J Physiol Sci (2010) 60:273–282
DOI 10.1007/s12576-010-0095-x

ORIGINAL PAPER

HCO_3^--dependent transient acidification induced by ionomycin in rat submandibular acinar cells

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Received: 8 April 2010 / Accepted: 27 April 2010 / Published online: 22 May 2010

Abstract Ionomycin (IM, 5 μM), which exchanges 1 Ca^{2+} for 1 H^+, changed intracellular pH (pH_i) with Ca^{2+} entry into rat submandibular acinar cells. IM-induced changes in pH_i consisted of two components: the first is an HCO_3^--dependent transient pH_i decrease, and the second is an HCO_3^--independent gradual pH_i increase. IM (1 μM), which activates store-operated Ca^{2+} channels, induced an HCO_3^--dependent and transient pH_i decrease without any HCO_3^--independent pH_i increase. Thus, a gradual pH_i increase was induced by the Ca^{2+}/H^+ exchange. The HCO_3^--dependent and transient pH_i decrease induced by IM was abolished by acetazolamide, but not by methyl isobutyl amiloride (MIA) or diisothiocyanatostilbene disulfonate (DIDS), suggesting that the Na^+/H^+ exchange, the Cl^-/HCO_3^- exchange, or the Na^+-HCO_3^- cotransport induces no transient pH_i decrease. Thapsigargin induced no transient pH_i decrease. Thus, IM, not Ca^{2+} entry, reduced pH_i transiently. IM reacts with Ca^{2+} to produce H^+ in the presence of CO_2/HCO_3^- : $[H-IM]^- + Ca^{2+} + CO_2 \rightleftarrows [H-Ca-IM]^+ \cdot HCO_3^- + H^+$. In this reaction, a monoprotonated IM reacts with Ca^{2+} and CO_2 to produce an electroneutral IM complex and H^+, and then H^+ is removed from the cells via CO_2 production. Thus, IM transiently decreased pH_i. In conclusion, in rat submandibular acinar cells IM (5 μM) transiently reduces pH_i because of its chemical characteristics, with HCO_3^- dependence, and increases pH_i by exchanging Ca^{2+} for H^+, which is independent of HCO_3^-.

Keywords Ionomycin · Intracellular pH · Intracellular Ca^{2+} concentration · Submandibular acinar cell · Bicarbonate

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Introduction

Ionomycin (IM) is widely used in experiments to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and is reported to increase intracellular $[Ca^{2+}]_i$ via two Ca^{2+} entry pathways. One is electrogenic Ca^{2+}/H^+ exchange (1 Ca^{2+} for 1 H^+), which is mediated by IM in phospholipid membranes and vesicles [1–4] and in PC12 cells and lymphocytes [5, 6]. The other is the store-operated Ca^{2+} channels (SOCs), which is activated in many cell types by depleting Ca^{2+} from the intracellular stores. In salivary cells [7–15], IM is used to activate SOCs, similarly to thapsigargin (TG) and acetylcholine (ACh).

However, it remains uncertain which concentration of IM activates Ca^{2+}/H^+ exchange in rat submandibular acinar cells. We examined Ca^{2+} entry via SOCs in rat submandibular acinar cells, using many agonists including IM. In the course of the experiments, a high concentration of IM, for example 5 μM, increased intracellular Ca^{2+}

concentration ($[Ca^{2+}]_i$) to an extremely high value, which is not inhibited by 1 μM Gd^{3+}, and induced depolarization (Yoshida, unpublished observation). However, a low concentration of IM, for example 1 μM, increased $[Ca^{2+}]_i$ moderately, which was inhibited by 1 μM Gd^{3+}. These findings suggest that 5 μM IM may mediate the Ca^{2+}/H^+ exchange. A previous report also showed that 6 μM IM induced electrogenic Ca^{2+} entry into lymphocytes, suggesting that 6 μM IM mediates the Ca^{2+}/H^+ exchange [6].

To clarify whether 5 μM IM exchanges 1 Ca^{2+} for 1 H^+, we measured pH_i in rat submandibular acinar cells stimulated with 5 μM IM. Unexpectedly, 5 μM IM induced a transient pH_i decrease: a rapid pH_i decrease followed by a pH_i increase. Moreover, the transient pH_i decrease induced by 5 μM IM was HCO_3^--dependent, because no transient pH_i decrease was noted and only a gradual pH_i was observed in the presence of a carbonic anhydrase inhibitor (200 μM acetazolamide). Thus, a gradual pH_i increase seems to be induced by the Ca^{2+}/H^+ exchange. There are two possible means of inducing a transient pH_i decrease. One is activation of ion transporters, for example H^+ extrusion, HCO_3^- secretion, and HCO_3^- uptake. The other is a chemical characteristic of IM [5]. In this study, we examined why IM induced a transient pH_i decrease in rat submandibular acinar cells.

Materials and methods

Solutions and chemicals

The control solution contained (mM): NaCl 121, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 25, NaHEPES 5, HHEPES 5, and glucose 5. The control solutions were aerated with 95% O_2 and 5% CO_2. To prepare Ca^{2+}-free solution, $CaCl_2$ was excluded from the solution (nominally Ca^{2+}-free). We did not use EGTA to chelate Ca^{2+}, because EGTA also chelates Gd^{3+}. There was no difference between $[Ca^{2+}]_i$ increases after re-introduction of Ca^{2+} using Ca^{2+}-free solution with or without EGTA. HCO_3^--free solution contained (mM): NaCl 146, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1.5, NaHEPES 5, HHEPES 5, and glucose 5, and was aerated with 100% O_2. The pHs of the solutions were adjusted to 6.8, 7.4, or 8.0 by the addition of 1 M HCl or 1 M NaOH, as appropriate. Ionomycin, TG, methyl isobutyl amiloride (MIA) and diisothiocyanatostilbene disulfonate (DIDS), bovine serum albumin (BSA), and collagenase were obtained from Wako Pure Chemical Industries (Osaka, Japan). The reagents were dissolved in dimethyl sulfoxide (DMSO) and stored for stock solutions. Stock solutions were diluted to their final concentrations just before the experiments. DMSO concentrations never exceeded 0.1%. DMSO (0.1%) has no effects on $[Ca^{2+}]_i$ and pH_i. All the experiments were performed at 37°C.

