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Increase in muscarinic stimulation-induced Ca^{2+} response by adenovirus-mediated Stim1-mKO1 gene transfer to rat submandibular acinar cells *in vivo*

Takao Morita *, Akihiro Nezu, Yosuke Tojyo, Akihiko Tanimura

Department of Pharmacology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

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ABSTRACT

Adenoviruses have been used for gene transfer to salivary gland cells *in vivo*. Their use to study the function of salivary acinar cells was limited by a severe inflammatory response and by the destruction of fluid-secreting acinar cells. In the present study, low doses of adenovirus were administered to express Stim1-mKO1 by retrograde ductal injection to submandibular glands. The approach succeeded in increasing muscarinic stimulation-induced Ca^{2+} responses in acinar cells without inflammation or decreased salivary secretions. This increased Ca^{2+} response was notable upon weak muscarinic stimulation and was attributed to increased Ca^{2+} release from internal stores and increased Ca^{2+} entry. The basal Ca^{2+} level was higher in Stim1-mKO1-expressing cells than in mKO1-expressing and non-expressing cells. Exposure of permeabilized submandibular acinar cells, where Ca^{2+} concentration was fixed at 50 nM, to inositol 1,4,5-trisphosphate (IP_3) produced similar effects on the release of Ca^{2+} from stores in Stim1-mKO1-expressing and non-expressing cells. The low toxicity and relative specificity to acinar cells of the mild gene transfer method described herein are particularly useful for studying the molecular functions of salivary acinar cells *in vivo*, and may be applied to increase salivary secretions in experimental animals and human in future.

1. Introduction

The salivary gland, an exocrine organ that secretes fluid, electrolytes, and proteins into the oral cavity, consists of specialized epithelial cells organized into two major domains, acini and ducts. Acinar cells produce the primary saliva, an isotonic plasma-like fluid rich in Na^+ and Cl^- ions. This fluid secretion is regulated primarily by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [1–3]. Ductal cells subsequently re-absorb Na^+ and Cl^- ions from the primary saliva and excrete K^+ and HCO_3^- ions, which results in the production of a hypotonic final saliva [4,5]. The final saliva is secreted into the oral cavity through ductal orifices that open directly into the oral cavity. The duct orifice of each major salivary gland (parotid, submandibular, and sublingual glands), which is directly contiguous with virtually every epithelial cell in each gland, is readily visible in the mouth; this feature makes possible the noninvasive administration of drugs, such as steroids, to salivary glands. In addition, retrograde ductal injection of viral vectors to salivary glands has been used for *in vivo* gene transfer to study and/or modulate the functions of salivary glands [6–8].

Two types of viral vectors, adenovirus and adeno-associated virus (AAV), have been used for the gene transfer of aquaporin-1, growth hormone, and erythropoietin to salivary glands [7,9–12]. Adenoviral vectors are the most effective means of transferring genes to salivary glands, although severe inflammation and destruction of fluid-secreting acinar cells were reported [13]. Although the use of AAV vectors leads to long-term transgene expression and low immunogenicity, gene expression is primarily limited to ductal cells [9,10,14]. Therefore, these methods have not been used to study functional salivary acinar cells.

Recently, we succeeded in expressing orange fluorescent protein (mKO1)-tagged Stim1 (Stim1-mKO1) in rat submandibular acinar cells without inflammation by retrograde adenovirus injection [15]. This method is particularly useful to study the functions of particular molecules and/or to modulate or enhance the functions of salivary acinar cells. Stim1 is an ER-resident protein and is essential for the activation of store-operated Ca^{2+} entry (SOCE) [16–18]. We previously reported that the exogenously expressed Stim1-mKO1 was functional and enhanced SOCE [15].

In the present study, overexpression of Stim1-mKO1 in submandibular acinar cells increased Ca^{2+} release from internal stores and increased Ca^{2+} entry. This increased Ca^{2+} release was most notable upon weak muscarinic stimulation. Based on these results,

* Corresponding author. Fax: +81 133 23 1399.
E-mail address: moritat@hoku-iryo-u.ac.jp (T. Morita).

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we discuss the future application of gene transfer to salivary acinar cells for increasing salivary fluid secretions.

2. Materials and methods

2.1. Materials and media

Fura-2/acetoxymethyl ester (Fura-2/AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Mag-fura-2/AM was purchased from Molecular Probes (Eugene, OR, United States). Trypsin, collagenase, and carbachol (CCh) were purchased from Sigma (St Louis, MO, United States). Inositol 1,4,5-trisphosphate (IP_3) was purchased from Alexis (San Diego, CA, United States).

