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# The high-affinity immunoglobulin E receptor (FcεRI) regulates mitochondrial calcium uptake and a dihydropyridine receptor-mediated calcium influx in mast cells: Role of the FcεRIβ chain immunoreceptor tyrosine-based activation motif

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ABSTRACT

A growing body of evidence suggests that mitochondria take up calcium upon receptor (agonist) stimulation and that this contributes to the dynamics of spatiotemporal calcium signaling. We have previously shown that engagement of the high-affinity receptor for immunoglobulin E (FcεRI) stimulates mitochondrial calcium ($[Ca^{2+}]_m$) uptake in mast cells. The present study was undertaken to investigate the mechanisms and biological significance of FcεRI regulation of $[Ca^{2+}]_m$. Antigen stimulated $[Ca^{2+}]_m$ uptake in a dose-dependent manner with a minimal effective dose of 0.03–3 ng/ml. This $[Ca^{2+}]_m$ uptake took place immediately, reaching its peak within minutes and was inhibited by the src family kinase inhibitor PP1 and phosphatidylinositol-3-kinase inhibitor wortmannin. Analyses using mast cells expressing the wild-type or the mutated type of the FcεRIβ immunoreceptor tyrosine-based activation motif (ITAM) in which all tyrosine residues were replaced by phenylalanine revealed that the FcεRIβ ITAM is essential for a sustained $[Ca^{2+}]_m$ uptake. The FcεRIβ ITAM was essential for overall calcium response upon weak FcεRI stimulation (at low antigen concentration), while upon strong stimulation (at high antigen concentration) it appeared necessary selectively to an immediate calcium response that was sensitive to the dihydropyridine receptor (DHPR) antagonist nifedipine and wortmannin but not to the store-operated calcium entry (SOCE) antagonists such as 2-aminoethoxyphenyl borate and SK&F96365. These data demonstrate that the FcεRIβ regulates $[Ca^{2+}]_m$ uptake in mast cells via the ITAM and suggest that this plays a key role in regulating calcium influx especially that induced via a DHPR-mediated calcium channel.



## 1. Introduction

Mast cells play a central role in allergic and inflammatory reactions. Mast cells and several tumor cell lines such as RBL-2H3 express the high affinity IgE receptor (FcεRI) on their surfaces, and antigen cross-linking of IgE-bound FcεRI initiates a cascade of intracellular signaling events that lead to mast cell effector responses, including the release of preformed

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granular substances such as histamine, β-hexosaminidase, and proteases, new synthesis and release of leukotrienes (LTs), prostagalandins, cytokines and chemokines [1]. These chemical mediators cause various pathophysiological events contributing to acute and chronic allergic and inflammatory reactions. FcεRI is a member of the immunoglobulin superfamily of antigen receptors, which also includes T and B cell receptors (TCR and BCR), and the signal transduction pathways present in mast cells have many similarities to those in T and B cells. FcεRI is a tetramer of α-, β-, and γ-chain homodimers, of which the α-chain binds IgE, while the β- and γ-chain mediate intracellular signaling through the receptor. Like TCR and BCR, FcεRI lacks intrinsic enzyme activity, but its β- and γ-chain contain the immunoreceptor tyrosine-based activation motifs (ITAMs), which are critical for cell activation through cell surface receptors [1–3]. The FcεRIβ ITAM is recognized as an important site of interaction with the src family tyrosine kinase Lyn for signal transduction [4]. The Lyn kinase weakly binds to the FcεRIβ ITAM in resting cells and is further recruited after receptor aggregation [5–7]. The Lyn kinase is required for an initial step of activation in which it phosphorylates the ITAMs of the β- and γ-chain [8,9]. The requirements for this initial process are not fully understood. However, it has been thought that lipid rafts, microdomains in the plasma membrane that are rich in sphingolipids and cholesterol, are required as a platform where the protein–protein interaction between Lyn kinase and the FcεRI may be enhanced [10].