Cell preparation and fluorescence measurements

Male rats (Slc:Wistar/ST; Japan SLC, 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 (60–70 mg kg^{-1}). The submandibular glands were removed from the rats for cell isolation, and the rats were then killed by the additional injection of pentobarbital sodium (100 mg kg^{-1}). The experiments were approved by the Animal Research Committee of Osaka Medical College, and the rats were cared for according to the guidelines of this committee. The procedures for the cell preparations have already been described in detail [13]. The submandibular glands were minced with a collagenase solution (0.1%) and then incubated (37°C) for 15 min. The digested tissue was filtered through a Nylon mesh (150 μm^2). The filtrate was centrifuged at 300 rpm (20*g*) for 1 min, washed three times, with centrifugation, 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, USA) to allow 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^{-1}. The fura 2 (340:380 nm) and BCECF (490:450 nm) fluorescence ratios were calculated and stored in the image-analysis system [13–15]. The calibration curve for pH_i was obtained from the F_{490}/F_{450} values of the BCECF-loaded cells, which were perfused with solution II containing nigericin (10 μg/ml). The pH of solution II was set at 6.6, 7.0, 7.2, 7.4, 7.8, or 8.0. Solution II contained (mM): KCl 130, NaCl 20, $MgSO_4$ 1, and HEPES 10. One experiment was performed using 6–12 coverslips from 3 to 4 animals. A typical response obtained from 7 to 9 cells in a coverslip is shown in the figures. To compare values among experiments, mean values obtained from 3 to 6 coverslips were used.

Activation of Ca^{2+} entry

Before addition of IM, cells were perfused with a Ca^{2+}-free solution for 5 min. IM was then added and cells were

Table 1 Time constants of pH_i increase following the re-introduction of Ca^{2+}

Experiments	τ_1 (min)	τ_2 (min)	n
IM (5 μM)	0.55 ± 0.10	1.73 ± 0.27	6
IM (5 μM) + MIA	0.95 ± 0.13*	2.36 ± 0.42	4
IM (5 μM) + DIDS	0.37 ± 0.04	0.81 ± 0.11*	4
IM (5 μM) + ACZ	–	1.15 ± 0.02	4
IM (5 μM) + HCO_3^--free	0.92 ± 0.12*	3.01 ± 0.56	7
IM (1 μM)	0.62 ± 0.4	–	7
IM (1 μM) + HCO_3^--free	1.58 ± 0.34*		3

Values are expressed as means ± SE

* Significantly different compared with values obtained by use of IM alone (5 μM) ($p < 0.05$)

incubated for an additional 10 min. The Ca^{2+}-free solution was suddenly replaced with the control solution (1.5 mM Ca^{2+}) maintaining an IM concentration (re-introduction of Ca^{2+}). The re-introduction of Ca^{2+} enables us to observe only a Ca^{2+} entry, because intracellular stores have already been depleted [11–13].

Calculation of time constant (τ)

To compare pH_i increase among different experiments, we calculated the time constant (τ) of changes in $[H^+]_i$. The changes in $[H^+]_i$ are expressed by Eq. 1:

$$\left([H^+]_{it} - [H^+]_{i\infty}\right) = \left([H^+]_{i0} - [H^+]_{i\infty}\right) \cdot \exp(-t/\tau) \quad (1)$$

where "t" is the time, and the subscripts "0" and "∞" indicate $[H^+]_i$ at $t = 0$ and $t = \infty$, respectively.

Equation 1 can be rewritten as Eq. 2:

$$\mathrm{Ln}\left(\left([H^+]_{it} - [H^+]_{i\infty}\right)/\left([H^+]_{i0} - [H^+]_{i\infty}\right)\right) = -1/\tau \cdot t \quad (2)$$

When "ln $(([H^+]_{it} - [H^+]_{i\infty})/([H^+]_{i0} - [H^+]_{i\infty}))$" is plotted against t (τ plot), the slope is $-1/\tau$. In this study we used τ as an index of the rate of pH_i increase. Therefore, we compared the pH_i increases following the re-introduction of Ca^{2+} among experiments by using τ (Table 1).

The statistical significance of the differences between the means was assessed using paired and unpaired Student's t tests, as appropriate. Differences were considered significant at $p < 0.05$.

Results

Intracellular pH changes induced by IM in the presence and the absence of HCO_3^-

Changes in intracellular pH (pH_i) were measured in submandibular acinar cells using 5 and 1 μM IM (Fig. 1). In the control solution, the pH_is of rat submandibular acinar cells were ~7.4 (Fig. 1).

In the presence of HCO_3^-, the switch to Ca^{2+}-free solution did not induce any pH_i change; then, further addition of 5 μM IM induced a small transient pH_i decrease, the duration of which was ~10 min (Fig. 1a). Re-introduction of Ca^{2+} induced a rapid and transient pH_i decrease followed by a gradual pH_i increase. The pH_is 1 and 3 min after re-introduction of Ca^{2+} were 6.89 ± 0.04 and 7.50 ± 0.03 ($n = 6$), respectively. The pH_i 3 min after re-introduction of Ca^{2+} was higher than that before re-introduction of Ca^{2+} (Fig. 1a). The time constant of the pH_i increase was calculated from the τ plot (Fig. 1b). The τ plot showed that the pH_i increase consisted of two components; a rapid increase (the first phase within 0.5 min after the rapid pH_i decrease) followed by a gradual increase (the second phase within 0.5–2.5 min after the rapid pH_i decrease). The time constants of the first phase (τ_1) and the second phase (τ_2) are shown in Table 1.

Similar experiments were carried out using 1 μM IM. The switch to Ca^{2+}-free solution and addition of 1 μM IM did not change pH_i. The re-introduction of Ca^{2+} induced a rapid transient pH_i decrease followed by a plateau. The pH_is 1 and 4 min after the re-introduction of Ca^{2+} were 7.02 ± 0.03 and 7.32 ± 0.03 ($n = 7$), respectively (Fig. 1c). The final pH_i during 1 μM IM stimulation was low compared with that during 5 μM IM stimulation ($p < 0.05$). The τ plot showed that the pH_i increase consisted of one component without any gradual increase (Fig. 1d; Table 1). The value of τ_1 obtained by use of 1 μM IM was not significantly different from that obtained by use of 5 μM IM ($p > 0.05$) (Table 1).