Modified Hanks' balanced salt solution buffered with Hepes (HBSS–H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 4.2 mM $NaHCO_3$, and 20 mM Hepes–NaOH (pH 7.4). Nominally Ca^{2+}-free HBSS–H was identical in composition to HBSS–H, except for the omission of $CaCl_2$. PO_4^{3-}-, SO_4^{2-}-, CO_3^{2-}-free HBSS–H medium contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgCl_2$, 5.6 mM glucose, and 20 mM Hepes–NaOH (pH 7.4).

Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM Hepes (pH 7.3 with KOH), 330 μM $CaCl_2$, and 1 mM O,O′-bis(2-aminoethyl)ethyleneglycol-N,N,N′-N′-tetraacetic acid (free Ca^{2+} concentration was 50 nM).

2.2. Cell culture

HEK293A cells were cultured in Dulbecco's Modified Eagle's Medium with high glucose (Sigma) supplemented with 10% fetal bovine serum, 0.1 mM MEM non-essential amino acids (Gibco BRL, Rockville, MD, United States), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL).

2.3. Retrograde ductal injection of adenovirus

Construction of the Ad-mKO1 and Ad-Stim1-mKO1 adenoviruses was performed as described previously [15]. Male Wistar ST rats (12–18 weeks) were anesthetized with pentobarbital sodium (45 mg/kg body weight, i.p.; Somnopentyl, Kyoritsu Seiyaku Corporation, Tokyo, Japan). A fine polyethylene tube (Intramedic PE-10, BD Diagnostic Systems, Sparks, MD, outer diameter = 0.61 mm) was inserted intraorally in the orifice of one side of the submandibular duct. A total of $0.5–1 \times 10^8$ viral particles (in 50–100 μl culture medium) of either Ad-mKO1 or Ad-Stim1-mKO1 were injected retrogradely through the cannula into the submandibular gland, as described previously [15]. Leakage was prevented by sealing the tube with quick-drying glue, and the tube was left in the ducts until the rats emerged from anesthesia (3–4 h). Experimental procedures performed on animals were approved by the Animal Care and Use Committee of the Health Sciences University of Hokkaido and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Health Sciences University of Hokkaido.

2.4. Preparation of submandibular acinar cells

Submandibular acinar cells were prepared using trypsin and collagenase, as described previously [19], with modifications. Briefly, the submandibular gland on the side injected was minced and incubated in HBSS–H containing trypsin (0.5 mg/ml) for 5 min at 37 °C. The minced tissues were then incubated in HBSS–H containing collagenase (0.33 mg/ml) for 6 min at 37 °C. The dispersed acinar cells were filtered through a nylon mesh, washed twice with HBSS–H, and suspended in HBSS–H containing 0.1% bovine serum albumin (BSA).

2.5. $[Ca^{2+}]_i$ and $[Ca^{2+}]_L$ imaging

To monitor $[Ca^{2+}]_i$, dispersed submandibular cells were incubated for 30 min at room temperature with 2 μM fura-2/AM in HBSS–H containing 0.1% BSA. Fura-2-loaded dispersed submandibular cells were washed with HBSS–H and allowed to attach to glass coverslips pre-coated with Cell-Tak (BD Biosciences, Bedford, MA, United States) and placed at the bottom of a small recording chamber just prior to the experiments. Fura-2-loaded cells were washed with HBSS–H and allowed to rest for at least 30 min prior to Ca^{2+} measurements. Ca^{2+} responses were monitored in PO_4^{3-}-, SO_4^{2-}-, CO_3^{2-}-free HBSS–H at room temperature.

For luminal Ca^{2+} concentration ($[Ca^{2+}]_L$) monitoring, cells were loaded with mag-fura-2 and permeabilized, essentially as described previously [20]. Briefly, cells were incubated with 8 μM mag-fura-2/AM in HBSS–H containing 0.2% BSA for 45 min at 37 °C in the dark. The mag-fura-2-loaded cells were washed with BSA-free HBSS–H and allowed to attach to Cell-Tak-coated recording chambers. The attached cells were washed with ICM, followed by exposure to ICM containing 100 μg/ml (w/v) saponin (ICN, Cleveland, OH, United States) for 1–3 min. The permeabilized cells were washed with ICM and then incubated in ICM containing 3 mM ATP and 1.4 mM $MgCl_2$ for at least 5 min.