In nonexcitable cells including mast cells, store-operated calcium entry (SOCE) or capacitative calcium entry is considered the most important route of calcium influx [11,12]. SOCE is activated by the emptying of intracellular calcium stores through inositol-1,4,5-triphosphate ($IP_3$), which is produced upon the activation of antigen receptors by mechanisms yet to be resolved. SOCE is also activated when intracellular calcium stores are emptied pharmacologically, for instance with thapsigargin (TG) [12–15]. The molecular entities of SOCE are still unclear, and they are still defined only electrophysiologically, in terms of the current passing through the store-operated calcium channels (SOCs). The best characterized SOC current in hematopoietic cells is $I_{CRAC}$, the current passing through calcium release-activated calcium (CRAC) channels, which was first identified in rat basophilic leukemia (RBL) cells and subsequently in Jurkat T cells [16,17].

It is well-known that mitochondria function as a cell's powerhouse, but over the past decade, it has been shown that they also serve as important regulators of calcium signaling in a wide variety of cells [18,19]. It is widely accepted that mitochondria act as calcium buffers; that is, they take up calcium and release it depending on the cytosolic calcium ($[Ca^{2+}]_i$) levels, thereby maintaining homeostasis of cellular calcium levels. Uptake of mitochondrial calcium ($[Ca^{2+}]_m$) takes place through a uniporter in the inner membrane driven by the large negative mitochondrial membrane potential ($\Delta\Psi_m$), while the accumulated $[Ca^{2+}]_m$ is released slowly through $Na^+$-dependent and -independent exchanges [20]. A growing body of evidence suggests that mitochondria contribute to the dynamics of spatiotemporal calcium signaling upon receptor (agonist) stimulation.

Turning to the immune system, the roles of mitochondria in T cells have been extensively studied; it has been shown that mitochondria have several important actions that are relevant to T cell activation and function [21]. It has been demonstrated that T cell mitochondria have a fundamental role in maintaining high-sustained rates of calcium influx through CRAC channels [22,23]. In T cells stimulated with CD3 or TG, agents that depolarize mitochondria (including mitochondrial uncouplers, CCCP, antimycin A + oligomycin, and myxothiazol) reduce calcium influx through CRAC channels. The resulting changes in $[Ca^{2+}]_i$ are associated with reduced accumulation of the transcription factor NF-AT in the nucleus [24]. Similar mitochondrial control of CRAC channel gating has been reported in RBL cells [25]. Thus mitochondria not only act as calcium buffers but also play a key role in regulating calcium signaling in immune cells.

We have previously shown that both FcεRI stimulation and TG provoke $[Ca^{2+}]_m$ uptake or release, but their effects are distinct in terms of the dependence of the permeability transition pore (PTP) [26]. This indicates that FcεRI signaling affects the mode of the regulation of $[Ca^{2+}]_m$ homeostasis. Herein, we demonstrate for the first time that the FcεRIβ ITAM regulates $[Ca^{2+}]_m$ uptake upon FcεRI stimulation, which plays a key role in regulating antigen-induced calcium influx via a dihydropyridine receptor (DHPR)-mediated calcium channel.

## 2. Materials and methods

### 2.1. Materials

Nifedipine and CGP-37157 were obtained from Calbiochem (San Diego, CA, USA). 2-Aminoethoxydiphenyl borate (2-APB) and thapsigargin (TG) were obtained from Sigma (St. Louis, MO, USA). The monoclonal anti-trinitrophenyl (TNP) IgE antibody (clone IgE-3) was from BD PharMingen Japan (Tokyo, Japan). TNP-bovine serum albumin (BSA) conjugate (25 molecules of TNP coupled to 1 molecule of BSA) was purchased from Cosmo Bio Co. (Tokyo, Japan). Wortmannin, LY294002, bongkrekic acid, and SK&F96365 were obtained from BioMol (Plymouth Meeting, PA, USA). Fluo3-acetoxyl-methyl ester (fluo3/AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Rhod-2/AM was obtained from Invitrogen Corp. (Carlsbad, CA, USA).