The experiments were also performed in the absence of HCO_3^- (Fig. 2). The switch to HCO_3^--free solution transiently increased pH_i, which then plateaued. The pH_is 2 and 5 min after the switch were 7.60 ± 0.04 and 7.41 ± 0.03 ($n = 7$), respectively. Then, the switch to the Ca^{2+}-free solution and addition of 5 μM IM did not change pH_i. The re-introduction of Ca^{2+} induced a rapid transient pH_i decrease followed by a gradual pH_i increase (Fig. 2a). The pH_is 1 and 5 min after the re-introduction of Ca^{2+} were 7.15 ± 0.03 and 7.48 ± 0.04 ($n = 7$), respectively. The τ plot showed that the pH_i increase still consisted of two components (Table 1) (data not shown). The τ_1 in the absence of HCO_3^- was significantly greater than that in the presence of HCO_3^- ($p < 0.05$), whereas τ_2 was not affected by HCO_3^--free solution ($p > 0.05$) (Table 1). Thus, the gradual pH_i increase stimulated by 5 μM IM is not induced by HCO_3^- entry, because there is no HCO_3^- outside the cells.

The experiments were also carried out using 1 μM IM (Fig. 2b). The switch to HCO_3^--free solution increased pH_i transiently. Then, the switch to Ca^{2+}-free solution and

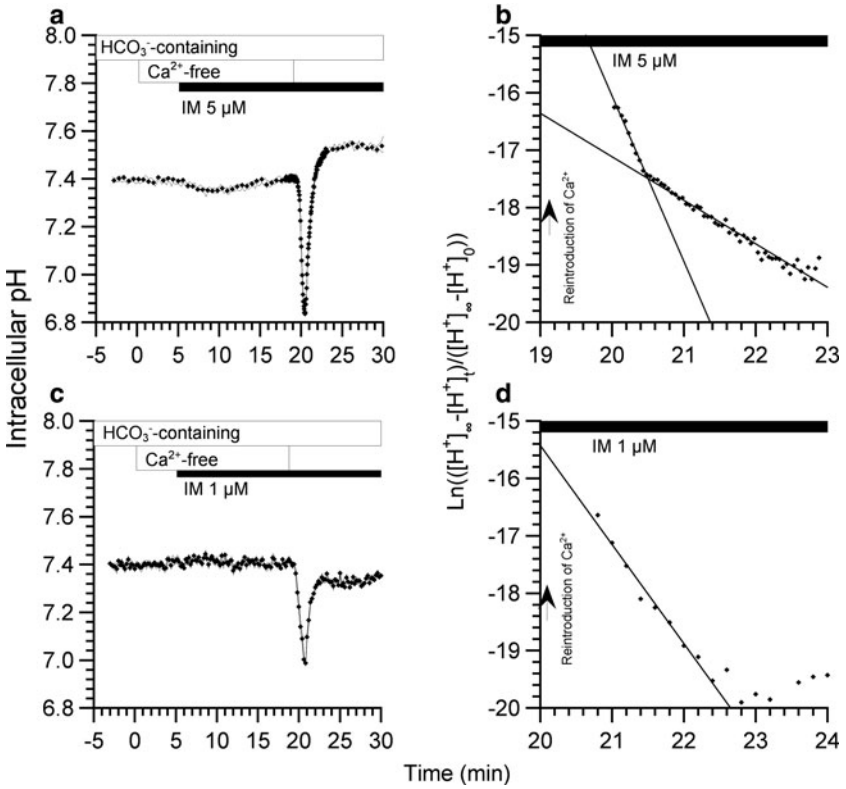

Fig. 1 Intracellular pH (pH_i) changes induced by ionomycin (IM) in the presence of HCO_3^-. **a** 5 μM IM in HCO_3^--containing solution. IM (5 μM) induced no pH_i decrease in Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a large transient pH_i decrease followed by a rapid pH_i increase. The plateau pH_i was slightly higher than that before re-introduction of Ca^{2+}. **b** τ Plot of **a**. The pH_is for 4 min after re-introduction of Ca^{2+} were used for the τ plot. The τ plot clearly shows that the pH_i increase following the rapid pH_i decrease consisted of two phases: the first phase followed by the second phase. **c** 1 μM IM in HCO_3^--containing solution. The re-introduction of Ca^{2+} induced a large transient pH_i decrease followed by a rapid pH_i increase. The plateau pH_i was slightly lower than that before the re-introduction of Ca^{2+}. **d** τ plot of **c**. The pH_is for 4 min after re-introduction of Ca^{2+} were used. The τ plot revealed that the pH_i increase following the rapid pH_i decrease consisted of the single component during 1 μM IM stimulation

further addition of 1 μM IM induced no pH_i change. The re-introduction of Ca^{2+} induced a rapid pH_i decrease followed by a small pH_i increase. The pH_is 1.5 and 5 min after re-introduction of Ca^{2+} were 7.17 ± 0.03 and 7.25 ± 0.02 ($n = 7$), respectively (Fig. 2b). The τ plot showed that the first phase was still observed during 1 μM IM in the absence of HCO_3 and the τ_1 was significantly greater than that in the presence of HCO_3^- ($p < 0.05$) (Table 1). Moreover, the extent of the rapid pH_i decrease was less in the absence of HCO_3^- than in the presence of HCO_3^-. These observations suggest that the transient pH_i decrease is accelerated by the presence of HCO_3^-.

Experiments were carried out using thapsigargin (TG, 4 μM). In HCO_3^--containing solution, the switch to the Ca^{2+}-free solution and the addition of TG did not change pH_i. On re-introduction of Ca^{2+} pH_i decreased gradually and plateaued within 3 min (Fig. 3a). In HCO_3^--free solution, the switch to Ca^{2+}-free solution and addition of TG did not change pH_i. On re-introduction of Ca^{2+} pH_i decreased gradually (Fig. 3b). Thus, TG did not induce any rapid transient pH_i decrease after re-introduction of Ca^{2+}. Thus, the transient pH_i decrease induced by IM is not caused by an $[Ca^{2+}]_i$ increase.

