Mitochondria Take Up Ca^{2+} in Two Steps Dependently on Store-Operated Ca^{2+} Entry in Mast Cells

Marie Takekawa,^a Tadahide Furuno,^a Naohide Hirashima,^b and Mamoru Nakanishi^{*a}

^aSchool of Pharmacy, Aichi Gakuin University; 1–100 Kusumoto-cho, Chikusa-ku, Nagoya 464–8650, Japan; and

^bGraduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan. Received September 30, 2011; accepted May 28, 2012; published online May 30, 2012

The ability of mitochondria to take up Ca^{2+} has important functional implications for modulation of cellular Ca^{2+} signaling. Mitochondrial Ca^{2+} uptake is stimulated by an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$). Here, we found that the increase in mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) occurs in two steps in a single antigen-activated mast cell in the presence of extracellular Ca^{2+} (1.0 mM). The two-step elevation of $[\text{Ca}^{2+}]_m$ was also observed after adding thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase. The proportion of mitochondria showing the two-step Ca^{2+} elevation dropped off in direct accord with decrease in extracellular Ca^{2+} concentration. The second step of the $[\text{Ca}^{2+}]_m$ increase was suppressed significantly in the absence of extracellular Ca^{2+} and in knockdown cells of stromal interaction molecule 1 (STIM1), an essential molecule on endoplasmic reticulum (ER) membrane for store-operated Ca^{2+} entry, in the presence of extracellular Ca^{2+} (1.0 mM), while the first elevation was not affected in either case. The results indicate that mitochondria take up cytosolic Ca^{2+} in two steps; first and second uptakes are derived from the Ca^{2+} release from ER and the Ca^{2+} influx through store-operated Ca^{2+} channels, respectively. Additionally, rotenone and antimycin A, which are inhibitors of mitochondrial electron transport complex I and III, respectively, diminished mitochondrial Ca^{2+} uptake and significantly suppressed degranulation stimulated with antigen. The mitochondrial Ca^{2+} uptake may modulate mast cell function by regulating the $[\text{Ca}^{2+}]_c$.

Key words mitochondria; calcium ion; mast cell; stromal interaction molecule 1; Rhod-2; degranulation

Ca^{2+} is a highly versatile second messenger that regulates a variety of cellular responses in eukaryotic organisms.¹⁾ In resting cells, the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) is maintained at a low level through energy-dependent pumping activity of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPases (SERCA) and plasma membrane Ca^{2+} -ATPases (PMCA) to exclude cytosolic Ca^{2+} into the endoplasmic reticulum (ER) and out of the plasma membrane, respectively. To trigger $[\text{Ca}^{2+}]_c$ elevation, an agonist bound to receptors induces the activation of phospholipase C (PLC) and consequent generation of inositol 1,4,5-triphosphate (IP_3) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate. The increase of IP_3 leads to the release of Ca^{2+} from the ER, and the decrease of Ca^{2+} in ER induces the opening of Ca^{2+} channels on the plasma membrane and, subsequently, store-operated Ca^{2+} entry (SOCE) through a stromal interaction molecule 1 (STIM1) in the ER and an Orai Ca^{2+} channel in the plasma membrane.²⁾ The increased $[\text{Ca}^{2+}]_c$ remains constant at a high level after stimulation by an equilibrium between the amount of Ca^{2+} influx and Ca^{2+} export. The PMCA is considered to be intimately involved with Ca^{2+} export because its dissociation constant for Ca^{2+} drops by *ca.* 100-fold under activated condition compared to resting state.³⁾

Mitochondria not only generate ATP but also regulate cellular calcium signals by accumulating and releasing Ca^{2+} .⁴⁾ The ability of mitochondria to act as Ca^{2+} buffers has important consequences for the pattern of calcium signaling. Mitochondrial Ca^{2+} uptake is mainly accomplished by the Ca^{2+} uniporter, and Ca^{2+} efflux occurs through the $\text{Na}^+/\text{Ca}^{2+}$ and $\text{H}^+/\text{Ca}^{2+}$ exchangers. This mitochondrial Ca^{2+} buffering ability has different consequences for cellular response.⁵⁾ Mitochondrial Ca^{2+} accumulation was influenced by the membrane potential across the inner membrane. Therefore inhibitors of

mitochondrial electron transport complex I or III including rotenone and antimycin A have been used to study the effect of mitochondrial Ca^{2+} uptake on cellular function because they cause a decreased membrane potential and a lesser degree of depolarization that complicates the interpretation of effect of mitochondrial Ca^{2+} handling.⁶⁾ Recently, new mitochondrial innermembrane proteins involved in mitochondrial Ca^{2+} uptake were identified; the mitochondrial Ca^{2+} uptake 1 (MICU1) played a role in Ca^{2+} sensing and Ca^{2+} uptake,⁷⁾ and the mitochondrial Ca^{2+} uniporter (MCU) functioned as the elusive Ca^{2+} uniporter of the mitochondrial membrane.^{8,9)}