### 2.2. RBL-2H3 cells

The RBL-2H3 cells were obtained from the National Institute of Health Sciences (Japanese Collection of Research Biosources [JCRB]; Cell No. JCRB0023) and grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA) in an atmosphere containing 5% $CO_2$. Cells were harvested by incubating them in Hank's balanced salt solution (HBSS) [pH 7.4] containing 1 mM ethylenediamine tetraacetate (EDTA) and 0.25% trypsin for 5 min at 37 °C. For IgE sensitization, cells suspended in complete DMEM were plated on a 100-mm culture dish ($5 \times 10^6$ cells) or in a 24-well plate ($2 \times 10^5$ cells/well) and incubated with anti-TNP IgE (1 μg/dish or 0.1 μg/well) at 37 °C overnight. IgE-sensitized cells were washed with PBS and suspended in HBSS. For measurements, IgE-sensitized cells

were stimulated with antigen (TNP-BSA) at the indicated concentrations at 37 °C for the time indicated.

### 2.3. BMMCs culture

All animal experiments were performed according to Nihon University guidelines. The β-chain-deficient ($\beta^{-/-}$) mice on the chimeric $BDF_1$ and 129 background described previously [27] were bred and kept in the animal facility under the specific pathogen-free conditions. Mouse bone marrow-derived mast cells (BMMCs) were prepared from femurs of 4- to 8-week-old $\beta^{-/-}$ mice as previously described [26]. Cells were cultured in the RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin and streptomycin (Invitrogen), $5 \times 10^{-5}$ M mercaptoethanol (2-ME; Wako Pure Chemicals, Osaka, Japan), 100 μg/ml sodium pyruvate (Invitrogen), 1% minimal essential medium (MEM) nonessential amino acid solution (Invitrogen), and 5 ng/ml recombinant IL-3 (Pepro Tech Inc., Rocky Hill, NJ, USA) in a 5% $CO_2$-containing atmosphere at 37 °C. After 4–6 weeks of culture, the cells were stained for a cell surface expression of FcεRI, and BMMCs were used for experiments after 4–8 weeks of culture (>95% mast cells). For retroviral transfection, BMMCs were cultured in the presence of 100 ng/ml recombinant stem cell factor (Pepro Tech) for another week. An ecotropic retrovirus packaging cell line, PLAT-E (kindly gifted by T. Kitamura) was maintained in Dulbecco's minimal essential medium (DMEM, Sigma) supplemented with 10% FBS, 1 μg/ml puromycin (Clontech, San Jose, CA, USA) and 10 μg/ml Blasticidin S (Kaken Pharmaceutical Co., Tokyo, Japan). For some experiments BMMCs were also generated from 4- to 8-week-old C57BL/6 mice and cultured as described above.

### 2.4. cDNA constructions and transfections

The mouse β-chain cDNA was constructed as described previously [28]. Phenylalanine substitutions for tyrosine on the β-chain ITAM (Y210F/Y216F/Y220F) were generated by using a PCR-based, site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutation of the β-chain was confirmed by DNA sequencing. Then, β-chain cDNAs were subcloned into the EcoRI site of the retroviral vector pMx-puro as previously described. These plasmids were transfected into PLAT-E packaging cells with FuGENE 6 (Roche Diagnostics, Basel, Switzerland) to generate retroviruses. BMMCs were infected with the retroviruses for 48 h in the presence of 1 μg/ml polybrene (Sigma). Cells expressing maker gene were selected by 1.2 μg/ml puromycin.

### 2.5. Flow cytometry analysis for FcεRI expression

For an evaluation of FcεRI expression on the cell surface, BMMCs were stained with 1 mg/ml fluorescein isothiocyanate-conjugated anti-TNP IgE at 4 °C for 30 min, and then washed twice with PBS containing 0.1% sodium azide. The stained cells were analyzed with FACSCalibur (BD Biosciences, San Jose, CA, USA). $\beta^{-/-}$ BMMCs were used as negative controls.