Effects of acetazolamide on the transient pH_i decrease

We also examined the effects of acetazolamide (100 μM), an inhibitor of carbonic anhydrase. Because the absence of HCO_3^- decreased the extent of rapid transient pH_i decrease and increased the value of τ_1 (Figs. 1, 2; Table 1). Experiments were carried out in the presence of HCO_3^-. In Ca^{2+}-free solution, the addition of acetazolamide and the further addition of 5 μM IM induced no pH_i change. Re-introduction of Ca^{2+} induced only a small pH_i decrease followed by a gradual pH_i increase (Fig. 4a). The τ plot showed that the pH_i increase consisted of one component (Fig. 4b). The time constant of the gradual pH_i increase was similar to τ_2 obtained by use of 5 μM IM alone (Table 1). Experiments were carried out using 1 μM IM (Fig. 4c). In a Ca^{2+}-free solution, addition of acetazolamide and IM (1 μM) induced no pH_i change. The re-introduction of Ca^{2+} induced no transient pH_i decrease,

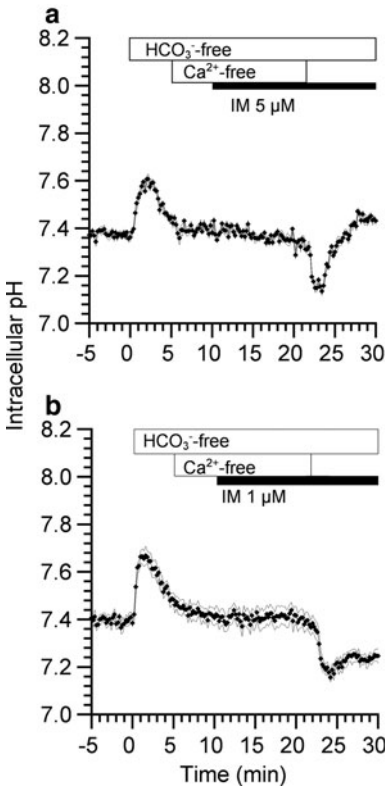

Fig. 2 Intracellular pH (pH_i) changes induced by ionomycin (IM) in the absence of HCO_3^-. **a** 5 μM IM in HCO_3^--free solution. IM (5 μM) induced no change in pH_i in Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a rapid pH_i decrease followed by a gradual pH_i increase. **b** 1 μM IM in HCO_3^--free solution. When control solution was switched to HCO_3^--free solution, pH_i transiently increased from 7.4 to 7.7 and then plateaued (pH_i 7.5). The switch to Ca^{2+}-free solution and addition of IM (1 μM) did not change pH_i. The re-introduction of Ca^{2+} induced a rapid pH_i decrease without any pH_i increase

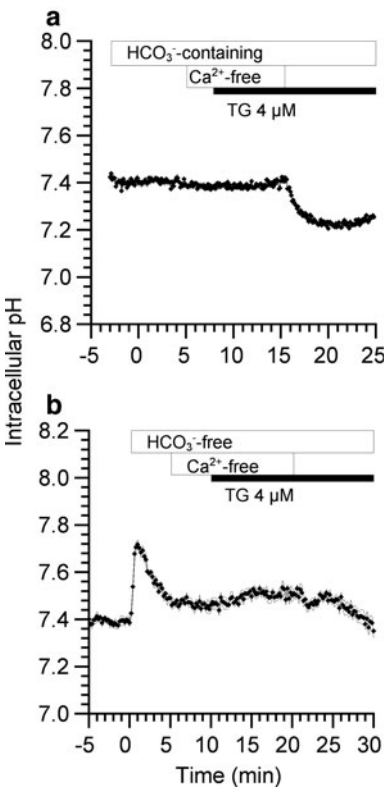

Fig. 3 Intracellular pH (pH_i) changes induced by thapsigargin (TG). **a** HCO_3^- containing solution. TG (4 μM) induced no pH_i decrease in Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a gradual pH_i decrease without any rapid pH_i increase. **b** HCO_3^--free solution. The switch to HCO_3^--free solution, increased pH_i transiently, and it then plateaued (pH_i 7.5). The switch to Ca^{2+}-free solution and addition of IM (1 μM) did not change pH_i. The re-introduction of Ca^{2+} induced a gradual pH_i decrease without any pH_i increase

and then pH_i decreased slightly. Thus, acetazolamide abolished the transient pH_i decrease after the re-introduction of Ca^{2+} in IM-treated cells, suggesting that the rapid transient pH_i decrease induced by IM was coupled with the reaction ($H^+ + HCO_3^- \rightleftarrows CO_2$).

Effects of Na^+/H^+ exchange, Na^+-HCO_3^- cotransport, and HCO_3^- on the transient pH_i decrease

To examine the effects of Na^+/H^+ exchange on the rapid pH_i decrease followed by the rapid increase in the presence of HCO_3^-, an inhibitor of the Na^+/H^+ exchange (MIA, 10 μM) was used (Fig. 5a). After switching to Ca^{2+}-free solution, addition of MIA and IM induced no pH_i change. Re-introduction of Ca^{2+} induced a large rapid pH_i decrease (pH_i 6.53 ± 0.03, $n = 3$), followed by a pH_i increase (pH_i 7.38 ± 0.03, 5 min after re-introduction of Ca^{2+}). The τ plot showed that the pH_i increase consisted of two components (Table 1), and the value of τ_1 is significantly greater than that obtained by use of 5 μM IM alone ($p < 0.05$) (Table 1). Thus, MIA enhanced the extent of rapid pH_i decrease (Fig. 5a) and delayed a pH_i recovery in the rapid transient pH_i decrease, although MIA did not significantly increase τ_2 ($p > 0.05$). An increase in $[Ca^{2+}]_i$ may activate H^+ extrusion via Na^+/H^+ exchangers in submandibular acinar cells, the inhibition of which enhances acidification.

