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ORIGINAL PAPER

Membrane potential modulation of ionomycin-stimulated Ca^{2+} entry via Ca^{2+}/H^+ exchange and SOC in rat submandibular acinar cells

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Abstract Ionomycin (IM) at 5 μM mediates the Ca^{2+}/H^+ exchange, while IM at 1 μM activates the store-operated Ca^{2+} entry channels (SOCs). In this study, the effects of depolarization on both pathways were examined in rat submandibular acinar cells by increasing extracellular K^+ concentration ($[K^+]_o$). IM (5 μM, the Ca^{2+}/H^+ exchange) increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to an extremely high value at 151 mM $[K^+]_o$. However, with increasing $[K^+]_o$, the rates of Ca^{2+} entry decreased in a linear relationship. The reversal potential (E_{rev}) for the Ca^{2+}/H^+ exchange was +93 mV, suggesting that IM (5 μM) exchanges 1 Ca^{2+} for 1 H^+. Thus, depolarization decreases the Ca^{2+} influx via the Ca^{2+}/H^+ exchange because of its electrogenicity (1 Ca^{2+} for 1 H^+). On the other hand, IM (1 μM, the SOCs) abolished an increase in $[Ca^{2+}]_i$ at 151 mM $[K^+]_o$. With increasing $[K^+]_o$, the rate of Ca^{2+} entry immediately decreased linearly. The E_{rev} for the SOC was +3.7 mV, suggesting that the SOCs are nonselective cation channels and less selective for Ca^{2+} over Na^+ ($P_{Ca}/P_{Na} = 8.2$). Moreover, an increase in extracellular Ca^{2+} concentration (20 mM) enhanced the Ca^{2+} entry via the SOCs at 151 mM $[K^+]_o$, suggesting depolarization does not inhibit the SOCs and decreases the driving force for the Ca^{2+} entry. This suggests that membrane potential changes induced by a secretory stimulation finely regulate the $[Ca^{2+}]_i$ via the SOCs in rat submandibular acinar cells. In conclusion, IM increases $[Ca^{2+}]_i$ via two pathways depending on its concentration, the exchange of 1 Ca^{2+} for 1 H^+ at 5 μM and the SOCs at 1 μM.

Keywords Ionomycin · Thapsigargin · Gd^{3+} · Intracellular Ca^{2+} concentration · Store-operated Ca^{2+} entry

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Introduction

Ionomycin (IM) is known to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in many cell types, including salivary acinar cells, and is used in a variety of experiments. The $[Ca^{2+}]_i$ increase stimulated by IM was reported to be due to the exchange of 1 Ca^{2+} for 1 H^+, which was concluded to be electrogenic [1–5].

On the other hand, IM is also known to activate store-operated Ca^{2+} channels (SOCs), that is, IM depletes Ca^{2+} from intracellular stores [4, 6, 7], which triggers Ca^{2+} entry via SOCs [4, 6–10]. The electrophysiological characteristics of the SOC in salivary cells have been reported to be different from those of the typical calcium release-activated Ca^{2+} channels (CRAC) in leukemia cells [8–14]. The

transient receptor potential canonical 1 (TRPC1) of ion channel proteins is also reported to be an essential component of the SOC in salivary acinar cells [11–13]. Patch clamp studies demonstrated that the SOCs are nonselective cation channels in salivary gland cells. In human submandibular gland (HSG) cells, the SOC current (I_{SOC}) was relatively inwardly rectifying and relatively selective for Ca^{2+} over Na^+ [reversal potential (E_{rev}) = +25 mV] [13, 14]. In human parotid gland (HSY) cells and mouse submandibular acinar cells, the I_{SOC} is almost linear and less selective for Ca^{2+} over Na^+ (E_{rev} = +3 mV) [13, 14]. Thus, two types of SOCs were reported in salivary acinar cells.

The SOCs in salivary cells are modulated by many cellular events stimulated by $[Ca^{2+}]_i$ increases, including membrane potential. In salivary acinar cells, depolarization was reported to decrease store-operated Ca^{2+} entry (SOCE) [8, 15] and to induce $[Ca^{2+}]_i$ oscillations (at 20–30 mM $[K^+]_o$) [16, 17].

To examine the effects of depolarization on SOCE, we measured the $[Ca^{2+}]_i$ in IM-stimulated rat submandibular acinar cells with increasing extracellular K^+ concentration ($[K^+]_o$). In the course of experiments, a high $[K^+]_o$, for example 150 mM, did not suppress increases in $[Ca^{2+}]_i$ stimulated by 5 μM IM, whereas it abolished those stimulated by 1 μM IM. Moreover, Gd^{3+} (1 μM) inhibited an increase in $[Ca^{2+}]_i$ stimulated by 1 μM, but not by 5 μM [5]. Thus, IM has two actions depending on its concentration, suggesting that 5 μM IM mediates the Ca^{2+}/H^+ exchange and 1 μM IM activates the SOCs [5].

In this study, we examined the effects of depolarization on the IM-stimulated Ca^{2+} entry pathways (the Ca^{2+}/H^+ exchange and the SOC) by measuring the rate of $[Ca^{2+}]_i$ increase (Ca^{2+} influx) with increasing $[K^+]_o$. The goal of this study is to confirm IM increases $[Ca^{2+}]_i$ via two pathways depending on its concentration, the electrogenic Ca^{2+}/H^+ exchange at 5 μM and the SOCs at 1 μM.

Materials and methods

Solutions and chemicals

The control solution contained (in mM): NaCl 121, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 25, NaHEPES 5, HHEPES 5, and glucose 5; the KCl solution contained (in mM): KCl 125.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $KHCO_3$ 25, KHEPES 5, HHEPES 5, and glucose 5. The pH of the solutions was adjusted to 7.4 by adding 1 M HCl. To increase the K^+ concentration of test solutions, an appropriate amount of KCl solution was added to the control solution. The solutions were aerated with a gas mixture of 95% O_2 and 5% CO_2. To prepare a Ca^{2+}-free solution, $CaCl_2$ was excluded from the solutions (nominal Ca^{2+}-free). We did not use EGTA to chelate Ca^{2+}, since EGTA also chelates Gd^{3+}. There was no difference between $[Ca^{2+}]_i$ increases following the reintroduction of Ca^{2+}, when we used a Ca^{2+}-free solution with or without EGTA [5]. A high Ca^{2+} solution (20 mM Ca^{2+}, HCO_3^--free) contained (in mM): NaCl 146, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 20, NaHEPES 5, HHEPES 5, and glucose 5; the KCl solution containing high Ca^{2+} contained (in mM): KCl 151.5, $MgCl_2$ 1, $CaCl_2$ 20, KHEPES 5, HHEPES 5, and glucose 5. The pH of the solutions was adjusted to 7.4 by adding 1 M HCl. To prepare a Ca^{2+} free solution for high Ca^{2+} experiments, 20 mM $CaCl_2$ was excluded from the high Ca^{2+} solutions, and 30 mM NaCl or 30 mM KCl was added to adjust the osmolarity. The high Ca^{2+} solutions (HCO_3^--free) were aerated with 100% O_2. IM, thapsigargin (TG), bovine serum albumin (BSA), and collagenase were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The reagents were diluted to their final concentrations just before the experiments. All the experiments were performed at 37°C.