### 2.6. Measurement of $[Ca^{2+}]_i$

Measurement of the cytosolic calcium concentration ($[Ca^{2+}]_i$) was performed using the calcium-reactive fluorescence probe Fluo3/AM as previously described [29]. Briefly, the cell suspension ($1 \times 10^6$ ml$^{-1}$) was incubated with 4 μM Fluo3/AM for 30 min at 37 °C, then washed with HBSS and resuspended in the solution supplemented with 1 mM $CaCl_2$. To study calcium store release and calcium entry separately, we resuspended aliquots of the Fluo3-loaded cells in HBSS supplemented with 1 mM ethyleneglycol tetraacetic acid (EGTA) in place of 1 mM $CaCl_2$. Fluo3 fluorescence was monitored at 5-s intervals for up to 3 min by a microplate fluorometer (Fluoroskan Ascent CF; Labsystems, Helsinki, Finland, excitation and emission at 485 and 527 nm, respectively). $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where $K_d$ is the dissociation constant of the calcium-Fluo3 complex (450 nM), $F_{max}$ the maximum fluorescence (obtained by treating cells with 5 μM A23187), and $F_{min}$ represents the minimum fluorescence (obtained for A23187-treated cells in the presence of 10 mM EGTA). $F$ is the actual sample fluorescence.

### 2.7. Measurement of $[Ca^{2+}]_m$

Measurement of the mitochondrial calcium ($[Ca^{2+}]_m$) was performed using the mitochondrially localizing calcium-reactive fluorescence probe rhod-2/AM as described in our earlier publication [26]. To improve the discrimination between cytosolic and mitochondrially localized dyes [30], 5 μM rhod-2/AM was reduced to the colorless, nonfluorescent dihydrorhod-2/AM by sodium borohydride according to the manufacturer's protocols. Cells were loaded with the dye for 40 min at 37 °C, washed, and resuspended in HBSS in a 24-well plate. After the cells were stimulated, rhod-2 fluorescence was monitored at 5-s intervals for up to 3 min in a microplate fluorometer (Fluoroskan Ascent CF; excitation and emission at 544 and 590 nm, respectively) using 5 μM A23187 as a positive control and 5 μM A23187 in the presence of 10 mM EGTA as a negative control.

### 2.8. Statistical analysis

The Student's $t$-test was performed to determine statistical significance among the experimental groups; $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Mast cell mitochondria take up calcium in response to antigen via the calcium uniporter

We have previously shown that FcεRI stimulation affected $[Ca^{2+}]_m$ levels in the tumor mast cell line RBL-2H3 [26]. First we confirmed the previous data with the potential expansion to primary mast cells. Analysis using rhod-2/AM, a calcium-sensitive fluorescent indicator that accumulates in mitochondria depending on the $\Delta\Psi_m$ [31] showed that antigen cross-linking of IgE-bound FcεRI resulted in a substantial