We examined the effects of the Cl^-/HCO_3^- exchange on the rapid transient pH_i decrease followed by a gradual pH_i increase by using an inhibitor of Cl^-/HCO_3^- exchange (DIDS, 200 μM) (Fig. 5b). After the switch to Ca^{2+}-free solution, addition of DIDS and IM induced no pH_i change. Re-introduction of Ca^{2+} induced a rapid transient pH_i decrease followed by pH_i recovery. The pH_is 1.5 and 5 min after the re-introduction of Ca^{2+} were 6.84 ± 0.01 and 7.54 ± 0.01 ($n = 4$), respectively. The τ plot showed that the pH_i increase consisted of two components (Table 1). The value of τ_1 was similar to that for 5 μM IM alone ($p > 0.05$), but that of τ_2 was significantly reduced ($p < 0.05$). A decrease in τ_2 value induced by DIDS may

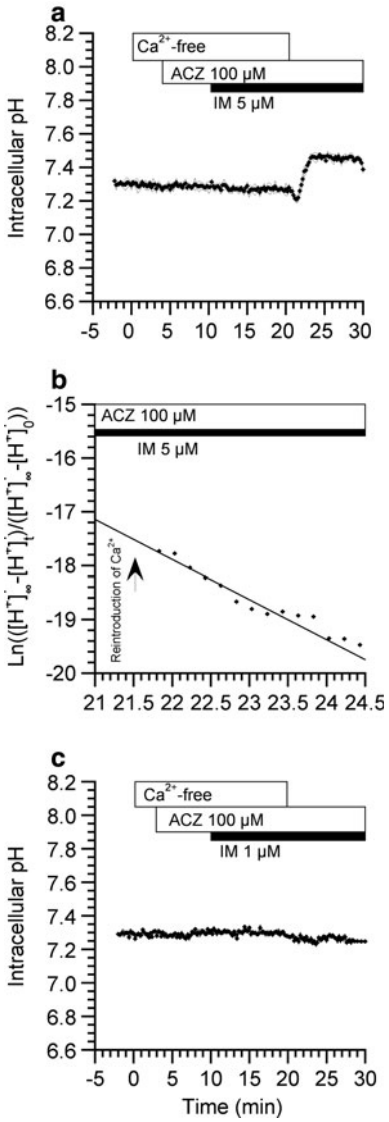

Fig. 4 Effects of 100 μM acetazolamide (ACZ, an inhibitor of carbonic anhydrase) on pH_i changes induced by IM. **a** 5 μM IM. The addition of ACZ and the further addition of 5 μM IM have no effect on pH_i in Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a small pH_i decrease followed by a gradual pH_i increase. **b** τ plot of **a**. The pH_is for 4 min after the re-introduction of Ca^{2+} were used. The τ plot revealed that the pH_i increase after re-introduction of Ca^{2+} consisted of the single component with ACZ. **c** 1 μM IM. ACZ abolished the pHi changes induced by 1 μM IM

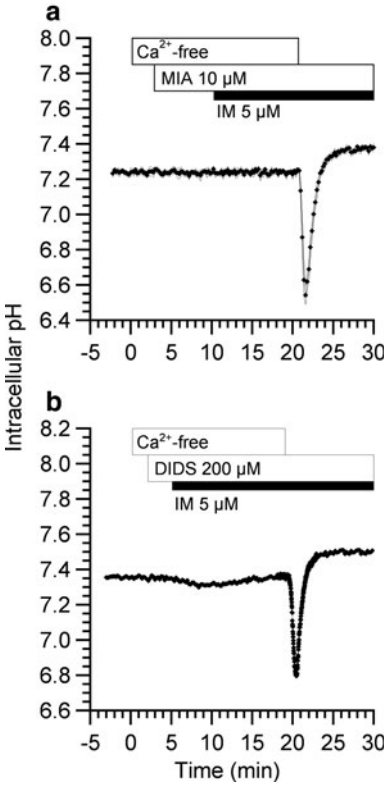

Fig. 5 Effects of MIA and DIDS on pHi changes induced by 5 μM IM. **a** MIA (10 μM, an inhibitor of Na^+/H^+ exchange) has no effect on pH_i. Re-introduction of Ca^{2+} induced a rapid pH_i decrease followed by a rapid pH_i increase. The pH_i decrease stimulated by 5 μM IM was enhanced by MIA in submandibular acinar cells. **b** DIDS (200 μM, an inhibitor of Cl^-/HCO_3^- exchange and Na^+-HCO_3^- cotransport). DIDS did not inhibit the rapid transient pHi decrease. The pH_i increase after the pH_i decrease seems to be faster with DIDS than that without DIDS

be caused by inhibition of Cl^-/HCO_3^- exchange. Inhibition of the Cl^-/HCO_3^- exchange by DIDS increases $[HCO_3^-]_i$ which accelerates pH_i increase.

DIDS also inhibits Na^+-HCO_3^- cotransport. If Na^+-HCO_3^- cotransport is active in submandibular acinar cells, inhibition of the cotransport increases the extent of rapid pH_i decrease and delays pH_i increase. Because DIDS did not increase the extent of rapid pH_i decrease and accelerated pH_i increase in this study, activity of Na^+-HCO_3^- cotransport seems to be low in rat submandibular acinar cells.

IM-induced Ca^{2+} entry

$[Ca^{2+}]_i$ was measured in rat submandibular acinar cells using 5 and 1 μM IM (Fig. 6). Experiments were carried out in the presence of HCO_3^-. Cells were first perfused with control solution, and then with Ca^{2+}-free solution. The switch to Ca^{2+}-free solution slightly reduced $[Ca^{2+}]_i$, which plateaued within 3 min (data not shown). Addition of 5 μM IM induced a transient $[Ca^{2+}]_i$ increase in the Ca^{2+}-free solution. The re-introduction of Ca^{2+} immediately increased $[Ca^{2+}]_i$, which plateaued within 3 min. The F_{340}/F_{380} plateau was extremely high (Fig. 6a). The large $[Ca^{2+}]_i$ increase following re-introduction of Ca^{2+} was not inhibited by 1 μM Gd^{3+}, although 1 μM Gd^{3+} reduced the rate of Ca^{2+} entry and the sustained $[Ca^{2+}]_i$ by ~10% (Fig. 6a).