Cell preparation and fluorescence measurements

Male rats (Slc: Wistar/ST, Japan SLC Inc., Hamamatsu, Japan) weighing 150–200 g were purchased and fed a standard pellet diet and water. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal 60–70 mg/kg). The submandibular glands were removed from the animals for cell isolation, and then the animals were killed by an additional injection of pentobarbital sodium (Nembutal 100 mg/kg). Experiments were approved by the Animal Research Committee of Osaka Medical College, and the animals were cared for according to the guidelines of this committee. The procedures for the cell preparations have already been described in detail [5, 16]. The submandibular glands were washed with the control solution to remove blood. Collagenase (0.1%) was dissolved in the control solution containing 2% BSA. The glands were minced in a collagenase solution with fine forceps and then incubated (37°C) for 15 min. The digested tissue was filtered through a nylon mesh with a pore size of 150-μm squares to remove undigested tissue. The filtrate was centrifuged at 300 rpm (10*g*) for 2 min, washed three times with centrifugation between each wash, and suspended in the control solution containing 2% BSA.

The isolated cells were loaded with fura 2-AM (2.5 μM, Dojindo, Kumamoto, Japan) or BCECF-AM (2.5 μM, Dojindo, Kumamoto, Japan) for 25 min at room temperature (23°C) and washed with the control solution.

They were then mounted on a coverslip precoated with neutralized Cell-Tak (Becton-Dickinson Labware, Bedford, MA) to allow the cells to firmly adhere to the coverslip. The coverslip with cells was set in a perfusion chamber, which was mounted on the stage of an inverted microscope (TE2000, Nikon, Tokyo, Japan) connected to an image analysis system (Aqua Cosmos, Hamamatsu Photonics, Hamamatsu, Japan). The capacity of the chamber was approximately 100 μl, and the perfusion rate was 500 μl/min. The fura 2 fluorescence ratios (340:380 nm) were calculated and stored in the image analysis system. One experiment was performed using 6–12 coverslips from 2 to 4 animals. A typical response obtained from five to seven cells on a coverslip is shown in the figures (mean ± SEM). To compare the values among experiments, averaged values calculated from five to six coverslips obtained from two to three animals were used.

Activation of Ca^{2+} entry

To observe Ca^{2+} entry, Ca^{2+} was reintroduced (reintroduction of Ca^{2+}) [5, 6]. The procedure for "the reintroduction of Ca^{2+}" was as follows. Before the start of experiments, the cells were perfused with control solution for 5 min. Following the control perfusion, the cells were perfused with a Ca^{2+}-free solution for 5 min and stimulated with IM for a further 10 min. Then, the Ca^{2+}-free solution was suddenly switched to a Ca^{2+}-containing solution, keeping the IM concentration (the reintroduction of Ca^{2+}). The reintroduction of Ca^{2+} is a method for stimulating a rapid Ca^{2+} entry without Ca^{2+} release from stores.

Membrane potential measurements

Membrane potential was measured by a gramicidin-perforated whole cell patch method. The details have already been described in a previous report [18]. Briefly, gramicidin D (Sigma) was dissolved in methanol (10 mg/ml) and diluted 100 times with a standard KCl-rich pipette solution. The KCl-rich pipette solution contained (in mM): KCl 150 and HEPES 10. The pH of the solution was adjusted to 7.4 using KOH. After making the perforated patch configuration, the membrane potential was measured using a patch clamp amplifier (model CEZ-2400, Nihon Kohden, Tokyo, Japan). The membrane potentials of the cells were measured under an unstimulated condition and 5 μM IM- or 4 μM TG-stimulated condition with 4.5 mM $[K^+]_o$ and 151 mM $[K^+]_o$.

The statistical significance of the difference between the mean values was assessed using Student's t test. Differences were considered significant at $p < 0.05$.

Results

Effects of high $[K^+]_o$ on IM-stimulated $[Ca^{2+}]_i$

During the control perfusion, the F340/F380 of the submandibular acinar cells were 1.2–1.3. The switch to a Ca^{2+}-free solution slightly decreased and plateaued $[Ca^{2+}]_i$ within 2 min in rat submandibular acinar cells (data not shown). The addition of 1 μM IM induced a small transient increase in $[Ca^{2+}]_i$. The reintroduction of Ca^{2+} induced a rapid transient increase followed by a sustained increase in $[Ca^{2+}]_i$ (Fig. 1a). Experiments were carried out using a KCl solution ($[K^+]_o = 151$ mM). The KCl solution abolished the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} in cells stimulated with 1 μM IM (Fig. 1a). Gd^{3+} (1 μM) similarly inhibited the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} in cells stimulated with 1 μM IM (data not shown), as previously reported [5].

The experiments were carried out using 5 μM IM. The switch to Ca^{2+}-free solution decreased $[Ca^{2+}]_i$ slightly, and then, the addition of 5 μM IM induced a large transient $[Ca^{2+}]_i$ increase. The reintroduction of Ca^{2+} immediately increased $[Ca^{2+}]_i$ to an extremely high value (F340/F380 = ~5) (Fig. 1b). Experiments were carried out using the KCl solution ($[K^+]_o = 151$ mM). The KCl solution did not inhibit the $[Ca^{2+}]_i$ increase following the reintroduction Ca^{2+} in cells stimulated by 5 μM IM, although the plateaus $[Ca^{2+}]_i$s were decreased by 10–15% (F340/F380 = ~4.4) (Fig. 1b). However, the rate of $[Ca^{2+}]_i$ increase was decreased by ~50%, compared with those of the control experiments. Gd^{3+} (1 μM) did not inhibit the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} in cells stimulated by 5 μM IM (data not shown), as previously reported [5].