To monitor fura-2 or mag-fura-2 fluorescence, cells were alternately excited at 345 and 380 nm, and emission signals were recorded at 500–530 nm using the Argus–HiSCA imaging system (Hamamatsu Photonics, Shizuoka, Japan) attached to an inverted fluorescence microscope equipped with a Nikon Fluor 40× objective (for fura-2) or with a Nikon UV-Fluor 100× objective (for mag-fura-2). To identify mKO1- and Stim1-mKO1-expressing cells, mKO1 fluorescence images were captured at an excitation wavelength of 540 nm and emission wavelength >605 nm. All experiments were performed at room temperature.

2.6. Statistical analysis

Results are presented as means ± SEM and represent data collected from all cells examined in at least three independent experiments. Statistical significance was assessed using Student's *t*-test. $P < 0.05$ (*) and $P < 0.01$ (**) were considered significant.

3. Results

Fig. 1A shows typical Ca^{2+} responses in Stim1-mKO1-expressing and non-expressing submandibular acinar cells upon weak muscarinic stimulation with 1 μM carbachol (CCh) in Ca^{2+}-free medium and subsequent addition of extracellular Ca^{2+}. In this experimental condition, expression of Stim1-mKO1 was found in ~20% of submandibular acinar cells, and 65% of Stim1-mKO1-expressing cells showed a transient increase in Ca^{2+} with 1 μM CCh in the absence of extracellular Ca^{2+}. This weak CCh stimulation induced a very small increase in Ca^{2+} in only 30% of non-expressing cells. Subsequent addition of extracellular Ca^{2+} induced a sustained Ca^{2+} elevation in 86% of Stim1-mKO1-expressing cells and 46% of non-expressing cells.

Quantitative analysis indicated that CCh-induced (1 μM) Ca^{2+} release and subsequent Ca^{2+} entry were significantly larger in Stim1-mKO1-expressing cells than in mKO1-expressing and non-expressing cells. This result suggests that the overexpression of Stim1 increased the Ca^{2+} release from intracellular Ca^{2+} stores and the Ca^{2+} entry through SOCE. The basal Ca^{2+} level was higher