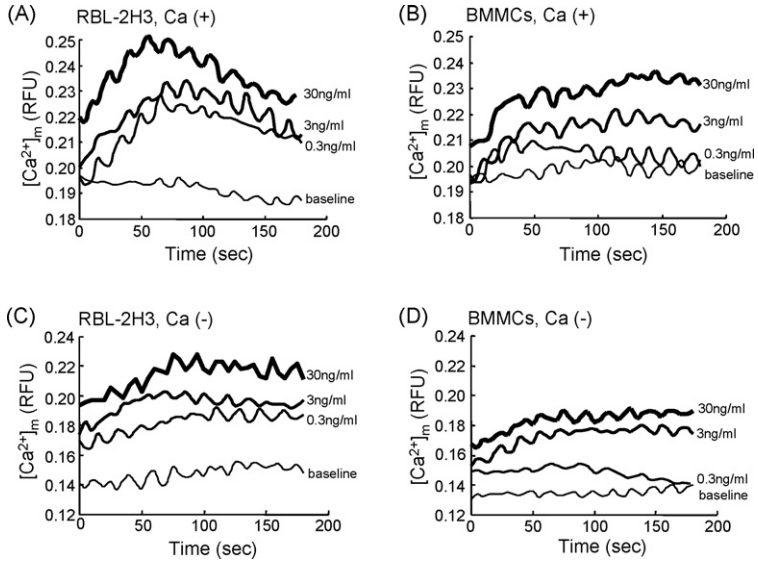


**Fig. 1 – FcεRI stimulation induces $[Ca^{2+}]_m$ uptake in mast cells. (A and B) RBL-2H3 cells (A) and BMMCs (B) ($1 \times 10^6$ $ml^{-1}$) were loaded with nonfluorescent dihydrorhod-2/AM for 40 min at 37 °C, washed, and resuspended in calcium-containing medium [Ca(+), HBSS supplemented with 1 mM $CaCl_2$] in a 24-well plate. These cells were stimulated with TNP-BSA at the indicated concentrations, and then rhod-2 fluorescence was measured for up to 3 min in a microplate fluorometer. (C and D) The dihydrorhod-2-loaded RBL-2H3 cells (C) and BMMCs (D) were suspended in nominally calcium-free buffer [Ca(−), HBSS supplemented with 1 mM EGTA in place of 1 mM $CaCl_2$] and stimulated with TNP-BSA at the indicated concentrations, and then fluorescence was measured as described above. Results expressed in A and B, and C and D were obtained in parallel, and expressed in relative fluorescence units (RFU). Data are representative of three to six independent experiments.**

increase in $[Ca^{2+}]_m$ in RBL-2H3 cells (Fig. 1A). This response was observed immediately, reaching its peak within minutes. Antigen at concentrations of ≥0.3 ng/ml showed the effect in a dose-dependent manner. Antigen induced a more sustained $[Ca^{2+}]_m$ uptake in BMMCs (Fig. 1B). As the concentration of antigen increased, the $[Ca^{2+}]_m$ uptake took place more rapidly and persistently, but the time course of the response varied in different experiments. In both cell types antigen could evoke $[Ca^{2+}]_m$ uptake substantially in a nominally calcium-free medium, though the effect was significantly smaller than that seen in a calcium-containing medium (Fig. 1C and D), indicating that mast cell mitochondria can take up calcium from both extracellular spaces and intracellular stores. According to the ''mitochondrial calcium cycling'' theory, mitochondria take up calcium via the calcium uniporter and release the accumulated calcium via the mitochondrial $Na^+/Ca^{2+}$ exchanger (mNCX) or $H^+/Ca^{2+}$ exchanger. We therefore elucidated the possible involvement of these pathways in antigen-induced $[Ca^{2+}]_m$ uptake by using selective pharmacological inhibitors. Treatment with ruthenium red, an inhibitor of the calcium uniporter completely blocked the response, but CGP-37157, a selective inhibitor of the mNCX did not reduce it (Figs. 2A and B). On the other hand, bongkrekic acid, a PTP antagonist significantly enhanced antigen-induced $[Ca^{2+}]_m$ uptake, and atractyloside, a PTP agonist completely abolished it (Fig. 2C); this is consistent with our previous finding that the PTP plays a role in regulating $[Ca^{2+}]_m$ [26]. Fig. 2D shows statistical analysis data. The data indicate that, in response to antigen, mast cell mitochondria take up calcium via the calcium uniporter and that this $[Ca^{2+}]_m$ uptake is negatively regulated by the PTP but not the mNCX.