The effects of 1 and 5 μM IM on the intracellular Ca^{2+} stores were examined (Fig. 1c, d). The addition of 1 or 5 μM IM induced a transient $[Ca^{2+}]_i$ increase in a Ca^{2+} free solution, and then, the further addition of 10 μM ACh induced no $[Ca^{2+}]_i$ increase. Thus, IM at both concentrations completely depletes Ca^{2+} from internal stores, indicating that IM (1 and 5 μM) activates the SOCs. Then, the reintroduction of Ca^{2+} increased $[Ca^{2+}]_i$ in both cells stimulated with 1 and 5 μM IM (Fig. 1c, d). Increases in $[Ca^{2+}]_i$ shown in panels c and d were similar to those shown in panels a and b.

Effects of high $[K^+]_o$ on TG-stimulated $[Ca^{2+}]_i$

Experiments were also carried out using 4 μM TG (Fig. 2). The addition of TG (4 μM) in a Ca^{2+}-free solution induced a slight and transient $[Ca^{2+}]_i$ increase. The reintroduction of Ca^{2+} induced a biphasic increase in $[Ca^{2+}]_i$ in cells stimulated with 4 μM TG, similarly to those stimulated

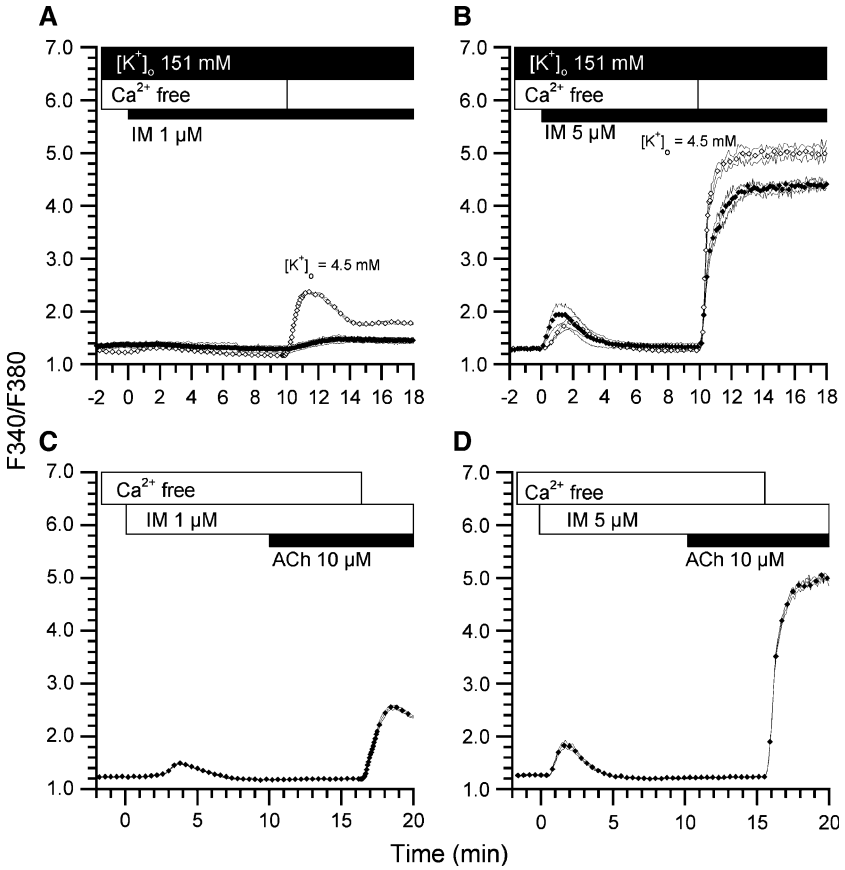

Fig. 1 $[Ca^{2+}]_i$ increases following the reintroduction of Ca^{2+} during IM stimulation. **a** IM (1 μM). The addition of 1 μM IM in the Ca^{2+}-free solution increased $[Ca^{2+}]_i$ slightly and transiently. The reintroduction of Ca^{2+} induced a transient increase followed by a sustained increase in $[Ca^{2+}]_i$. An increase of $[K^+]_o$ (151 mM) abolished the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+}. **b** IM (5 μM). The addition of 5 μM IM in the Ca^{2+}-free solution increased $[Ca^{2+}]_i$ transiently. The reintroduction of Ca^{2+} induced a sustained increase in $[Ca^{2+}]_i$. The final $[Ca^{2+}]_i$s were much higher than that of 1 μM IM. An increase of $[K^+]_o$ (151 mM) still increased $[Ca^{2+}]_i$ following the reintroduction of Ca^{2+}, although the final $[Ca^{2+}]_i$ decreased by 10%. **c**, **d** Effects of 1 or 5 μM IM on internal Ca^{2+} stores. The addition of 1 or 5 μM IM increased $[Ca^{2+}]_i$ transiently, and then the further addition of 10 μM ACh did not induce any $[Ca^{2+}]_i$ increase. The reintroduction of Ca^{2+} increased $[Ca^{2+}]_i$

with 1 μM IM. Experiments were carried out using the KCl solution. The KCl solution abolished the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} in cells stimulated with 4 μM TG (Fig. 2). The $[Ca^{2+}]_i$ increase stimulated with 4 μM TG was inhibited by 1 μM Gd^{3+} (data not shown), as previously reported [5]. Thus, $[Ca^{2+}]_i$ increases stimulated by 4 μM TG are similar to those stimulated by 1 μM IM, indicating that 1 μM IM activates SOCs.

Effects of $[K^+]_o$ on $[Ca^{2+}]_i$ increase

The rates of $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} were measured at various $[K^+]_o$s in cells stimulated with 1 μM IM. The $[K^+]_o$s used ranged from 4.5 to 151 mM. The experimental protocol used is shown in Fig. 1. Cells were treated with 1 μM IM for 10 min in a Ca^{2+}-free solution, and then the Ca^{2+} was reintroduced. Figure 3 shows increases in $[Ca^{2+}]_i$ for 1.5 min following the reintroduction of Ca^{2+} at three $[K^+]_o$s (4.5, 25 and 151 mM). In cells stimulated by 1 μM IM, the rate of Ca^{2+} entry and the peak $[Ca^{2+}]_i$ decreased to ~30 and ~50% at 25 mM $[K^+]_o$, respectively. No $[Ca^{2+}]_i$ increase was detected at 151 mM $[K^+]_o$. Similar results were obtained in cells stimulated by 4 μM TG instead of 1 μM IM (data not shown).

