (IP3) and consequent activation of IP3 receptors (IP3R) located in the endoplasmic reticulum (ER). Release of Ca2+ from the ER via IP3R is essential for fluid secretion of salivary glands (4). Elevation of intracellular Ca2+ concentration ([Ca2+]i) activates Ca2+-dependent Cl− channels located in the luminal membrane, which causes efflux of Cl− to the lumen (5-7). Electrochemical gradient produced by Cl− induces transport of Na+ from blood plasma to the lumen through tight junctions. The resulting osmotic gradient for NaCl evokes transepithelial movement of water (8).

In addition of release of Ca2+ from the ER, depletion of ER-stored Ca2+ leads to activation of plasma membrane Ca2+ channels that mediate influx of Ca2+ from the extracellular fluid into cells, which is called store-operated Ca2+ entry (SOCE) (9). The magnitude of SOCE is also one of the factors that determine the saliva flow rate (10). As the Ca2+...
sensor of the intracellular compartments and plasma membrane Ca$^{2+}$ channel, stromal interaction molecule (STIM) and Orai were identified, respectively (11-13). It is widely accepted that both Ca$^{2+}$ release from the ER and SOCE in salivary acinar cells regulate saliva fluid secretion.

In this study, in order to find factors that determine the saliva flow rate, we compared Ca$^{2+}$ mobilization and gene expression involved in SOCE in submandibular gland cells of the two strains.

**Materials and Methods**

**Preparation of submandibular gland cells**

All animal experiments were carried out in accordance with institutional and national guidelines for the care and use of experimental animals and were approved by the Experimental Animal Committee of the Nihon University School of Dentistry at Matsudo (AP16MD020). Submandibular glands were removed from male C57BL/6 and C3H mice under anesthesia with a combination of 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. The cells were isolated from submandibular glands as reported previously (14). Briefly, finely minced glands were digested with collagenase and hyaluronidase in Hanks’ balanced salt solution containing 20 mM HEPES-NaOH, pH 7.4 and 0.5% bovine serum albumin at 36°C for 30 min.

**Measurement of intracellular Ca$^{2+}$ mobilization**

Isolated submandibular gland cells were loaded with 2 μM fura-2 AM (Thermo Fisher) in loading buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2.5 mM CaCl$_2$, 10 mM glucose, 1.25 mM probenecid). When thapsigargin was used, the cells were washed and suspended with Ca$^{2+}$-free buffer. The fluorescence of the fura-2-loaded cells was measured by using CAF-110 (Jasco, Tokyo, Japan) with excitation at 340 and 380 nm, and emission at 510 nm. Elevation of the intracellular Ca$^{2+}$ concentration was evaluated as the increase in the ratio of fluorescence intensities at 340 nm and 380 nm (F340/F380). The increase in F340/F380 before and at times indicated in the figure legends after stimulation was shown as ΔF340/F380, which reflects increase in intracellular Ca$^{2+}$ concentration.

**RNA preparation and real time RT-PCR analysis of mRNA expression**

Total RNA was isolated from the submandibular gland cells using the Qiazol reagent and RNeasy Mini kits (Qiagen, Hilden, Germany). Amounts of RNA were quantified by measuring the absorbance at 260 nm. Expression levels were determined with One Step TB Green PrimeScript PLUS kit (Takara Bio, Kusatsu, Japan). *Gadph* was used as a reference. Primer pairs for amplification of STIM1, STIM2 and Orai1 were as follows: *Stim1*: forward 5’- CAG TAG TGT ACA ACT GGA GTG TTG TG -3’, reverse 5’- GGT TAC TGC TAG CCT TGG CAT G -3’ with a predicted size of 130 bp; *Stim2*: forward 5’- ATG CAG AGC AGG AAC TGG AG -3’, reverse 5’- CGT GTG TTA GCT GAA GCC ATT TC -3’ with a predicted size of 119 bp; *Orai1*: forward 5’- GCT AAG GAG TAA GGA GTT TGA G -3’, reverse 5’- GAA TGG GGA CAG TTT CTC TC -3’ with a predicted size of 96 bp. PCR products were evaluated by melting curve analysis according to the manufacturer’s instructions and by examining the sizes of the PCR products separated on 2.0% agarose gels. Relative RNA equivalents for each sample were obtained by normalizing to *Gadph* levels. Each sample was run in duplicate to determine the sample reproducibility, and the average relative RNA equivalents per sample pair were used for further analysis.

**Statistical analyses**

All values are reported as means ± SEM. Statistical analyses of the differences in the means of experimental pairs were evaluated by an unpaired t-test. The P values obtained are indicated in the figure legends when statistically significant.