### 3.2. *FcεRI-delivered signals are necessary for sustained $[Ca^{2+}]_m$ uptake*

Because the endoplasmic reticulum (ER) $Ca^{2+}$-ATPase sequesters calcium leaked from intracellular stores back into the organelles, pharmacological inhibitors of this pump such as TG can induce a release of calcium, ultimately resulting in the depletion of intracellular calcium stores. This leads to an influx of external calcium via SOC channels, thereby evoking mast cell activation independently of the generation of the second messenger $IP_3$ [32]. TG could induce a robust $[Ca^{2+}]_m$ uptake in both RBL-2H3 cells and BMMCs (Fig. 3A and B). The peak $[Ca^{2+}]_m$ levels seen with 1 μM TG was usually significantly higher than those seen with 30 ng/ml antigen, an optimal dose for cell activation. Moreover, both external and internal calcium were incorporated into the mitochondria in response to TG, though the contribution of the external calcium appeared to be more prominent in BMMCs than in RBL-2H3 cells (Fig. 3A and B). However, the effect of TG was usually less persistent than that of antigen; after stimulation with TG, $[Ca^{2+}]_m$ returned to the resting levels within minutes (compare Fig. 2 with Fig. 3). In addition, both bongkrekic acid and atractyloside had a minimal effect on TG-induced $[Ca^{2+}]_m$ uptake (Fig. 3C), indicating that the PTP plays a minor role. This strongly suggests that some FcεRI-delivered signals that are bypassed by TG may be necessary for sustained $[Ca^{2+}]_m$ uptake and the dependence of the PTP.

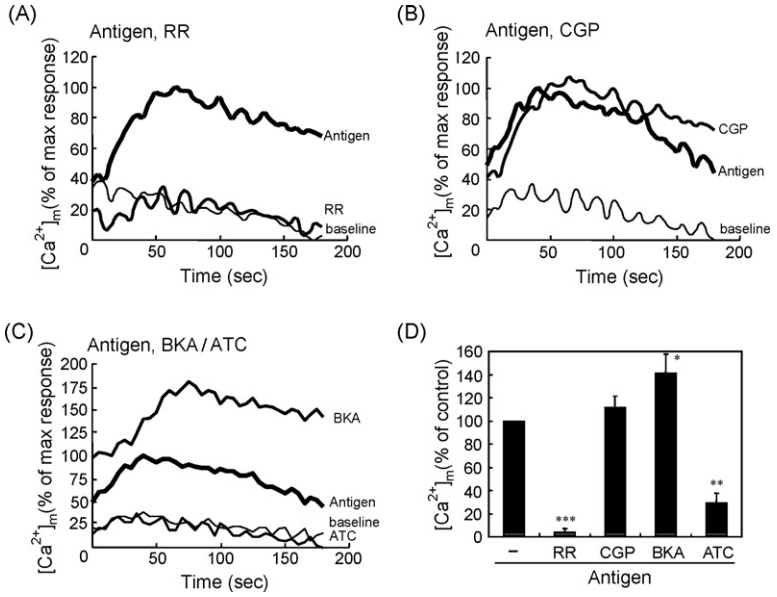


**Fig. 2 – FcεRI-mediated $[Ca^{2+}]_m$ uptake is regulated by calcium uniporter and the mPTP but not the mNCX. The dihydrorhod-2-loaded RBL-2H3 cells ($1 \times 10^6$ ml$^{-1}$) were treated with 3 μM ruthenium red (RR, A), 10 μM CGP-37157 (CGP, B), 1 μM bongkrekic acid (BKA, C) or with 10 μM atractyloside (ATC, C) and immediately stimulated with 30 ng/ml TNP-BSA, and then fluorescence was measured as described above. Results are expressed as a percentage where the maximal response induced by antigen alone is 100, and are representative of three to five independent experiments. (D) Statistical analysis data of the maximal $[Ca^{2+}]_m$. The data represent the mean ± S.E.M.**