Experiments were also carried out using 5 μM IM (Fig. 3b). Cells were treated with 5 μM IM for 10 min in a Ca^{2+}-free solution, and then the Ca^{2+} was reintroduced. The reintroduction of Ca^{2+} increased $[Ca^{2+}]_i$ to a high value (F340/F380 = 4.5–5). However, the rates of Ca^{2+} entry stimulated by 5 μM IM decreased with increasing $[K^+]_o$s from 4.5 to 151 mM (Fig. 3b). The peak $[Ca^{2+}]_i$ (5 min after the reintroduction of Ca^{2+}) was decreased by ~10% at 151 mM $[K^+]_o$ (Figs. 1b, 3b).

The results were summarized in Fig. 4. In panel a, the rates of Ca^{2+} entry following the reintroduction of Ca^{2+}

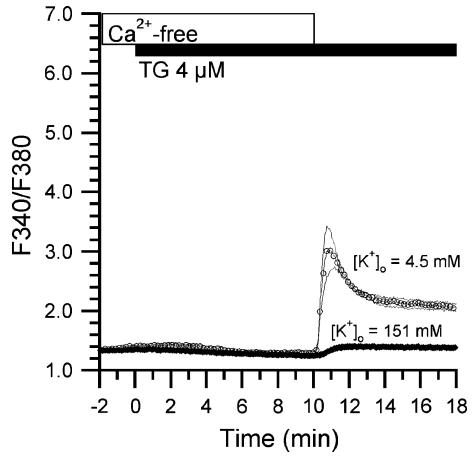

Fig. 2 $[Ca^{2+}]_i$ increases following the reintroduction of Ca^{2+} during 4 μM TG stimulation. The addition of 4 μM TG in the Ca^{2+}-free solution increased $[Ca^{2+}]_i$ slightly and transiently. The reintroduction of Ca^{2+} induced a transient increase followed by a sustained increase in $[Ca^{2+}]_i$. An increase of $[K^+]_o$ (151 mM) abolished the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+}

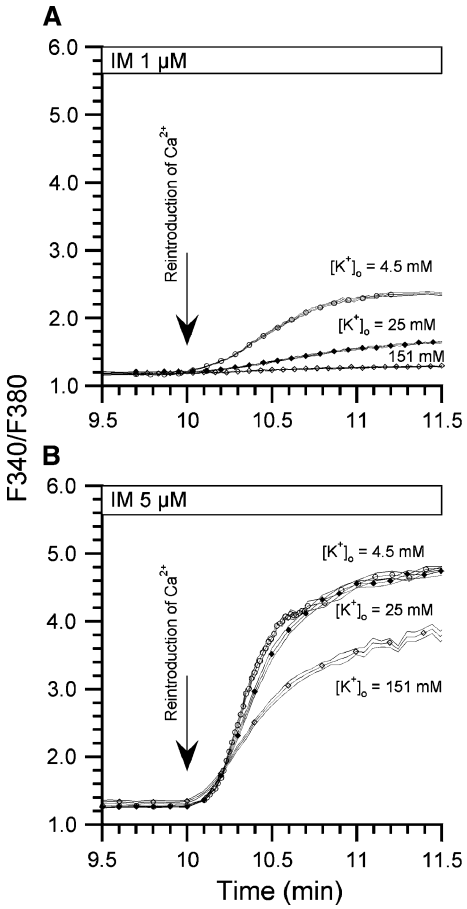

Fig. 3 Effects of depolarization on $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} during IM stimulation. The experimental protocol is shown in Figs. 1 and 2. Cells were perfused with a Ca^{2+}-free solution for 5 min prior to the IM addition. Cells were treated with IM for 10 min, and then Ca^{2+} (1.5 mM) was reintroduced. In these figures, changes in the $[Ca^{2+}]_i$ increase for 2 min were shown (9.5–11.5 min from the start of IM stimulation). The figure clearly shows decreases in the rate of $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} with increasing $[K^+]_o$. **a** IM (1 μM). With increasing $[K^+]_o$, the rate of $[Ca^{2+}]_i$ increase decreased, and the final $[Ca^{2+}]_i$ also decreased. **b** IM (5 μM). With increasing $[K^+]_o$, the rate of $[Ca^{2+}]_i$ increase decreased. The final $[Ca^{2+}]_i$s decreased by 10% at 151 mM $[K^+]_o$

were plotted against $[K^+]_o$s. In cells stimulated with 5 μM IM, the rates of Ca^{2+} entry decreased in a linear relationship with increasing $[K^+]_o$. The intercept of the *x* axis ($[K^+]_o$) is 4.5 M. $[K^+]_i$ is 125 mM, which was calculated from the membrane potential at 151 mM $[K^+]_o$ (+5 mV) as shown in Figs. 6 and 7. The E_{rev} was calculated to be +93 mV [$=-60 \times \log(0.125/4.5)$]. In cells stimulated with 1 μM IM, the rates of Ca^{2+} entry also linearly decreased with increasing $[K^+]_o$, and the intercept of the *x* axis ($[K^+]_o$) was 144 mM (Fig. 4a). The E_{rev} was calculated to be +3.7 mV. Experiments were carried out using 4 μM TG. In cells stimulated with 4 μM TG, the rates of Ca^{2+} entry also linearly decreased, and the intercept of the *x* axis ($[K^+]_o$) was 132 mM. The E_{rev} was calculated to be +1.4 mV. These results suggest that the SOCs of rat submandibular acinar cells are nonselective cation channels and less selective for Ca^{2+} over Na^+ [12–14]. Depolarization markedly decreased the driving force for Ca^{2+} entry via SOCs in submandibular acinar cells.

The peak $[Ca^{2+}]_i$s within 5 min following the reintroduction of Ca^{2+} were plotted against $[K^+]_o$s in Fig. 4b. In cells stimulated with 5 μM IM, the peak $[Ca^{2+}]_i$s were decreased slightly with increasing $[K^+]_o$. In cells stimulated with 1 μM IM or 4 μM TG, the peak $[Ca^{2+}]_i$s decreased in a linear relationship with increasing $[K^+]_o$. The regression line of 1 μM IM shifted upward compared with that of 4 μM TG, although the *x* intercepts of both lines were not significantly different ($p > 0.05$) (Fig. 4b). IM (1 μM) may mediate the Ca^{2+}/H^+ exchange, although the $[Ca^{2+}]_i$ increases mediated by the Ca^{2+}/H^+ exchange were negligibly small in cells stimulated by 1 μM IM.