Similar experiments were carried out using 1 μM IM and 4 μM TG. Addition of 1 μM IM induced a small transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a biphasic $[Ca^{2+}]_i$ increase, that is, a rapid transient increase followed by a

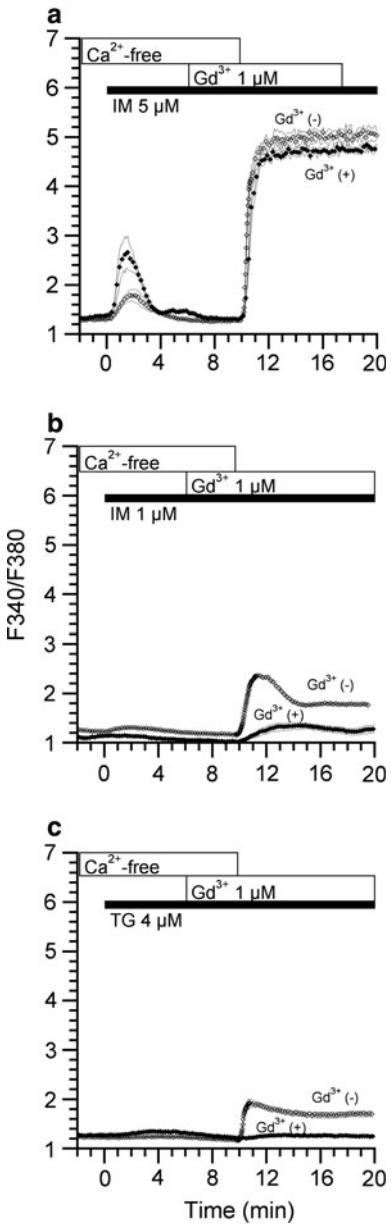

Fig. 6 IM-induced $[Ca^{2+}]_i$ increases after re-introduction of Ca^{2+}. **a** IM (5 μM). Experiments were carried out using HCO_3^--containing solution. Addition of 5 μM IM induced a large transient increase in fura 2 fluorescence ratio (F_{340}/F_{380}) in Ca^{2+}-free solution. Re-introduction of Ca^{2+} increased F_{340}/F_{380}, which plateaued within 3 min. The plateau level was extremely high. This increase was not inhibited by 1 μM Gd^{3+}. **b** and **c** IM (1 μM, **b**) and TG (4 μM, **c**). Addition of 1 μM IM or 4 μM TG induced a small transient increase in F_{340}/F_{380} in Ca^{2+}-free solution. Re-introduction of Ca^{2+} induced a biphasic increase in F_{340}/F_{380}, which was inhibited by 1 μM Gd^{3+}

sustained increase in $[Ca^{2+}]_i$ (Fig. 6b). The biphasic $[Ca^{2+}]_i$ increase after re-introduction of Ca^{2+} was inhibited by 1 μM Gd^{3+} (Fig. 6b). The sustained F_{340}/F_{380} stimulated with 1 μM IM was much less than that stimulated with 5 μM IM (Fig. 6a, b). Similar results were obtained by use of 4 μM TG (Fig. 6c).

Effects of pH_o on the rate of $[Ca^{2+}]_i$ increase

Experiments were carried out using the same procedure as shown in Fig. 6. The pH_os used were 6.8, 7.4, and 8.0. When 5 μM IM was used for stimulation, re-introduction of Ca^{2+} immediately increased and plateaued $[Ca^{2+}]_i$ (F_{340}/F_{380}). The final plateaus of $[Ca^{2+}]_i$ were similar at the three pH_os. The rate of Ca^{2+} entry was calculated from the rate of F_{340}/F_{380} increase (/min). The rate of Ca^{2+} entry increased with changing pH_o from 6.8 to 8.0 (Fig. 7a, c). In contrast, when 1 μM IM was used for stimulation, re-introduction of Ca^{2+} immediately increased and then gradually reduced F_{340}/F_{380}. Increases in $[Ca^{2+}]_i$ and the rates of Ca^{2+} entry were almost the same at the three pH_os (Fig. 7a, c). The rates of Ca^{2+} entry were plotted against pH_os. In cells stimulated with 5 μM IM, a pH_o increase enhanced Ca^{2+} influx whereas in cells stimulated with 1 μM IM or 4 μM TG, it did not affect Ca^{2+} influx (Fig. 7c). Thus, Ca^{2+} influxes stimulated by 5 μM IM were because of the Ca^{2+}/H^+ exchange.

Ca^{2+} entry induced by IM in the absence of HCO_3^-

$[Ca^{2+}]_i$ was also measured using 5 and 1 μM IM in HCO_3^--free solution (Fig. 8). The switch to Ca^{2+}-free solution slightly reduced $[Ca^{2+}]_i$, which plateaued within 3 min (data not shown). Addition of 5 μM IM induced a transient $[Ca^{2+}]_i$ increase in the Ca^{2+}-free solution. Re-introduction of Ca^{2+} immediately increased $[Ca^{2+}]_i$ to an extremely high value within 3 min (Fig. 8a). The large $[Ca^{2+}]_i$ increase with 5 μM IM was not inhibited by 1 μM Gd^{3+}, similarly to Fig. 6a (data not shown).

Addition of 1 μM IM induced a small transient increase in $[Ca^{2+}]_i$ in the Ca^{2+}-free solution. The re-introduction of Ca^{2+} induced a biphasic $[Ca^{2+}]_i$ increase, that is, a rapid transient increase followed by a sustained increase in $[Ca^{2+}]_i$ (Fig. 8b). The biphasic $[Ca^{2+}]_i$ increase following to the re-introduction of Ca^{2+} was inhibited by 1 μM Gd^{3+}, similarly to Fig. 6b (data not shown). Similar results were obtained by addition of 4 μM TG (data not shown). Thus, HCO_3^--free solution did not affect $[Ca^{2+}]_i$ increases after re-introduction of Ca^{2+} during stimulation with 1 or 5 μM IM. The rates of Ca^{2+} entry in the absence of HCO_3^- at 7.4 pH_o are plotted in Fig. 7. The rates of $[Ca^{2+}]_i$ increase in the absence of HCO_3^- were similar to those in the presence of HCO_3^- at pH_o 7.4.