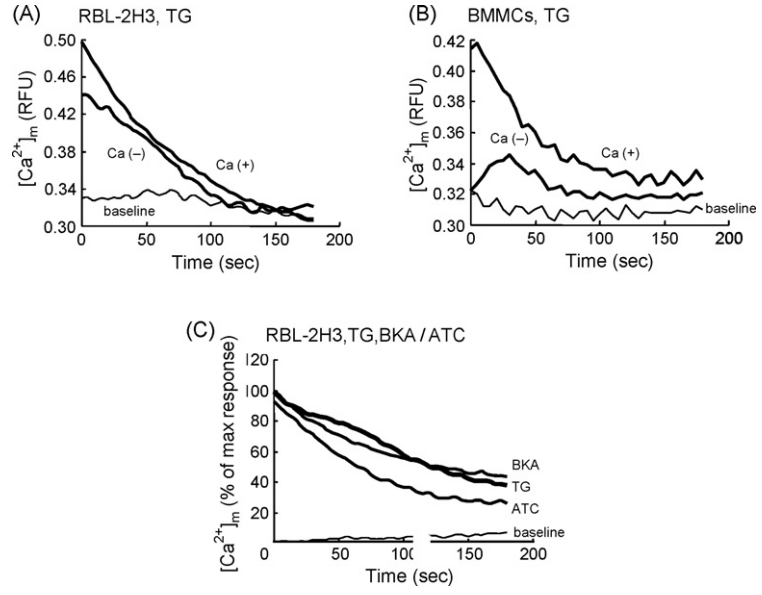


**Fig. 3 – TG induces $[Ca^{2+}]_m$ uptake that is not regulated by the mPTP. (A and B) The dihydrorhod-2-loaded RBL-2H3 cells (A) and BMMCs (B) ($1 \times 10^6$ ml$^{-1}$) were suspended in calcium-containing medium [Ca(+)] or nominally calcium-free medium [Ca(−)]. The cells were stimulated with 1 μM thapsigargin (TG), and then fluorescence was measured for up to 3 min in a microplate fluorometer. (C) After dihydrorhod-2 loading, RBL-2H3 cells ($1 \times 10^6$ ml$^{-1}$) were treated with 1 μM bongkrekic acid >μM atractyloside (ATC, C)/>μM atractyloside (ATC) and immediately stimulated with 1 μM TG, and then fluorescence was measured. Results are expressed in RFU or a percentage where the maximal response induced by TG alone is 100, and are representative of three to five independent experiments.**

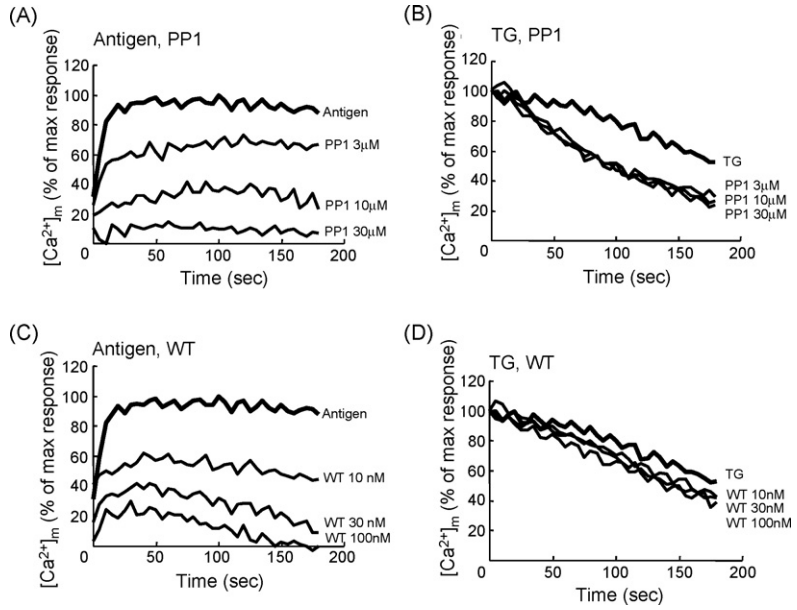


**Fig. 4 – The role of src family kinase and PI3K activity in FcεRI-mediated $[Ca^{2+}]_m$ uptake. The dihydrorhod-2-loaded RBL-2H3 cells were treated with PP1 at the indicated concentrations (A and B) or with wortmannin at the indicated concentrations (WT, C and D) and immediately stimulated with 30 ng/ml TNP-BSA (A and C) or 1 μM TG (B and D), and then fluorescence was measured. Results are expressed as a percentage where the maximal response induced by antigen or TG alone is 100, and are representative of three to five independent experiments.**