Effects of high $[Ca^{2+}]_o$ on Ca^{2+} entry

To examine the effects of an increase in the driving force for Ca^{2+} entry via SOCs during depolarization, a high Ca^{2+} solution ($[Ca^{2+}]_o = 20$ mM) was used. In cells stimulated with 4 μM TG, a high $[Ca^{2+}]_o$ (20 mM) enhanced the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} at 151 mM $[K^+]_o$, suggesting that depolarization does not inhibit SOCs (Fig. 5a). The rate of Ca^{2+} entry was plotted against $[K^+]_o$ in Fig. 5b. With increasing $[K^+]_o$, the rates of Ca^{2+} entry linearly decreased (Fig. 5b). The intercept of the *x* axis ($[K^+]_o$) was 188 mM. Assuming the intracellular osmolarity was the same as the extracellular

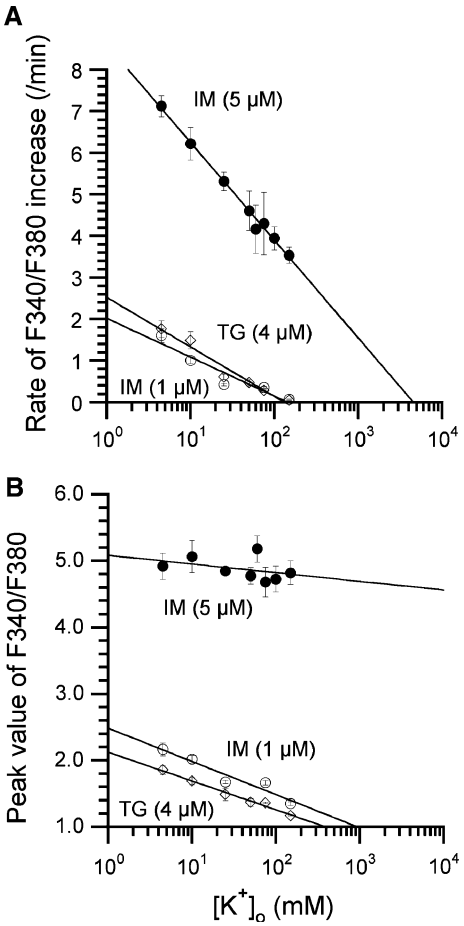

Fig. 4 Effects of $[K^+]_o$ on $[Ca^{2+}]_i$ increase stimulated by 1 and 5 μM IM. **a** Effects of $[K^+]_o$ on the rate of F340/F380 increase (Ca^{2+} influx). During 5 μM IM stimulation, the rate of $[Ca^{2+}]_i$ increase linearly decreased. The intercept of the *x* axis was 4.5 M. During 1 μM IM and 4 μM TG stimulation, the rates of $[Ca^{2+}]_i$ increase linearly decreased. The intercepts of the *x* axis were 144 mM during IM stimulation and 131 mM during TG stimulation. **b** Effects of $[K^+]_o$ on the sustained F340/F380. With increasing $[K^+]_o$, the final $[Ca^{2+}]_i$s are almost constant during 5 μM IM stimulation. However, with increasing $[K^+]_o$, the final $[Ca^{2+}]_i$ linearly decreased

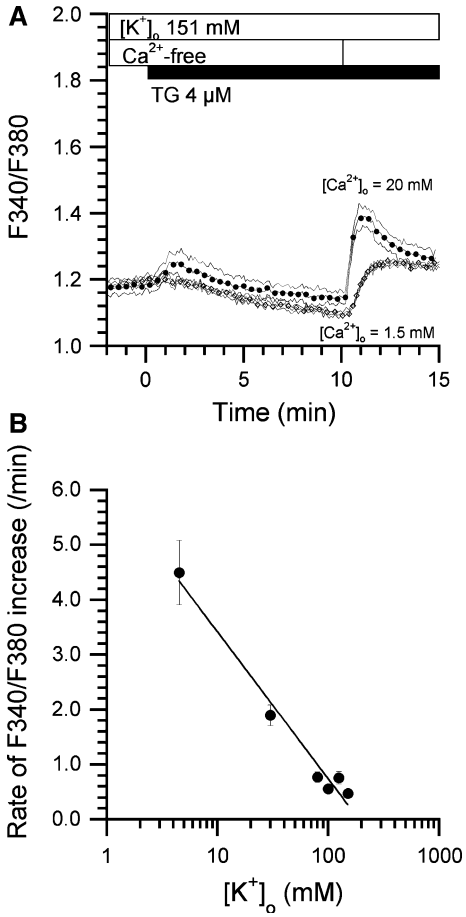

Fig. 5 Effects of a high $[Ca^{2+}]_o$ (20 mM). Experiments were carried out using 4 μM TG in a HCO_3^--free solution. In these experiments, the $[Ca^{2+}]_o$ used was 20 mM to increase the driving force for Ca^{2+} entry. **a** An increase in $[Ca^{2+}]_o$ (20 mM) enhanced the $[Ca^{2+}]_i$ increase following the reintroduction of Ca^{2+} at 151 mM $[K^+]_o$. **b** Effects of $[K^+]_o$ on the rate of F340/F380 increase (Ca^{2+} influx). With increasing $[K^+]_o$, the rates of $[Ca^{2+}]_i$ increase linearly decreased, and the intercept of the *x* axis was 188 mM

osmolarity, $[K^+]_i$ was estimated to be 150 mM (=125 × 180/151). The E_{rev} calculated was +5.9 mV.