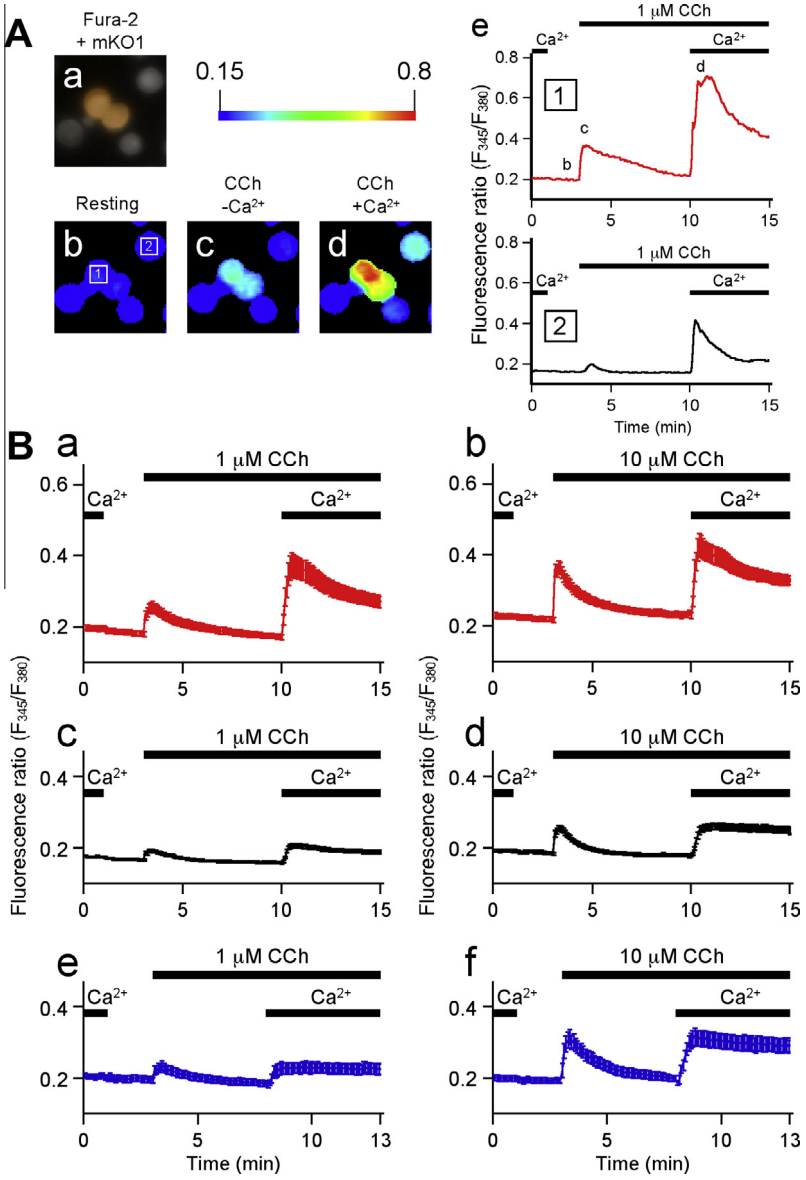

Fig. 1. CCh-induced Ca^{2+} responses in Ad-Stim1-mKO1-infected and Ad-mKO1-infected rat submandibular acinar cells. Dispersed submandibular acinar cells were exposed to 1 or 10 μM CCh in Ca^{2+}-free medium, after which 1.3 mM Ca^{2+} was added. (A) (a) Merged fluorescence image of Stim1-mKO1 and fura-2; (b–d) pseudocolor images of the fluorescence ratio of fura-2 obtained at the time points indicated in (e); (e) time-dependent changes in the fluorescence ratio of fura-2 in Stim1-mKO1-expressing cell (cell 1) and non-expressing cell (cell 2) depicted in the panel (b). (B) Effects of 1 μM (a, c, e) or 10 μM (b, d, f) CCh and subsequent Ca^{2+} addition to submandibular acinar cells. Traces shown are the means ± SEM of the fluorescence ratios of fura-2 in Stim1-mKO1-positive (a, b; red), Stim1-mKO1-negative (c, d; black), and mKO1-positive (e, f; blue) cells. Horizontal bars indicate the presence of 1 or 10 μM CCh and 1.3 mM Ca^{2+}.

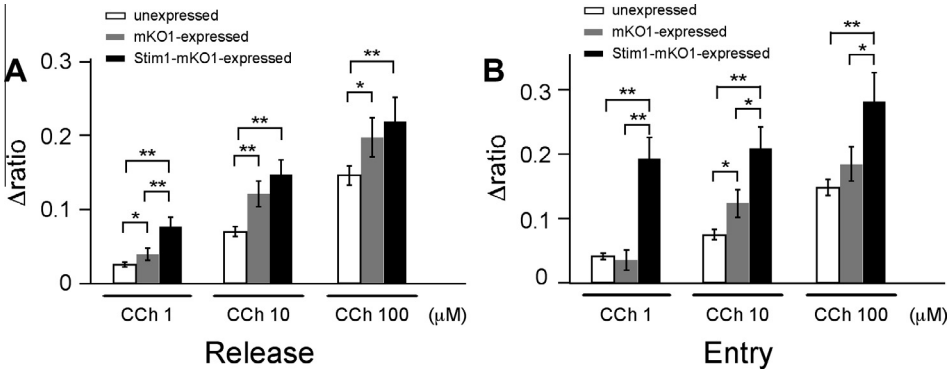

Fig. 2. CCh-induced Ca^{2+} release and entry in Ad-Stim1-mKO1-infected and Ad-mKO1-infected rat submandibular acinar cells. CCh-induced Ca^{2+} release and entry in Stim1-mKO1-positive (black column), Stim1-mKO1-negative (open column), and mKO1-positive (gray column) cells are summarized. Columns show the maximal increases in fluorescence ratios of fura-2 with CCh-induced Ca^{2+} release in Ca^{2+}-free medium and subsequent addition of Ca^{2+}. Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

in Stim1-mKO1-expressing cells than in mKO1-expressing and non-expressing cells.

Increased Ca^{2+} release and Ca^{2+} entry in Stim1-mKO1-expressing cells was also observed after stimulation with 10 μM CCh (Fig. 1B), though the responses were less obvious than those elicited by 1 μM CCh. Ionomycin-induced Ca^{2+} release from Ca^{2+} stores and Ca^{2+} entry were not altered by the expression of Stim1-mKO1 (data not shown).

Fig. 2 summarizes the effect of 1, 10, and 100 μM CCh on Ca^{2+} release and Ca^{2+} entry in Stim1-mKO1-expressing, mKO1-expressing, and non-expressing cells. These results indicate that the overexpression of Stim1-mKO1 increased Ca^{2+} entry after muscarinic stimulation at all CCh concentrations tested. In addition, Stim1-mKO1 increased Ca^{2+} release with weak (1 μM CCh) muscarinic stimulation.