Mast cells express high-affinity immunoglobulin E (IgE) receptors (FcεRI) on their surface, and the aggregation of FcεRI triggers cellular activation. Antigen-induced crosslinking of FcεRI induces the activation of several protein tyrosine kinases including Lyn, Fyn, Syk, and Btk.¹⁰⁾ These events lead to phosphorylation of the linker for activation of T cells (LAT), and the phosphorylated LAT recruits PLCγ and several adaptor molecules.¹¹⁾ The phosphorylation of recruited PLCγ produces IP_3 and diacylglycerol, which elicit the increase in $[\text{Ca}^{2+}]_c$ and protein kinase C (PKC) activity, respectively. Especially, STIM1/Orai-mediated SOCE is essential for mast cell function because downstream signaling of $[\text{Ca}^{2+}]_c$ increase and PKC activation is important to cellular function such as degranulation, lipid mediator release, and cytokine production in mast cells.

Accordingly, the manner in which mitochondria contribute to the intracellular Ca^{2+} signaling in mast cells is interesting. However, it is controversial whether mitochondria release Ca^{2+} or take up Ca^{2+} in activated mast cells.^{12,13)} Here, we observed simultaneous Ca^{2+} mobilization in individual mitochondria and in the cytoplasm in an activated single mast cell. We found that mitochondria captured Ca^{2+} from the cytoplasm in

*To whom correspondence should be addressed. e-mail: mamoru@dpc.agu.ac.jp

two steps during cellular activation and mitochondrial Ca^{2+} uptake plays a role in mast cell function through cytoplasmic Ca^{2+} buffering.

MATERIALS AND METHODS

Materials Rhod 2-AM and Fura 2-AM were obtained from Dojindo (Kumamoto, Japan) and MitoTracker Green FM was from Invitrogen (Carlsbad, CA, U.S.A.). Rotenone and antimycin A were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti-dinitrophenyl (DNP) monoclonal IgE (IgE-53-569) and DNP-conjugated bovine serum albumin (BSA) (DNP-BSA) were prepared as described in our previous paper.¹⁴⁾ In our present experiments, an average of seven DNP groups was conjugated with a BSA molecule.

Cell Culture and Silencing Plasmid Transfection A rat mast cell line RBL-2H3 cells were cultured in an atmosphere of 5% CO_2 at 37°C in minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (Roche, Mannheim, Germany). The pSilencer™ 4.1-CMV *neo* plasmid vector (Ambion, Austin, TX, U.S.A.) was used for silencing STIM1 expression. The oligonucleotide pair, 5'-GAT CCG GCT GAT TAC GTA TGT GGA TTC AAG AGA TCC ACA TAC GTA ATC AGC CACA-3' (STIM1 short interfering RNA (siRNA) sense) and 5'-AGC TTG TGG CTG ATT ACG TAT GTG GAT CTC TTG AAT CCA CAT ACG TAA TCA GCCG-3' (STIM1 siRNA antisense), was inserted into the silencing vector according to the manufacturer's protocol. The cells were electroporated in cold K^+ -phosphate buffered saline (PBS) with 20 μg of the silencing plasmid at 300 V and 950 μF using Gene Pulser (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The RBL-2H3 cells that were transfected with the plasmid were maintained in culture dishes for a few days and were used for the experiments. The stable transfectant cells were obtained by selection with G418 antibiotic (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Measurements of Cytosolic and Mitochondrial Ca^{2+} In measuring $[\text{Ca}^{2+}]_c$, RBL-2H3 cells (1×10^6 cells) were loaded with Fura 2-AM (1 μM) and anti-DNP IgE (mouse ascites; 1:5000) for 30 min at 37°C and then washed twice with *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (10 mM Hepes [pH 7.2], 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl_2 , 0.1% glucose, 0.1% BSA, and 0.01% sulfinpyrazone). The Ca^{2+} concentration in Hepes buffer was adjusted by CaCl_2 and ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA). The Fura 2 fluorescence intensity was measured at excitation wavelengths of 340 nm and 360 nm and an emission wavelength of 500 nm by a spectrofluorometer (RF-5300PC, Shimadzu, Kyoto, Japan) at 37°C. The data (F_{340}/F_{360}) were calculated by dividing the fluorescence intensity (F_{340}) at 340 nm by F (F_{360}) at 360 nm. Ratios were converted to Ca^{2+} concentrations by a procedure described previously.¹⁵⁾

In measuring cytosolic and mitochondrial Ca^{2+} simultaneously, RBL-2H3 cells were loaded with Fura 2-AM (1 μM), Rhod 2-AM (1.7 μM), and anti-DNP IgE (1:5000) for 30 min at 37°C, and then washed twice with Hepes buffer. The fluorescence intensities of Fura 2 and Rhod 2 were measured with an excitation wavelength of 340 nm and 550 nm and an emission wavelength of 500 nm and 570 nm, respectively, by a spectrofluorometer at 37°C. The data (F/F_0) were shown by dividing

the fluorescence intensity (F) by it (F_0) at a resting level ($t=0$).