Membrane potential measurement

The membrane potential of submandibular acinar cells was measured using a whole cell patch-clamp technique [18]. The resting membrane potential was approximately −40 to −50 mV. The addition of 5 μM IM induced depolarization following an immediate hyperpolarization (−80 mV), and the final membrane potential was −10 to −20 mV (Fig. 6a). However, the addition of 4 μM TG induced a gradual depolarization following an immediate hyperpolarization (−80 mV), and the final membrane potential was similar to that before the addition of 4 μM TG (−40 mV) (Fig. 6c). The switch to a high-K^+ solution (151 mM) induced an immediate depolarization (+5 mV), and the further addition of 4 μM TG or 5 μM IM had no effect on the membrane potential. On returning to the control solution, the membrane potential shifted to −10 mV during stimulation with 5 μM IM (Fig. 6b) and to −60 mV during stimulation with 4 μM TG (Fig. 6d). The final membrane potentials (12 min after the addition of IM or TG) are summarized in Fig. 7 (mean ± SEM, 5 cells obtained from 2 to 3 animals). IM (5 μM) and TG (4 μM) depolarized the membrane potentials to −18 and −37 mV, respectively. The depolarization induced by 5 μM IM was significantly larger than that induced by TG ($p < 0.05$), suggesting that the 5 μM IM mediated an electrogenic Ca^{2+}/H^+ exchange. With increasing $[K^+]_o$ from 4.5 to 151 mM, the membrane potentials depolarized immediately to +5 mV, and the further addition of IM or TG had no effect on the

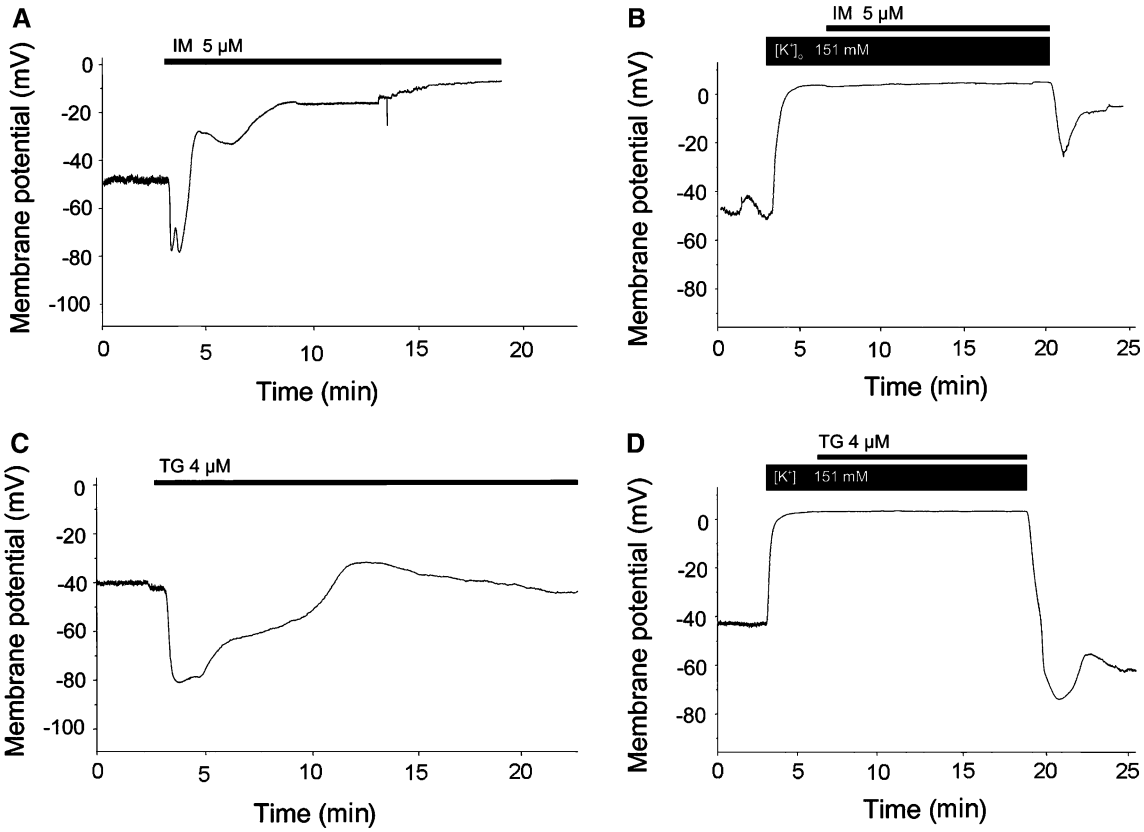

Fig. 6 Membrane potential changes induced by 4 μM TG and 5 μM IM. Membrane potentials were measured using a gramicidin-perforated whole cell patch clamp technique. **a** Stimulation with 5 μM IM at 4.5 mM $[K^+]_o$. **b** Stimulation with 5 μM IM at 151 mM $[K^+]_o$. **c** Stimulation with 4 μM TG at 4.5 mM $[K^+]_o$. **d** Stimulation with 4 μM TG at 151 mM $[K^+]_o$

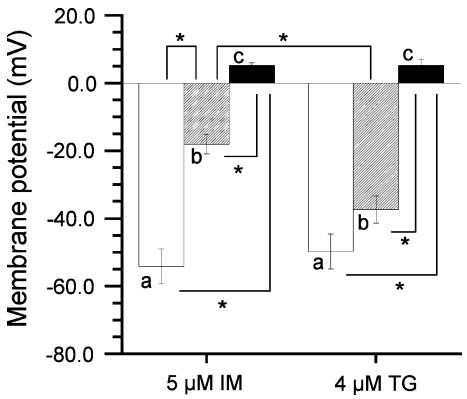

Fig. 7 Membrane potential changes induced by 4 μM TG and 5 μM IM. Membrane potentials before stimulation (*a*), 12 min after the addition of TG or IM at 4.5 mM $[K^+]_o$ (*b*) and at 151 mM $[K^+]_o$ (*c*) were shown in this figure. IM (5 μM) significantly depolarized the membrane potential. *Asterisks* indicate significantly different ($p < 0.05$)

membrane potentials (Fig. 7). From the membrane potential at 151 mM $[K^+]_o$, we evaluated the $[K^+]_i$ to be 125 mM.

Discussion

This study demonstrated that IM increases $[Ca^{2+}]_i$ via two pathways in rat submandibular acinar cells depending on its concentration. One is the Ca^{2+}/H^+ exchange, which is mediated by a high concentration of IM, such as 5 μM. The Ca^{2+} entry via the Ca^{2+}/H^+ exchange is insensitive to 1 μM Gd^{3+} and depolarization, and accompanied by membrane depolarization [3–5, 19]. A previous report demonstrated that an increase in extracellular pH enhances the rate of Ca^{2+} entry stimulated by 5 μM IM [5]. The Ca^{2+}/H^+ exchange increased $[Ca^{2+}]_i$s to an extremely high value. The other is the SOCs, which are activated by a low concentration of IM, such as 1 μM. The Ca^{2+} entry via SOCs is sensitive to 1 μM Gd^{3+} [5] and decreased by depolarization. Changes in extracellular pH have been shown to have no effect on the rate of Ca^{2+} entry [5]. $[Ca^{2+}]_i$ increases stimulated by 1 μM IM were similar to those stimulated by TG, which activated SOCs by depletion of Ca^{2+} from stores [6, 15].