Discussion

This study demonstrated that IM increases $[Ca^{2+}]_i$ via two pathways depending on its concentration. One is the SOCs activated by depletion of intracellular stores, which are

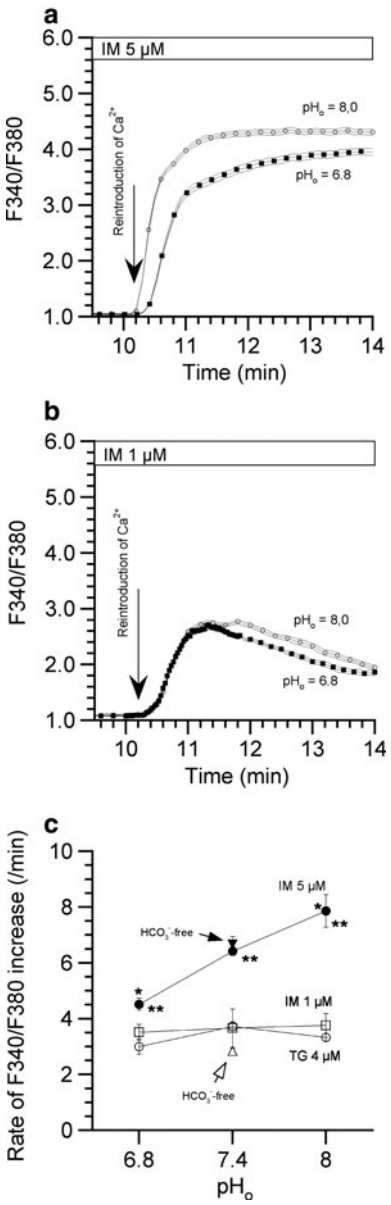

Fig. 7 Effects of extracellular pH (pH$_o$) on the rate of F_{340}/F_{380} increase (Ca^{2+} influx) stimulated by IM. Experiments were carried out using a HCO_3^--containing solution. **a** 5 μM IM: increases in F_{340}/F_{380} after re-introduction of Ca^{2+} at pH$_o$ of 8.0 and 6.8. Increases in F_{340}/F_{380} were enhanced at a pH$_o$ of 8.0. **b** 1 μM IM. Increases in F_{340}/F_{380} after re-introduction of Ca^{2+} at pH$_o$ of 8.0 and 6.8. Increases in F_{340}/F_{380} were not affected by pH$_o$. **c** Rates of the F_{340}/F_{380} increase were plotted against pH$_o$. Upon stimulation with 5 μM IM, the rates increased with increasing pH$_o$. However, upon stimulation with 1 μM IM, the rates remained almost constant with increasing pH$_o$. The rates of F_{340}/F_{380} increase obtained in HCO_3^--free solution (data calculated from Fig. 8) are also plotted in this figure

sensitive to Gd^{3+} and activated by a low concentration of IM (1 μM). The other is Ca^{2+}/H^+ exchange, which is Gd^{3+}-insensitive, accelerated by an increase of pH$_o$, and mediated by a high concentration of IM (5 μM), [5, 6, 17].

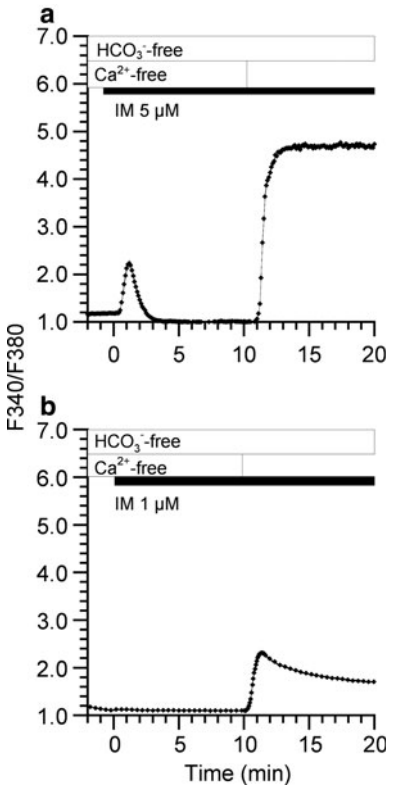

Fig. 8 IM-induced $[Ca^{2+}]_i$ increase after re-introduction of Ca^{2+} in HCO_3^--free solution. The re-introduction of Ca^{2+} increases $[Ca^{2+}]_i$ in rat submandibular acinar cells stimulated with 5 μM or 1 μM IM. Increases in $[Ca^{2+}]_i$ in the absence of HCO_3^- were similar to those in the presence of HCO_3^-. **a** 5 μM IM, **b** 1 μM IM

This study also demonstrated that 5 μM IM induced a large transient pH$_i$ decrease after re-introduction of Ca^{2+}. The pH$_i$ changes induced by 5 μM IM consisted of two phases. The first phase is a rapid transient pH$_i$ decrease, and the second phase is a gradual pH$_i$ increase. The rapid transient pH$_i$ decrease was reduced by HCO_3^--free solution and abolished by ACZ, whereas the gradual pH$_i$ increase was not affected by HCO_3^--free solution or ACZ. Thus, the rapid transient pH$_i$ decrease was HCO_3^--dependent and the gradual pH$_i$ increase was HCO_3^--independent.

IM (1 μM) induced a rapid transient pH$_i$ decrease, but not a gradual pH$_i$ increase. Because 5 μM IM, not 1 μM IM, mediates Ca^{2+}/H^+ exchange, the gradual pH$_i$ increase is induced by the Ca^{2+}/H^+ exchange. Moreover, TG, which increased $[Ca^{2+}]_i$, induced no rapid transient pH$_i$ decrease and no gradual pH$_i$ increase, although it gradually reduced pH$_i$. Thus, the rapid transient pH$_i$ decrease was induced by IM, not by an $[Ca^{2+}]_i$ increase.

On the other hand, the rapid transient pH$_i$ decrease followed by the gradual pH$_i$ increase was not abolished by MIA and DIDS, although MIA enhanced the extent of rapid pH$_i$ decrease and delayed the pH$_i$ recovery (an

increase in τ_1), and DIDS accelerated the pH_i recovery (a decrease in τ_2). Thus, Na^+/H^+ exchangers (H^+ extrusion) or Cl^-/HCO_3^- exchangers (HCO_3^- extrusion) do not cause the transient pH_i decrease stimulated by IM, although they modified the pH_i changes induced by IM. The pH_i changes in DIDS-treated cells also suggest that the activity of Na^+-HCO_3^- cotransporters (HCO_3^- uptake) in rat submandibular acinar cells is low, because DIDS did not enhance the rapid pH_i decrease and did not delay pH_i recovery. Moreover, an increase in $[Ca^{2+}]_i$ induced by TG did not induce any rapid transient pH_i decrease, especially in the absence of HCO_3^-. If a $[Ca^{2+}]_i$ increase stimulates HCO_3^- secretion via anion channels, TG should reduce pH_i rapidly, similarly to IM. These observations indicate that the rapid pH_i decrease is not induced by HCO_3^- secretion. The extent of pH_i decrease may affect pH_i recovery. The extent of rapid pH_i decrease with DIDS was less than that with MIA. However, pH_i recovery with DIDS was faster than with MIA. This may suggest that the gradients of H^+ between intra-cellular and extra-cellular spaces do not directly affect H^+ extrusion.