Microscopic Measurements RBL-2H3 cells were harvested from culture dishes, transferred to an observation chamber (Elecon, Chiba, Japan), and cultured overnight in a CO_2 incubator. For double staining, the cells were treated with MitoTracker Green FM (0.2 μM) and Rhod 2-AM (1.7 μM) for 30 min and washed with Hepes buffer. The preparations were viewed on a confocal laser scanning microscope (LSM-510; Zeiss, Oberkochen, Germany).^{14,16)} To detect MitoTracker Green and Rhod 2 fluorescence simultaneously, MitoTracker Green and Rhod 2 were excited at 488 nm (argon laser) and at 543 nm (He-Ne laser), and their fluorescence was observed with band pass filters of 500–530 nm and 565–615 nm, respectively. For measuring Ca^{2+} mobilization, the cells were treated with mouse anti-DNP IgE (1:5000) and Rhod 2-AM (1.7 μM) and washed with Hepes buffer. Rhod 2 was excited at 543 nm, and its fluorescence was detected by a long pass filter (>560 nm). Rhod 2 fluorescence was used to detect mitochondrial and cytosolic Ca^{2+} mobilization because most Rhod 2 distributed in mitochondria and a small diffused Rhod 2 resided in cytoplasm. During the experiments, the temperature of the observation chambers was maintained at 37°C. The data ($\Delta F = F - F_0$) were calculated by subtracting the fluorescence intensity (F_0) at resting level ($t=0$) from fluorescence intensity (F) after stimulation.

Western Blot Western blotting analysis was performed using previously described procedures.^{14,16)} To prepare whole cell lysates, collected RBL-2H3 cells were suspended in cold lysis buffer (20 mM Hepes [pH 7.9], 0.1% NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Na_3VO_4 , 10 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ leupeptin, and 10% glycerol) and allowed to stand on ice for 30 min. The suspension was clarified by centrifugation ($150000 \times g$, 20 min). The resulting supernatants were solubilized by treatment with Laemmli buffer at 100°C for 3 min and separated by electrophoresis on an 8% SDS-polyacrylamide gel. The electrophoresed proteins were transferred to a PVDF membrane with an electroblotter. After blocking with 0.5% casein, the membranes were probed with rabbit anti-STIM1 (1:500) (BD Biosciences, San Jose, CA, U.S.A.) or mouse anti- β -actin (1:40000) antibody, and treated with 1:1000 HRP-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), respectively.

β -Hexosaminidase Secretion Degranulation of RBL-2H3 cells was monitored by measuring the activity of the granule-stored enzyme β -hexosaminidase secreted into cell supernatants. The cells were plated in 24-well plates at 5×10^4 cells/well. The following day, the cells were sensitized with anti-DNP IgE (1:5000) for 30 min at 37°C, and monolayers were washed with Hepes buffer and incubated with DNP-BSA (50 ng/mL) for 30 min at 37°C. Following incubation, aliquots of the supernatants were transferred to a 96-well plate (20 μL /well) and incubated with 20 μL of substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 100 mM citrate [pH 4.5]) for 1 h at 37°C. After terminating the reaction with 160 μL of 167 mM Na_2CO_3 – NaHCO_3 buffer, the absorbance at 405 nm was measured using a microplate reader (model 680, Bio-Rad). The release activity relative to the total β -hexosaminidase content of the cells was determined by disrupting cells with 0.1% Triton X-100.¹⁶⁾ Each assay was performed in triplicate.

Tumor Necrosis Factor (TNF)- α Production RBL-2H3 cells (5×10^5 cells/well) were sensitized with anti-DNP IgE (1:5000) for 30 min at 37°C, and monolayers were washed with Hanks' balanced salt solution (HBSS) (Invitrogen) adjusting Ca^{2+} concentration and stimulated with DNP-BSA (50 ng/mL) for 3 h in HBSS at 37°C. Supernatants were collected and absolute amounts of TNF- α released by cells were determined using a mouse TNF- α immunoassay system (R&D Systems, Minneapolis, MN, U.S.A.).¹⁶⁾

Statistical Analysis A one-way analysis of variance (ANOVA) was used to compare differences between groups for analyzing degranulation and TNF- α production, and χ^2 -test was performed for analyzing responding cell rate. Results were considered statistically significant when $p < 0.05$.

RESULTS

Cytosolic and Mitochondrial Ca^{2+} Mobilization in Individual Mast Cells The Ca^{2+} -sensitive fluorescence indicator Rhod 2 accumulates in the mitochondria in various kinds of cells.^{17–21)} Here, we first confirmed that Rhod 2 localized in the mitochondria in RBL-2H3 cells. When RBL-2H3 cells were loaded with Rhod 2-AM and MitoTracker Green FM, an indicator that labels mitochondria independently of mitochondrial function,²²⁾ Rhod 2 fluorescence localized predominantly within mitochondria (Fig. 1A). This indicated that microscopic observation can distinctively measure mitochondrial Ca^{2+} mobilization in individual RBL-2H3 cells by Rhod 2 fluorescence. On the other hand, a small diffuse Rhod 2 cytosolic signal was also detected, suggesting that the Rhod 2 signal in the cytoplasm was helpful to monitor cytosolic Ca^{2+} mobilization.