In the Ca^{2+}/H^+ exchange (5 μM IM), Ca^{2+} influx linearly decreased with developing depolarization. The reversal potential (E_{rev}) was estimated from the rates of

Ca^{2+} entry with increasing $[K^+]_o$. The $[K^+]_o$, which abolished the driving force for Ca^{2+} entry via the Ca^{2+}/H^+ exchange, is estimated to be 4.5 M from the observation shown in Fig. 4a (the intercept of x axis), and $[K^+]_i$ is evaluated to be 125 mM from the membrane potential (+5 mV) at 151 mM $[K^+]_o$ (Fig. 7). The E_{rev} for the Ca^{2+}/H^+ exchange calculated was +93 mV [$=-60 \times \log(0.125/4.5)$].

In the simplest model for the IM-mediated Ca^{2+}/H^+ exchange, the E_{rev} was expressed using conductances of Ca^{2+} (g_{Ca}) and H^+ (g_H) as Eq. 1.

$$g_{Ca}(E_{rev} - E_{Ca}) = g_H(E_H - E_{rev}). \quad (1)$$

The equilibrium potential for Ca^{2+} (E_{Ca}) is calculated to be +140 mV, when $[Ca^{2+}]_i$ is 100 nM and $[Ca^{2+}]_o$ is 1.5 mM. The equilibrium potential for H^+ (E_H) is 0 mV, because intracellular pH (pH_i) is similar to extracellular pH (pH_o).

This leads to Eq. 2, because E_H is 0 mV.

$$E_{rev} = [g_{Ca}/(g_{Ca} + g_H)] \times E_{Ca} \quad (2)$$

E_{rev} for Ca^{2+}/H^+ exchange mediated by 5 μM IM is +91 mV, which was 65% of E_{Ca}. This indicates that the g_{Ca}/g_H is approximately 2/1. Since Ca^{2+} has two positive charges and H^+ has one positive charge, Ca^{2+} and H^+ appear to be equally contributed to the E_{rev}, suggesting that IM exchanges 1 Ca^{2+} for 1 H^+.

On the other hand, in the SOC stimulated by 1 μM IM or 4 μM TG, the Ca^{2+} influx decreased linearly with increasing $[K^+]_o$ from 4.5 to 151 mM. The E_{rev}s are +3.7 and +1.4 mV in 1 μM IM- and 4 μM TG-stimulated cells, respectively. Moreover, in TG-stimulated cells, an increase in $[Ca^{2+}]_o$ (20 mM) enhanced Ca^{2+} influx significantly at 151 mM $[K^+]_o$, indicating that depolarization does not inhibit SOCs, and the E_{rev} was +5.9 mV. Thus, an increase in $[Ca^{2+}]_o$ did not shift the E_{rev} significantly ($p > 0.05$). These E_{rev}s suggest that the SOCs are permeable to monovalent cations, such as Na^+ and K^+. The previous patch clamp studies demonstrated that the SOCs are permeable to not only Ca^{2+}, but also Na^+ and Cs^+ in salivary cells ($P_{Cs}/P_{Na} = 1$ in HSG cells and 1.3 in HSY cells) [11, 14].

In this study, we calculated P_{Ca}/P_{Na} [20], assuming that the SOCs are permeable to K^+ similarly to Cs^+ and the P_{Na}/P_K is 1.

$$P_{Ca}/P_{Na} = (1 + \exp(E_{rev}F/RT)) \times \left(([Na^+]_i + \alpha[K_+]_i) \times \exp(E_{rev} \times F/RT) - ([Na^+]_o + \alpha[K^+]_o)\right)/4/[Ca^{2+}]_o \quad (3)$$

In Eq. 3, α is P_K/P_{Na} (=1). We also assumed that the intracellular osmolarity is equal to the extracellular osmolarity. The calculated ratio (P_{Ca}/P_{Na}) was 8.2 at 1.5 mM $[Ca^{2+}]_o$ in rat submandibular acinar cells stimulated with 1 μM IM. In cells stimulated with 4 μM TG, the P_{Ca}/P_{Na}s were 2.6 at 1.5 mM $[Ca^{2+}]_o$ and 2.3 at 20 mM $[Ca^{2+}]_o$. Previous studies showed that the E_{rev}s have been reported to be +25 mV in HSG cells, +5 mV in HSY cells, and +3 mV in mouse submandibular acinar cells [13, 14], and that the permeability ratio of Ca^{2+} over Na^+ (P_{Ca}/P_{Na}) is 40 in HSG cells and 4.6 in HSY cells [13, 14]. The characteristics of the SOC in rat submandibular acinar cells are similar to those in HSY cells and mouse submandibular acinar cells, that is, the SOCs are less selective for Ca^{2+} over Na^+ [13, 14].

The SOCs of rat submandibular acinar cells have a linear current-voltage (I–V) relationship. Moreover, they are less selective for Ca^{2+} over Na^+ ($P_{Ca}/P_{Na} = 2$–8) compared with the typical CRAC of rat basophilic leukemia cells [8, 9, 14]. I_{CRAC} showed strong inward rectification and high selectivity for Ca^{2+} over Na^+ ($P_{Ca^{2+}}/P_{Na^+} > 500$). Thus, the SOCs of rat submandibular acinar cells are distinct from those of CRAC channels of T lymphocytes and other hematopoietic cells. The SOCs of rat submandibular acinar cells are likely to be TRPCs type, as shown in HSY cells and mouse submandibular acinar cells [11–14].

A dependence of IM-induced Ca^{2+} entry on the magnitude of depolarization has been studied mainly in artificial liposomes [3, 19], since artificial liposomes have simple properties unlike cells containing complicated biological properties. The extents of Ca^{2+} transport mediated via IM (Ca^{2+}/H^+ exchange) are reported to be a function of IM concentration [19]. The ratio of the Ca^{2+}/H^+ exchange in IM was varied according to the experimental conditions, such as the IM concentration and the external pH, especially in living cells [1–4, 19]. The experimental conditions making IM exchange 1 Ca^{2+} for 1 H^+ in living cells are still unknown. The present study demonstrated that a high concentration of IM (5 μM) exchanges 1 Ca^{2+} for 1 H^+ in rat submandibular acinar cells and that the stoichiometry is consistent with that obtained from studies of artificial liposomes or vesicles [1–3, 19].