As mentioned above, the gradual pH_i increase was induced by the Ca^{2+}/H^+ exchange mediated by 5 μM IM. However, the rapid transient pH_i decrease induced by IM is not explained by cellular ion transporters activated by an $[Ca^{2+}]_i$ increase. An increase in $[Ca^{2+}]_i$, which stimulates cellular metabolisms, increases CO_2 production, resulting in a pH_i decrease. However, the pH_i decrease induced by TG was much slower than that induced by 1 μM IM. These results suggest that a rapid transient pH_i decrease is induced by a chemical characteristic of IM.

The rapid transient pH_i decrease induced by IM was HCO_3^--dependent as described above. The reactions of IM were considered as follows in the absence of both Ca^{2+} and HCO_3^- [5, 17].

$$[H_2 - IM] \rightleftarrows [H - IM]^- + H^+ \quad (3)$$

$$[H - IM]^- \rightleftarrows IM^{2-} + H^+ \quad (4)$$

Given that the highest alkaline pk_a of IM is >8 [18–20], the complex $[H–IM]^-$ should be the most abundant species at a physiological pH. These IM complexes are considered to be membrane-permeable [5].

For IM interacting with Ca^{2+}, relatively lipophilic complexes that bear a positive charge are monoprotonated forms of the 1:1 species ($[H–Ca–IM]^+$). Therefore, addition of Ca^{2+} induces reaction 5 at a physiological pH.

$$[H - IM]^- + Ca^{2+} \rightleftarrows [H - Ca - IM]^+ \quad (5)$$

Thus, addition of Ca^{2+} (re-introduction of Ca^{2+}) does not change pH_i in the absence of HCO_3^-. However, in the presence of HCO_3^-, reaction 5 is coupled with reaction 6.

$$CO_2 \rightleftarrows HCO_3^- + H^+ \quad (6)$$

The coupling of two reactions (5 and 6) produces an electroneutral Ca^{2+} complex, $[[H–Ca–IM]^+ \cdot HCO_3^-]$ [20] as shown in reaction 7.

$$[H - IM]^- + Ca^{2+} + CO_2 \rightleftarrows [[H - Ca - IM]^+ \cdot HCO_3^-] + H^+ \quad (7)$$

Thus, in the presence of HCO_3^-, a monoprotonated IM reacts with Ca^{2+} and CO_2 to produce an electroneutral Ca^{2+} complex and H^+, which rapidly reduces pH_i after re-introduction of Ca^{2+}. Previous reports suggest that, in IM-induced Ca^{2+} transport, electroneutral and electrogenic Ca^{2+} transports may occur, involving $[[H–Ca–IM]^+ \cdot X^-]$ and $[H–Ca–IM]^+$ species, respectively [5, 20]. Then, H^+ reacts with HCO_3^- to produce CO_2 (reaction 6). CO_2 is removed from the cell, which results in the rapid pH_i increase. Our hypothesis, in which IM induces a rapid transient pH_i decrease mediated via reactions 6 and 7, was supported by the experiments using ACZ: inhibition of reaction 6 abolished the rapid transient pH_i decrease.

The presence of HCO_3^- has been reported to enhance a transient pH_i decrease after removal of NH_4^+ pulse in alveolar type II cells [16]. The CO_2/HCO_3^- reaction also enhanced the transient pH_i decrease after removal of the NH_4^+ pulse in ATII cells. Thus, the CO_2/HCO_3^- reaction (reaction 6) seems to be important role in pH_i regulation in cells, especially on acute acid loading.

TG or 1 μM IM caused a transient increase in $[Ca^{2+}]_i$ after re-introduction of Ca^{2+}, whereas 5 μM IM caused a sustained plateau increase in $[Ca^{2+}]_i$. During TG stimulation, an immediate increase in $[Ca^{2+}]_i$ after re-introduction of Ca^{2+} is considered to be due to Ca^{2+} entry but the plateau level is affected by Ca^{2+} extrusion pathways, most notably mitochondrial Ca^{2+} uptake and Ca^{2+} extrusion via plasma membrane Ca^{2+}-ATPase (PMCA). Because IM does not inhibit SERCA (sarcoendoplasmic reticulum Ca^{2+} ATPase), the transient increase in $[Ca^{2+}]_i$ induced by 1 μM IM seems to reflect the activation of mitochondrial Ca^{2+} uptake and PMCA. However, during stimulation with 5 μM IM, these clearance pathways seem to be inhibited. PMCA activities are known to be inhibited by cytosolic acidification [21, 22]. In this study, 5 μM IM was demonstrated to induce marked cytosolic acidification, which may inhibit PMCA. Moreover, at pH_o 6.8, the sustained $[Ca^{2+}]_i$ responses were similar to those at pH_o 7.4 and pH_o 8.0, that is, not transient. This may suggest that the sustained plateau is caused by the inhibition of PMCA by 5 μM IM-induced cytosolic acidification. However, in the presence of HCO_3^-, the cytosolic acidification induced by 5 μM IM was transient. After pH_i recovery, the sustained plateau $[Ca^{2+}]_i$ was not reduced. A high concentration of

IM, for example 5 μM, may induce acidification inside the mitochondria by H^+ uptake coupled with Ca^{2+} release via the IM-mediated Ca^{2+}/H^+ exchange. This may inhibit ATP synthesis resulting in inhibition of PMCA and SERCA. IM (5 μM) seems to inhibit Ca^{2+} extrusion pathways. Further studies will be needed to clarify this.

Acknowledgments This work is carried out in the Nakahari Project of the central research laboratory (Osaka Medical College), and is partially supported by Grants-in Aid for Scientific Research from the Japan Society of the Promotion of Science, to TN (21590243).

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