To study the relationship between mitochondrial Ca^{2+} mobilization and $[\text{Ca}^{2+}]_c$ in detail, we observed cytosolic and mitochondrial Ca^{2+} mobilization in a single RBL-2H3 cell. Imaging analysis indicated that while $[\text{Ca}^{2+}]_c$ increased and was maintained at a high level after antigen stimulation, $[\text{Ca}^{2+}]_m$ increased in concert with increase in $[\text{Ca}^{2+}]_c$ and then increased again approximately 50 s later (Fig. 1B) in the presence of 1 mM extracellular Ca^{2+} . On the other hand, the second elevation of $[\text{Ca}^{2+}]_m$ stimulated with antigen mostly disappeared in the absence of extracellular Ca^{2+} (Fig. 1C).

Next, to investigate the relation between the two-step increase in $[\text{Ca}^{2+}]_m$ and extracellular Ca^{2+} concentration, we measured cytosolic and mitochondrial Ca^{2+} mobilization at different extracellular Ca^{2+} concentration (0, 0.1, 0.5, 1.0 mM). Whereas the averaged $[\text{Ca}^{2+}]_c$ transiently increased and returned at the basal level at 0 and 0.1 mM Ca^{2+} of extracellular Ca^{2+} concentration after antigen stimulation, the time-course of $[\text{Ca}^{2+}]_c$ increase was maintained at the higher level in a similar extent at 0.5 mM and 1.0 mM Ca^{2+} (Fig. 1D). Single cell analysis showed that the rates of mitochondria showing second Ca^{2+} elevation dropped off in accord with the decrease in extracellular Ca^{2+} concentration (Table 1). Interestingly, the second mitochondrial Ca^{2+} uptake is observed at 1.0 mM extracellular Ca^{2+} close to the physiological concentration despite similar lasting $[\text{Ca}^{2+}]_c$ increases between 0.5 mM and 1.0 mM extracellular Ca^{2+} .

Involvement of STIM1 in Mitochondrial Ca^{2+} Mobilization In RBL-2H3 cells, the SOCE is necessary for maintaining $[\text{Ca}^{2+}]_c$ at a high level after antigen stimulation. Because

Fig. 1. Ca^{2+} Mobilization in Mitochondria and Cytosol of RBL-2H3 Cells

(A) Localization of Rhod 2 in mitochondria. Representative fluorescence images of RBL-2H3 cells loaded with MitoTracker green and Rhod 2 are shown. Bar = 5 μm . (B) A representative time-course of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) and mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) in a single RBL-2H3 cell after adding antigen (DNP-BSA; 50 ng/mL) in HEPES buffer including 1 mM Ca^{2+} . (C) A representative time-course of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ in a single RBL-2H3 cell after adding antigen (DNP-BSA; 50 ng/mL) in Ca^{2+} -free HEPES buffer. (D) The change in averaged $[\text{Ca}^{2+}]_c$ in Fura 2-loaded RBL-2H3 cells after adding antigen (DNP-BSA; 50 ng/mL) in HEPES buffer including 0, 0.1, 0.5, and 1.0 mM Ca^{2+} by a spectrofluorometer.

it has been demonstrated that two key proteins, STIM1 in the ER membrane and Orail in the plasma membrane, regulate SOCE,²⁾ we obtained STIM1-knockdown (STIM1-KD)

Table 1. Mitochondrial Ca^{2+} Elevation Patterns after Antigen (DNP-BSA; 50 ng/mL) Stimulation in RBL-2H3 Cells

Extracellular Ca^{2+} concentration	Number of mitochondria ^{a)}			
	Two-step Ca^{2+} elevation	One-step Ca^{2+} elevation	No Ca^{2+} elevation	Total
1.0 mM	58 (56)	44 (43)	1 (1)	103 (100)
0.5 mM ^{b)}	10 (10)	87 (85)	5 (5)	103 (100)
0.1 mM ^{b)}	8 (8)	91 (91)	1 (1)	100 (100)
0.0 mM ^{b)}	11 (10)	96 (90)	0 (0)	107 (100)

a) Indicates the number of mitochondria analyzed from 6–9 RBL-2H3 cells. Mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) was considered elevated when Rhod 2 fluorescence intensity in a single mitochondrion increased by >20% of basal intensity. The mitochondria in which the second $[\text{Ca}^{2+}]_m$ elevation occurred after more than 15 s from the first elevation was defined as a “two-step Ca^{2+} elevation.” The percentages of mitochondria to the total number are shown in parentheses. b) $p < 0.01$, by the χ^2 -test vs. 1.0 mM Ca^{2+} , between the two-step Ca^{2+} elevation and the one-step Ca^{2+} elevation.