**Results**

**Comparison of Ca$^{2+}$ response induced by carbacol**

Muscarinic stimulation increases the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), which is essential for fluid secretion of saliva. We examined Ca$^{2+}$ response in submandibular gland cells prepared from the two strains of mice. The values of basal F340/F380 ratio of B6 and C3H before stimulation were 0.745 ± 0.027 and 0.788 ± 0.021, respectively. When cells were stimulated by 1 μM carbacol (CCh), there is no significant difference between the peaks of Ca$^{2+}$ elevation in the two strains (Fig. 1A). However, the sustained phase at 2 min after addition of CCh was higher in C3H than in B6 although there is no significant difference (Fig. 1B). ΔF340/F380 in the sustained phase in C3H was kept 71.4% ± 8.3 of the peak while that of B6 was only 53.4% ± 9.3. In addition, the peak response and sustained
Fig. 1. Comparison of Ca\textsuperscript{2+} responses in submandibular gland cells of C3H and B6. A. Time course of Ca\textsuperscript{2+} response to 1 and 10 \textmu{}M CCh. The values are shown as the ratio of the fluorescence intensities at 510 nm following excitation at 340 nm and 380 nm (F340/F380). B. Comparison of the increase in F340/F380 (\Delta F340/F380) at sustained phase after stimulation by 1 \textmu{}M CCh between the two strains. The values (\Delta F340/F380) are shown as the difference from the basal level before stimulation and at 2 min after stimulation by 1 \textmu{}M CCh. Although there is no significant difference between the two strains, \Delta F340/F380 of the sustained phase in C3H tends to be higher than in B6 (n=8). C. Comparison of \Delta F340/F380 between the basal level and the peak response to 10 \textmu{}M CCh between the two strains. The peak response was significant higher in C3H than in B6 (*P<0.05; unpaired t-test, n=8).
phase induced by additive stimulation with 10 µM CCh were also higher in C3H than in B6 (Fig. 1A and C).

**Expression levels of genes involved in store-operated calcium entry**

Sustained phase of Ca\(^{2+}\) elevation induced by muscarinic stimulation is affected by SOCE. Because the sustained phase of Ca\(^{2+}\) level induced by 1 µM CCh in C3H tends to be higher than in B6, expression levels of genes that are involved in SOCE such as STIM1, STIM2 and Orai1 in submandibular gland cells were quantified. Although the expression of all the three genes was detected in submandibular gland cells of the two strains, there is no significant difference in expression levels between the two strains (Fig. 2).

**Comparison of Ca\(^{2+}\) elevation induced by thapsigargin**

We used thapsigargin, an inhibitor of ER Ca\(^{2+}\)-ATPase, to examine the capacity of intracellular Ca\(^{2+}\) pool and SOCE. When thapsigargin was added to the cells suspended in Ca\(^{2+}\)-free extracellular buffer, Ca\(^{2+}\) leaking out from the intracellular pools was no longer recovered, so [Ca\(^{2+}\)]\(_i\) gradually increased (Fig. 3A and B). We found that the increase in [Ca\(^{2+}\)]\(_i\) induced by thapsigargin was larger in C3H than in B6 (Fig. 3B and C). Addition of extracellular Ca\(^{2+}\) after depletion of intracellular pool induced rapid and large increase in [Ca\(^{2+}\)]\(_i\), which corresponds to SOCE. The magnitude of SOCE was not different between the two strains (Fig. 3A).

**Discussion**

In salivary glands, primary saliva is produced as isotonic fluid in acini. The primary saliva is then modified by removal and addition of ions during passing in the ducts without absorption and secretion of water. Therefore, the amount of the primary saliva produced in acini determines saliva flow rate. Increase of [Ca\(^{2+}\)]\(_i\) in acinar cells is the primary trigger for salivary fluid secretion. Ion transport initiated by increase of [Ca\(^{2+}\)]\(_i\) makes transepithelial salt gradients, which evokes water movement from blood to the acinar lumen (8). Therefore, understanding the molecular machinery to regulate [Ca\(^{2+}\)]\(_i\) is necessary to clarify the mechanism for regulation of saliva flow rate.

Muscarinic stimulation causes rapid increase and sustained elevation of [Ca\(^{2+}\)]\(_i\). The two phases of Ca\(^{2+}\) mobilization are considered to depend on release from intracellular pool and influx from extracellular space, respectively. In this study, there was no difference in the peak response upon stimulation with 1 µM CCh between the two strains. This process is dependent on activation of muscarinic receptors and IP\(_3Rs\), which may be comparable in both strains. Previous study showed that expression levels of muscarinic receptors in the two strains were comparable (3), which also supports this inference. In contrast, the sustained phase of Ca\(^{2+}\) mobilization tends to be higher in C3H than in B6. Moreover, the peak response to additional stimulation with 10 µM CCh rose higher than the first response with 1 µM CCh in C3H, while response to 10 µM CCh was lower than that of 1 µM in B6.

We also measured the effect of thapsigargin on the change of [Ca\(^{2+}\)]\(_i\). Thapsigargin inhibits the ER Ca\(^{2+}\) pump that plays a role in recovery of Ca\(^{2+}\) from cytosol to the ER Ca\(^{2+}\) pool (15). Addition of thapsigargin causes leak of Ca\(^{2+}\) from the ER and consequently increases [Ca\(^{2+}\)]\(_i\). In this study, the elevation of [Ca\(^{2+}\)]\(_i\) induced by thapsigargin was higher in C3H than in B6. Combined with the results of response to CCh, it is likely that the capacity of intracellular Ca\(^{2+}\) pool was larger in C3H than in B6. Strong and long stimulation may lead to depletion of the Ca\(^{2+}\) pool and resulting smaller response in B6. There is a possibility that the difference in capacity of Ca\(^{2+}\) pool is one of the reasons of the difference in Ca\(^{2+}\) response between the two strains.
We observed SOCE caused by addition of extracellular Ca\textsuperscript{2+} after depletion of intracellular pool by thapsigargin. There was no significant difference in rates and amounts of Ca\textsuperscript{2+} influx between the two strains. In addition, the expression levels of STIMs and Orai1 mRNAs were not significantly different between the two strains. These results suggest that factors other than SOCE is the cause of the difference in the sustained phase of Ca\textsuperscript{2+} mobilization.
In conclusion, there is a difference in Ca\(^{2+}\) mobilization between the two strains that have a different ability to secrete saliva. To clarify the molecular mechanism that makes the difference may be useful to understand the cause of hyposalivation in xerostomia patients.

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