The increase in Ca^{2+} release might be attributed to an increase in the sensitivity of intracellular Ca^{2+} stores. Thus, inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release was tested directly using permeabilized submandibular acinar cells with the low affinity Ca^{2+} indicator mag-fura-2. Fig. 3A shows the typical changes observed in mag-fura-2 ratio with a stepwise increase in IP_3 concentration in Stim1-mKO1-expressing and non-expressing cells; the concentration–response relationship of IP_3 and Ca^{2+} release showed no detectable difference between the two cell types (Fig. 3B).

4. Discussion

In the present study, a Stim1-mKO1 fluorescent fusion protein was expressed in the salivary glands of live animals by retrograde ductal injection of an adenoviral vector. Overexpression of Stim1-mKO1 increased the Ca^{2+} response to weak muscarinic stimulations in submandibular acinar cells.

Stim1 is an ER-resident protein, and it plays an essential role in SOCE [16–18]. Accordingly, increased Ca^{2+} entry was observed in Stim1-mKO1-expressing cells at all CCh concentrations tested. In addition to increased Ca^{2+} entry, results demonstrated increased Ca^{2+} release from intracellular stores in Stim1-mKO1-expressing cells compared to control cells. This increased Ca^{2+} release was notable upon weak muscarinic stimulation with 1 μM CCh, but not with higher concentrations of CCh. It is expected that this increased Ca^{2+} release led to a profound decrease in stored Ca^{2+} and caused a further increase in SOCE with weak muscarinic stimulation. This increased Ca^{2+} release upon weak muscarinic stimulation was found to correspond to an increase in the number of responding cells and in the magnitude of the Ca^{2+} response, suggesting enhanced sensitivity of muscarinic receptor-mediated Ca^{2+} release.

The activity of IP_3 receptors is modulated by cytosolic and luminal Ca^{2+} [21–24]. We showed here that the resting $[Ca^{2+}]_i$ in Stim1-mKO1-expressing cells was higher than that in mKO1-expressing and non-expressing cells; this small increase in resting $[Ca^{2+}]_i$ might potentiate the activity of the IP_3 receptor. The increased Ca^{2+} release upon overexpression of Stim1-mKO1 may simply be attributable to the increase in resting $[Ca^{2+}]_i$. Alternatively, recent reports have shown that Stim1 or Stim1-POST (partner of Stim1) complex interacted with plasma membrane Ca^{2+} pump (PMCA) and inhibited its activity [25,26]; this may attenuate Ca^{2+} clearance, resulting in increased Ca^{2+} responses upon Stim1 overexpression.

We also demonstrated that the direct effect of IP_3 on the Ca^{2+} release from permeabilized submandibular acinar cells was not altered by the overexpression of Stim1-mKO1. Unlike the Ca^{2+} release in intact cells, IP_3 receptors in permeabilized cells were exposed to ICM, where $[Ca^{2+}]_i$ was fixed at 50 nM; thus, the effect of cytosolic Ca^{2+} was eliminated. In addition, ionomycin-induced Ca^{2+} release was not increased by the expression of Stim1-mKO1. These results suggest that the content of the intracellular Ca^{2+} stores in submandibular acinar cells was not increased by the expression of Stim1-mKO1. Similarly, the results indicate that the increased Ca^{2+} release upon Stim1-mKO1 expression could not be attributed to differences in Ca^{2+} stores, such as higher expression of IP_3R or larger stored Ca^{2+} content. Together with these results, the increased Ca^{2+} release in Stim1-mKO1-expressing cells is likely to be attributable to the positive modulation of cytosolic Ca^{2+} by the small increase in resting Ca^{2+}, though we do not exclude a possible increase in IP_3 production as a result of activation of phospholipase C by intracellular Ca^{2+}. The precise mechanism of the