Fig. 2. Ca^{2+} Mobilization in Mitochondria of STIM1-Knockdown (STIM1-KD) RBL-2H3 Cells

(A) Western blotting analysis showing the expression of STIM1 in wild type and in STIM1-KD cells. (B) The change in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in the wild type and STIM1-KD RBL-2H3 cells after adding thapsigargin (TG) (20 nM) in Ca^{2+} -free HEPES buffer by a spectrofluorometer. Ca^{2+} (3 mM) was added to the cell suspension at 300 s. (C) A representative time-course of $[\text{Ca}^{2+}]_c$ and mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) in a single RBL-2H3 cell after adding thapsigargin (20 nM) in HEPES buffer including 1 mM Ca^{2+} . (D) A representative time-course of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ in a STIM1-KD RBL-2H3 cell after adding thapsigargin (20 nM) in HEPES buffer including 1 mM Ca^{2+} .

RBL-2H3 cells by introducing the silencing vector for STIM1 to investigate the effect of SOCE on mitochondrial Ca^{2+} mobilization (Fig. 2A). We here used thapsigargin, an inhibitor of SERCA, to confirm that SOCE was inhibited in STIM1-KD cells, because thapsigargin efficiently elicits in Ca^{2+} leak from the ER and SOCE through the plasma membrane. Adding thapsigargin induced a Ca^{2+} leak from the ER in STIM1-KD cells similar to wild type cells, while the subsequent SOCE was suppressed considerably in STIM1-KD cells compared to wild type cells (Fig. 2B). A microscopic analysis showed that the two-step elevation in $[\text{Ca}^{2+}]_m$ was also observed after adding thapsigargin in a wild type cell (Fig. 2C). However, in a STIM1-KD cell, the $[\text{Ca}^{2+}]_m$ rose in accordance with the increase in $[\text{Ca}^{2+}]_c$ but the subsequent elevation of $[\text{Ca}^{2+}]_m$ was hardly observed even in the presence of 1 mM Ca^{2+} (Fig. 2D). The proportions of mitochondria shown in the two-step Ca^{2+} elevation were 91% ($n=103$) and 19% ($n=102$) in wild type

cells and STIM1-KD cells, respectively. These results showed that mitochondrial Ca^{2+} uptake occurred in two steps in activated RBL-2H3 cells, suggesting that the first uptake may be due to Ca^{2+} release from the ER, and that the second uptake may be based on the STIM1-mediated SOCE.

Effects of Mitochondrial Ca^{2+} Uptake on Mast Cell Function Further, to study the role of mitochondrial Ca^{2+} uptake in mast cell function, we first measured the β -hexosaminidase release and TNF- α secretion after antigen stimulation at different extracellular Ca^{2+} concentration. Antigen elicited in the release of β -hexosaminidase and TNF- α to mast cells in a similar extent at 0.5 mM and 1.0 mM Ca^{2+} , but not at 0 and 0.1 mM Ca^{2+} (Figs. 3A,B). This suggested that SOCE was necessary for the release of β -hexosaminidase and TNF- α and that the maintenance of $[\text{Ca}^{2+}]_c$ at a proper level was important in mast cell function.

As described above, mitochondrial Ca^{2+} uptake was

Fig. 3. Effect of Mitochondrial Ca^{2+} Uptake on Mast Cell Function

(A) The percentages of released β -hexosaminidase to the total cell contents in a 30 min assay with antigen (DNP-BSA; 50 ng/mL) in RBL-2H3 cells. Results are expressed as mean $\pm$ S.E. ** $p < 0.01$ compared with 0 mM and 0.1 mM Ca^{2+} , respectively. (B) The amount of TNF- α production in a 3 h assay with antigen (DNP-BSA; 50 ng/mL) in RBL-2H3 cells. Results are expressed as mean $\pm$ S.E. *** $p < 0.01$ compared with 0 mM and 0.1 mM Ca^{2+} , respectively. (C) The change in Rhod 2 fluorescence intensity (F/F_0) in RBL-2H3 cells pretreated with no inhibitors and rotenone (1 μM) after adding antigen (DNP-BSA; 50 ng/mL) in HEPES buffer including 1 mM Ca^{2+} by a spectrofluorometer. (D) The time-course of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) and mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) in RBL-2H3 cells loaded with Fura 2 and Rhod 2 after the addition of antigen (DNP-BSA; 50 ng/mL) in Ca^{2+} -free HEPES buffer by a spectrofluorometer. Ca^{2+} (3 mM) was added to the cell suspension at 300 s. (E) The time-course of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ in rotenone (1 μM)-pretreated RBL-2H3 cells loaded with Fura 2 and Rhod 2 after the addition of antigen (DNP-BSA; 50 ng/mL) in Ca^{2+} -free HEPES buffer by a spectrofluorometer. Ca^{2+} (3 mM) was added to the cell suspension at 300 s. (F) The percentages of released β -hexosaminidase to total contents in cells for 30 min assay with antigen (DNP-BSA; 50 ng/mL) in RBL-2H3 cells without inhibitors, pretreated with rotenone (1 μM) for 30 min, and pretreated with antimycin A (1 $\mu\text{g/ml}$) for 30 min. Results are expressed as mean $\pm$ S.E. ** $p < 0.01$ compared with no inhibitors.