The present study also showed that the peak values stimulated by 1 μM IM are slightly higher than those stimulated by 4 μM TG (Fig. 4b), and the P_{Ca}/P_{Na} in 1 μM IM stimulated cells (=8.2) was higher than those in TG stimulated cells (2.2–2.6). This may suggest that 1 μM IM also mediates the Ca^{2+}/H^+ exchange, although the Ca^{2+}/H^+ exchange contributed little to the increase $[Ca^{2+}]_i$ in cells stimulated with 1 μM IM. At present, the reason why IM (1 μM) does not exchange Ca^{2+} for H^+ via the plasma membrane is unknown. IM (1 μM) transports Ca^{2+} via the ER membrane, since it completely depletes intracellular stores. IM may accumulate intracellular vesicles, not cytoplasma in cells stimulated by 1 μM IM.

In rat submandibular acinar cells, ACh stimulation induces a hyperpolarization followed by a depolarization [21, 22], as shown in the whole cell patch clamp recording of this study. These changes in membrane potential affect $[Ca^{2+}]_i$ by altering the driving force for Ca^{2+} entry via SOCs. This indicates that changes in membrane potential induced by ACh stimulation finely control $[Ca^{2+}]_i$ via SOCs, which regulates fluid secretion in the rat submandibular gland.

On the other hand, previous reports [16, 17] demonstrated that an increase in $[K^+]_o$, such as 25–30 mM, stimulates $[Ca^{2+}]_i$ oscillations in rat submandibular acinar cells with a long interval ($\sim$10 min). These oscillations were induced by changes in Ca^{2+} entry via the SOCs [16]. However, an increase in $[K^+]_o$ (30 mM) induced no oscillation in the membrane potential (data not shown). Depolarization affects many cellular events, such as ion fluxes and cell volume. These cellular events may modulate SOCs, which may cause $[Ca^{2+}]_i$ oscillations. Further studies will be needed to answer this.

References

1. Pfeiffer DR, Taylor RW, Lardy HA (1978) Ionophore A23187: cation binding and transport properties. Ann N Y Acad Sci 307:402–423
2. Kolber MA, Haynes DH (1981) Fluorescence study of the divalent cation-transport mechanism of ionophore A23187 in phospholipid membrane. Biophysical J 36:369–391
3. Fasolato C, Pozzan T (1989) Effect of membrane potential on divalent cation transport catalyzed by the "electroneutral" ionophores A23187 and ionomycin. J Biol Chem 264:19630–19636
4. Mason MJ, Grinstein S (1993) Ionomycin activates electrogenic Ca^{2+} influx in rat thymic lymphocytes. Biochem J 296:33–39
5. Yoshida H, Shimamoto C, Ito S, Daikoku E, Nakahari T (2010) HCO_3^--dependent transient acidification induced by ionomycin in rat submandibular acinar cell. J Physiol Sci. doi: 10.1007/s12576-010-0095-x
6. Watson EL, Wu Z, Jacobson KL, Storm DR, Singh JC, Ott SM (1998) Capacitative Ca^{2+} entry is involved in cAMP synthesis in mouse parotid acini. Am J Physiol Cell Physiol 43:C557–C565
7. Putney JW Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium 7:1–12
8. Parekh AB, Penner R (1997) Store depletion and calcium influx. Physiol Rev 77:601–930
9. Parekh AB, Putney JW Jr (2005) Store-operated calcium channels. Physiol Rev 85:757–810
10. Elliott AC (2001) Recent developments in non-excitable cell calcium entry. Cell Calcium 30:73–93
11. Ong HL, Cheng KT, Liu X, Bandyopadhyay BC, Paria BC, Soboloff J, Pani B, Gwack Y, Srikanth S, Singh BB, Gill D, Ambudkar IS (2007) Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx: evidence for similarities in store-operated and calcium release-activated calcium channel components. J Biol Chem 282:9105–9116
12. Liu X, Singh BB, Ambudkar IS (2003) TRPC1 is required for functional store-operated Ca^{2+} channels. J Biol Chem 278: 11337–11343
13. Liu X, Cheng KT, Bandyopadhyay BC, Pani B, Dietrich A, Paria BC, Swaim WD, Beech D, Yildrim E, Singh BB, Brinbaumer L, Ambudkar IS (2007) Attenuation of store-operated Ca^{2+} current impairs salivary gland fluid secretion in TRPC1(−/−) mice. Proc Natl Acad Sci USA 104:17542–17547
14. Liu X, Groschner K, Ambudkar IS (2004) Distinct Ca^{2+}-permeable cation currents are activated by internal Ca^{2+}-store depletion in RBL-2H3 cells and human salivary gland cells, HSG and HSY. J Membr Biol 200:93–104
15. Mertz LM, Baum BJ, Ambudkar IS (1992) Membrane potential modulates divalent cation entry in rat parotid acini. J Membr Biol 126:183–193
16. Yoshida H, Marunaka Y, Nakahari T (2003) $[Ca^{2+}]_i$ oscillations induced by high $[K^+]_o$ in acetylcholine-stimulated rat submandibular acinar cells: regulation by depolarization, cAMP and pertussis toxin. Exp Physiol 88:369–379
17. Ma CY, chen CY, Cui ZJ (2004) Selective use of a reserved mechanism for inducing calcium oscillations. Cell Signal 16:1435–1440
18. Hirono C, Nakamoto T, Sugita M, Iwasa Y, Akagawa Y, Shiba Y (2001) Gramicidin-perforated patch analysis on HCO_3^- secretion through a forskolin-activated anion channel in rat parotid intralobular duct cells. J Membr Biol 180:11–19
19. Erdahl WL, Chapman CJ, Taylor RW, Pfeiffer DR (1994) Ca^{2+} transport properties of ionophore A23187, ionomycin, and 4-BrA23187 in a well-defined model system. Biophys J 66:1678–1693
20. Voets T, Prenen J, Vriens J, Watanabe H, Janssens A, Wissenbach U, Bödding M, Droogmans G, Nilius B (2002) Molecular determinants of permeation through the cation channels TRPV4. J Biol Chem 277:33704–33710
21. Nishiyama A, Petersen OH (1974) Membrane potential and resistance measurement in acinar cells from salivary glands in vitro: effect of acetylcholine. J Physiol 242:173–188
22. Nishiyama A, Hayashi H, Takahashi H, Saito Y (1990) Electrophysiology of salivary acinar cells: microelectrode studies. In: Young JA, Wong PYD (eds) Epithelial secretion of water and electrolytes. Springer, Berlin, pp 189–206