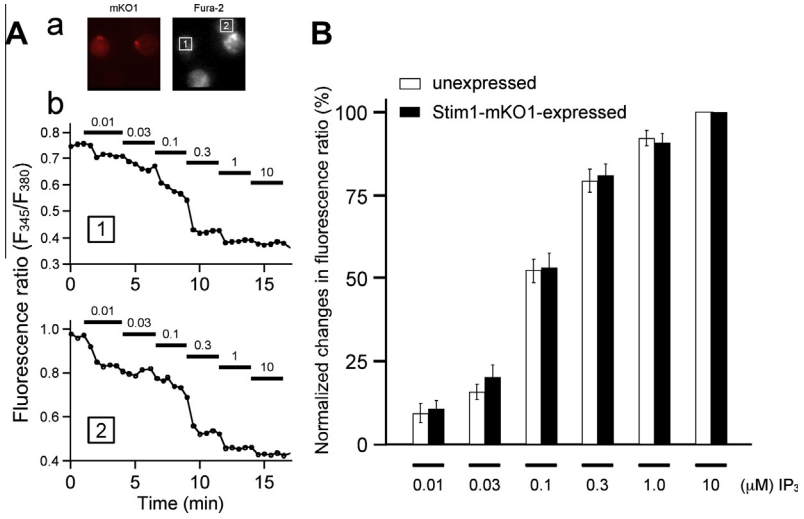

Fig. 3. IP_3-induced Ca^{2+} release in Ad-Stim1-mKO1-infected and saponin-permeabilized rat submandibular acinar cells. Dispersed submandibular acinar cells were loaded with mag-fura-2 and permeabilized with saponin. Cells were then stimulated with several concentrations of IP_3 (0.01–10 μM) in intracellular-like medium (ICM) containing ATP, Mg^{2+}, and 50 nM Ca^{2+}. (A) (a) Fluorescence images of Stim1-mKO1 and mag-fura-2; (b) time-dependent changes in the fluorescence ratio of mag-fura-2 in Stim1-mKO1-expressing cell (cell 1) and non-expressing cell (cell 2) depicted in the fluorescence image (a). The horizontal bars at the top of the figure indicate the concentrations of IP_3 in ICM. (B) Effect of different concentrations of IP_3 on Ca^{2+} release in saponin-permeabilized rat submandibular acinar cells. Normalized changes in fluorescence ratio of mag-fura-2 with various IP_3 concentrations were compared between Stim1-mKO1-expressing (closed column) and non-expressing (open column) cells. Changes in the fluorescence ratio of mag-fura-2 were normalized to the effects of IP_3 saturation with 10 μM. Data are shown as means ± SEM.

increased Ca^{2+} release in Stim1-mKO1-expressing cells should be investigated further.

Several potential clinical applications of gene transfer to ductal cells in salivary glands using adenovirus have been reported [7,27,28]. Retrograde administration of a high dose of adenovirus (10^9–10^{11} pfu/gland) into salivary glands permits the expression of exogenous genes in both acinar and ductal cells, but causes severe inflammation and destroys fluid-secreting acinar cells [13]. We administered a low dose of adenovirus (0.5×10^8 pfu) in a small volume (50 μl/gland), and succeeded in expressing Stim1-mKO1 in salivary acinar cells without inflammation or decrease in salivary secretions. In addition, gene transfer with low doses of adenovirus resulted in the relatively specific expression of Stim1-mKO1 in salivary acinar cells, with 20% of acinar cells expressing the transgene *vs.* 1–2% of ductal cells [15]. The enhanced sensitivity of Stim1-mKO1-expressing submandibular acinar cells to weak muscarinic stimulations further supports these observations. The low toxicity and the relative specificity to acinar cells of this mild gene transfer method are particularly useful for studying the molecular functions of salivary acinar cells *in vivo*, and for future clinical applications aimed at increasing salivary secretions. Presently, we are trying to express a Ca^{2+} biosensor in rat submandibular glands using this method, and to observe Ca^{2+} responses *in vivo* (personal communication).

Intracellular Ca^{2+}, via the activation of muscarinic receptors, triggers salivary fluid secretion. Our results showed that the overexpression of Stim1-mKO1 increased the Ca^{2+} response induced by weak muscarinic stimulation. Based on the idea that salivary secretion is induced by relatively weak Ca^{2+} responses [29,30], the overexpression of Stim1-mKO1 in submandibular acinar cells might increase salivary secretion, and the expression of Stim1 in salivary acinar cells may be a suitable gene therapy strategy for xerostomia (dry mouth) in the future. At this stage, the expression of Stim1-mKO1 was detected in only ~20% of acinar cells, and thus an increase in the number of expressing cells is required to increase salivary secretion with Stim1-mKO1 or Stim1.

Recently, several methods for enhanced adenoviral transduction *in vivo* and *ex vivo*, such as the use of magnetic nanoparticles and polyethylenimine, have been reported [31,32]. In addition, an alternative promoter might increase protein expression in salivary cells *in vivo*. Incorporation of these novel methods to our gene transfer protocol might improve the efficiency of expression of Stim1-mKO1 in living acinar cells and increase salivary secretion.

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