considered to be involved in the maintenance of $[\text{Ca}^{2+}]_c$ at a proper level for mast cell activation. Finally, to investigate whether inhibition of mitochondrial Ca^{2+} uptake affects mast cell degranulation, we studied the effect of rotenone and antimycin A, which are inhibitors of mitochondrial electron transport complex I and III, respectively, on the β -hexosaminidase release in RBL-2H3 cells. Cells were pretreated with rotenone and antimycin A for 30 min at 1 μM and 1 $\mu\text{g/ml}$, respectively, which are less than concentrations reported to alter hardly intracellular ATP levels.²³⁾ The pretreatment with rotenone diminished the mitochondrial Ca^{2+} uptake stimulated with antigen to a certain extent in the presence of extracellular 1 mM Ca^{2+} (Fig. 3C). Antimycin A also reduced the mitochondrial Ca^{2+} uptake and the averaged F/F_0 of Rhod 2 at 150 s after antigen addition were 1.32 ± 0.01 , $1.25 \pm 0.02^*$, and $1.24 \pm 0.01^{**}$ in RBL-2H3 cells pretreated with no inhibitors, rotenone, and antimycin A, respectively ($n=3$, * $p < 0.05$ and ** $p < 0.01$ vs. no inhibitors). Additionally, rotenone showed the inhibition of mitochondrial Ca^{2+} uptake after the Ca^{2+} entry rather than the Ca^{2+} release from ER (Figs. 3D,E). According to the suppression of mitochondrial Ca^{2+} uptake, the increase in $[\text{Ca}^{2+}]_c$ was enhanced after the addition of Ca^{2+} in RBL-2H3 cells pretreated with rotenone. Similar results were obtained in cells pretreated with antimycin A (data not shown). Based on the effect of these inhibitors on mitochondrial Ca^{2+} uptake, we then measured β -hexosaminidase release in RBL-2H3 cells pretreated with rotenone (1 μM) and antimycin A (1 $\mu\text{g/ml}$). Both inhibitors significantly inhibited the degranulation induced by antigen (Fig. 3F) possibly due to be over a suitable

level of $[\text{Ca}^{2+}]_c$ by the suppression of mitochondrial Ca^{2+} uptake. This suggested that the mitochondrial Ca^{2+} uptake regulated mast cell function through the cytosolic Ca^{2+} buffering.

DISCUSSION

Storage of mitochondrial Ca^{2+} is an important physiological process that is linked to many different cellular responses.^{24,25)} Ca^{2+} controls numerous mitochondrial functions, and Ca^{2+} handling by mitochondria influences cytosolic Ca^{2+} signals. The mitochondrial metabolic enzymes such as pyruvate dehydrogenase, ketoglutarate dehydrogenase, and isocitrate dehydrogenase are activated by Ca^{2+} , resulting in an increase in the respiratory rate, H^+ extrusion, and ATP production. In contrast, mitochondrial Ca^{2+} overload is associated with cell damage and death. An excessive $[\text{Ca}^{2+}]_m$ elevation causes the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol and results in activation of caspases that trigger apoptosis. The mitochondrial uptake and release of Ca^{2+} is closely associated with cytosolic Ca^{2+} homeostasis and leads to several intracellular effects. In modulating intracellular Ca^{2+} movement, mitochondrial Ca^{2+} buffering prevents the spread of intracellular Ca^{2+} signals.²⁶⁾ Local Ca^{2+} control by IP_3 is modulated by Ca^{2+} uptake and metabolic activity of the mitochondria located in the high- Ca^{2+} microdomain. The existence of a high- Ca^{2+} microdomain between the ER and mitochondria were actually presumed by recent experiments.^{27,28)} Mitochondria also prevent Ca^{2+} depletion in the ER by returning captured Ca^{2+} . In regulating SOCE,

translocation of mitochondria to the immunological synapse is necessary to maintain Ca^{2+} influx in T cells, and mitochondrial positioning determines the magnitude of local Ca^{2+} entry by reducing local Ca^{2+} -dependent channel inactivation.^{29,30} Accordingly, the intracellular Ca^{2+} mobilization coupling among mitochondria, ER, and Ca^{2+} channels plays a critical role in cell physiology.

Because the role of mitochondrial Ca^{2+} mobilization in mast cell function was controversial, we analyzed the relationship of individual $[\text{Ca}^{2+}]_m$ with $[\text{Ca}^{2+}]_c$ in a single RBL-2H3 cell using confocal laser scanning microscopy. We found that the antigen induced an increase in $[\text{Ca}^{2+}]_m$ in two steps though the $[\text{Ca}^{2+}]_c$ was maintained at a certain high level (Fig. 1B). The proportion of mitochondria taking up Ca^{2+} in two-step was significantly larger at 1.0 mM (56%) than 0, 0.1, and 0.5 mM of extracellular Ca^{2+} (ca. 10%) (Table 1). The second Ca^{2+} elevation in mitochondria mostly disappeared in STIM1-KD mast cells, which are deficient in SOCE, even in the presence of extracellular Ca^{2+} (Fig. 2D). Because STIM1/Orai1-mediated SOCE is required for lasting cytosolic Ca^{2+} signaling in mast cells,²⁾ it was suggested that the mitochondrial two-step Ca^{2+} uptake occurred when $[\text{Ca}^{2+}]_c$ was highly maintained in activated mast cells. Additionally, the probability of a mitochondrial two-step Ca^{2+} elevation was higher when adding thapsigargin (91%) than antigen (56%) in the presence of extracellular Ca^{2+} in wild type cells (see Results and Table 1). Further, the mitochondrial Ca^{2+} elevation was greatly suppressed in mast cells loaded with BAPTA, an intracellular Ca^{2+} chelator (data not shown). A recent report showed that MICU1 associated with mitochondrial innermembrane played a role in Ca^{2+} sensing and Ca^{2+} uptake.⁷⁾ From these findings, it was considered that the mitochondria took up an increased cytosolic Ca^{2+} , and the first and second elevation was based on IP_3 -induced Ca^{2+} release from ER and STIM/Orai-mediated Ca^{2+} entry from SOCE channels, respectively.

Some lines of evidence indicate that mitochondria contribute to the SOCE, degranulation, and histamine content in mast cell secretory granules.^{12,29,30} They suggest that mitochondrial Ca^{2+} elevation subsequently contributed to the regulation of cell function through cytosolic Ca^{2+} modulation. We here showed that all of $[\text{Ca}^{2+}]_c$ increase, degranulation, and $\text{TNF-}\alpha$ production were induced in a similar extent in the presence of 0.5 and 1.0 mM extracellular Ca^{2+} (Figs. 1D, 3A,B). It has been known that Ca^{2+} pumps such as SERCA and PMCA efficiently regulate the $[\text{Ca}^{2+}]_c$ down at a suitable level in activated cells.³⁾ We considered that mitochondria also regulated the $[\text{Ca}^{2+}]_c$ by taking up cytosolic Ca^{2+} when the excessive SOCE occurred, because the proportion of mitochondria taking up Ca^{2+} in two-step was significantly larger at 1.0 mM than 0.5 mM of extracellular Ca^{2+} (Table 1) as described above. Therefore, we analyzed the relation between mitochondrial Ca^{2+} uptake and mast cell function using mitochondrial electron transport complex inhibitors, rotenone and antimycin A at lower concentration. Though it was reported that antimycin A decreased the mitochondrial membrane potential but not rotenone,¹²⁾ both inhibitors diminished the mitochondrial Ca^{2+} uptake and the degranulation in a similar extent after antigen stimulation in the presence of 1 mM Ca^{2+} (Figs. 3C,F). This suggested that both inhibitors might suppressed the mitochondrial Ca^{2+} uptake independently of the mitochondrial membrane potential, and that the poor mitochondrial Ca^{2+}

uptake induced the inhibition of degranulation by the failure of cytosolic Ca^{2+} buffering in the presence of 1 mM extracellular Ca^{2+} .

Suzuki *et al.* previously showed that antigen stimulation induced Ca^{2+} release from mitochondria *via* mitochondrial permeability transition pore in RBL-2H3 cells.¹²⁾ However, we have never observed the decrease in Rhod 2 fluorescence after adding antigen or thapsigargin in our experimental condition. Mitochondria may be able to release and take up Ca^{2+} in activated mast cells in difference of conditions including concentrations of stimulants.

In conclusion, we showed here that mitochondria take up Ca^{2+} from the cytosol in activated mast cells in two steps and suggested that the mitochondrial Ca^{2+} uptake play a role in degranulation and cytokine production, probably through the cytosolic Ca^{2+} buffering. Although local Ca^{2+} communication exists between mitochondria and ER, the Ca^{2+} linking of mitochondria and SOCE and the mechanism of Ca^{2+} uptake and Ca^{2+} release in mitochondria are not fully understood. The results described here will provide useful information on the molecular mechanism of intracellular Ca^{2+} modulation in mast cell responses.

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REFERENCES

- 1) Clapham DE. Calcium signaling. *Cell*, **80**, 259–268 (1995).
- 2) Calloway N, Vig M, Kinet JP, Holowka D, Baird B. Molecular clustering of STIM1 with Orai1/CRACM1 at the plasma membrane depends dynamically on depletion of Ca^{2+} stores and on electrostatic interactions. *Mol. Biol. Cell*, **20**, 389–399 (2009).
- 3) Brini M, Carafoli E. Calcium pumps in health and disease. *Physiol. Rev.*, **89**, 1341–1378 (2009).
- 4) Gunter TE, Yule DI, Gunter KK, Eliseev RA, Salter JD. Calcium and mitochondria. *FEBS Lett.*, **567**, 96–102 (2004).
- 5) Santo-Domingo J, Demaurex N. Calcium uptake mechanisms of mitochondria. *Biochim. Biophys. Acta*, **1797**, 907–912 (2010).
- 6) Demaurex N, Poburko D, Frieden M. Regulation of plasma membrane calcium fluxes by mitochondria. *Biochim. Biophys. Acta*, **1787**, 1383–1394 (2009).
- 7) Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK. MICU1 encodes a mitochondrial EF hand protein required for Ca^{2+} uptake. *Nature*, **467**, 291–296 (2010).
- 8) De Stefani D, Raffaello A, Teardo E, Szabò I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature*, **476**, 336–340 (2011).
- 9) Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Kotliansky V, Mootha VK. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature*, **476**, 341–345 (2011).
- 10) Galli SJ, Kalesnikoff J, Grimaldeston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol.*, **23**, 749–786 (2005).
- 11) Rivera J. Molecular adapters in FcεRI signaling and the allergic response. *Curr. Opin. Immunol.*, **14**, 688–693 (2002).
- 12) Suzuki Y, Yoshimaru T, Inoue T, Ra C. Mitochondrial Ca^{2+} flux is

- a critical determinant of the Ca^{2+} dependence of mast cell degranulation. *J. Leukoc. Biol.*, **79**, 508–518 (2006).
- 13) Ma HT, Beaven MA. Regulation of Ca^{2+} signaling with particular focus on mast cells. *Crit. Rev. Immunol.*, **29**, 155–186 (2009).
 - 14) Furuno T, Hirashima N, Onizawa S, Sagiya N, Nakanishi M. Nuclear shuttling of mitogen-activated protein (MAP) kinase (extracellular signal-regulated kinase (ERK) 2) was dynamically controlled by MAP/ERK kinase after antigen stimulation in RBL-2H3 cells. *J. Immunol.*, **166**, 4416–4421 (2001).
 - 15) Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450 (1985).
 - 16) Nakamura R, Furuno T, Nakanishi M. The plasma membrane shuttling of CAPRI is related to regulation of mast cell activation. *Biochem. Biophys. Res. Commun.*, **347**, 363–368 (2006).
 - 17) Hajnóczky G, Robb-Gaspers LD, Seitz MB, Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*, **82**, 415–424 (1995).
 - 18) Jou MJ, Peng TI, Sheu SS. Histamine induces oscillations of mitochondrial free Ca^{2+} concentration in single cultured rat brain astrocytes. *J. Physiol.*, **497**, 299–308 (1996).
 - 19) Babcock DF, Herrington J, Goodwin PC, Park YB, Hille B. Mitochondrial participation in the intracellular Ca^{2+} network. *J. Cell Biol.*, **136**, 833–844 (1997).
 - 20) Boitier E, Rea R, Duchon MR. Mitochondria exert a negative feedback on the propagation of intracellular Ca^{2+} waves in rat cortical astrocytes. *J. Cell Biol.*, **145**, 795–808 (1999).
 - 21) Collins TJ, Lipp P, Berridge MJ, Bootman MD. Mitochondrial Ca^{2+} uptake depends on the spatial and temporal profile of cytosolic Ca^{2+} signals. *J. Biol. Chem.*, **276**, 26411–26420 (2001).
 - 22) Poot M, Zhang YZ, Krämer JA, Wells KS, Jones LJ, Hanzel DK, Lugade AG, Singer VL, Haugland RP. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J. Histochem. Cytochem.*, **44**, 1363–1372 (1996).
 - 23) Rimessi A, Giorgi C, Pinton P, Rizzuto R. The versatility of mitochondrial calcium signals: from stimulation of cell metabolism to induction of cell death. *Biochim. Biophys. Acta*, **1777**, 808–816 (2008).
 - 24) Tinel H, Cancela JM, Mogami H, Gerasimenko JV, Gerasimenko OV, Tepikin AV, Petersen OH. Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca^{2+} signals. *EMBO J.*, **18**, 4999–5008 (1999).
 - 25) Giacomello M, Drago I, Bortolozzi M, Scorsetto M, Gianelle A, Pizzo P, Pozzan T. Ca^{2+} hot spots on the mitochondrial surface are generated by Ca^{2+} mobilization from stores, but not by activation of store-operated Ca^{2+} channels. *Mol. Cell*, **38**, 280–290 (2010).
 - 26) Csordás G, Várnai P, Golenár T, Roy S, Purkins G, Schneider TG, Balla T, Hajnóczky G. Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol. Cell*, **39**, 121–132 (2010).
 - 27) Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, Hoth M. T cell activation requires mitochondrial translocation to the immunological synapse. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 14418–14423 (2007).
 - 28) Schwindling C, Quintana A, Krause E, Hoth M. Mitochondria positioning controls local calcium influx in T cells. *J. Immunol.*, **184**, 184–190 (2010).
 - 29) Gilbert JA, Parekh AB. Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca^{2+} current I_{CRAC} . *EMBO J.*, **19**, 6401–6407 (2000).
 - 30) Tagen M, Elorza A, Kempuraj D, Boucher W, Kepley CL, Shirihi OS, Theoharides TC. Mitochondrial uncoupling protein 2 inhibits mast cell activation and reduces histamine content. *J. Immunol.*, **183**, 6313–6319 (2009).