### 3.3. *The role of src family kinase and PI-3K activity in antigen-induced $[Ca^{2+}]_m$ uptake*

To gain insight into such early signals required for sustained $[Ca^{2+}]_m$ uptake, we examined the effect of divergent pharmacological inhibitors of selective signaling pathways on this response. As shown in Fig. 4A, treatment with PP1, a src family kinase inhibitor, reduced antigen-induced $[Ca^{2+}]_m$ uptake in a dose-dependent manner with a minimal effective dose of 3 μM and with a complete inhibition at 30 μM. On the other hand, PP1 treatment marginally inhibited TG-induced $[Ca^{2+}]_m$ uptake (Fig. 4B). Wortmannin, a selective inhibitor of PI-3K activity, also blocked antigen-induced $[Ca^{2+}]_m$ uptake in a dose-dependent manner with almost complete inhibition at 100 nM (Fig. 4C). In contrast, wortmannin up to 100 nM had no effect on TG-induced $[Ca^{2+}]_m$ uptake (Fig. 4D). Essentially similar results were obtained with BMMCs (data not shown). These results show that src family kinase and PI-3K activity are signals required for persistent $[Ca^{2+}]_m$ uptake.

### 3.4. *FcεRIβ ITAM is essential for antigen-induced $[Ca^{2+}]_m$ uptake*

Lyn is a major src family kinase found in mast cells. The kinase binds to the FcεRIβ loosely even in resting cells and is activated immediately upon FcεRI activation to bind to the FcεRIβ tightly and to transduce downstream signals [33]. It is established that the FcεRIβ ITAM is an important site of interaction with Lyn for signal transduction [33,34]. Given that the above data indicate the importance of a src family kinase, FcεRIβ ITAM might be critical for antigen-induced $[Ca^{2+}]_m$ uptake. As BMMCs from the FcεRIβ-deficient mice lack cell surface expression of FcεRI, we tested this possibility by analyzing BMMCs expressing the wild-type (YYY) or the mutated FcεRIβ in which all the three tyrosine residues in the ITAM were replaced by phenylalanine (FFF mutant). These mutant cells have been extensively characterized, and the mutant FFF ITAM-expressing cells are shown to lack an FcεRIβ-Lyn interaction via the ITAM [28,35]. When stimulated with antigen, a rapid and persistent $[Ca^{2+}]_m$ uptake was seen in the YYY ITAM-expressing cells (Fig. 5A). Antigen at concentrations of ≥3 ng/ml showed a significant effect. Compared to the YYY ITAM-expressing cells, the FFF ITAM-expressing cells displayed a smaller $[Ca^{2+}]_m$ uptake, which was seen only when they were stimulated with an optimal concentration of antigen (30 ng/ml) (Fig. 5A and B). In response to TG, by contrast, a comparable $[Ca^{2+}]_m$ uptake was seen in these two cell types (Fig. 5C). Fig. 5D shows statistical analysis data.

### 3.5. *The FcεRIβ ITAM is essential for an immediate DHP-sensitive calcium response*

To elucidate the potential role of $[Ca^{2+}]_m$ uptake in the regulation of calcium response, the YYY or the FFF ITAM-expressing cells were stimulated with varying concentrations of antigen, and $[Ca^{2+}]_i$ was measured. In the YYY ITAM-expressing cells, antigen caused a substantial $[Ca^{2+}]_i$ increase in a dose-dependent manner with a minimal effective dose of 0.3 ng/ml (Fig. 6A). On the other hand, suboptimal concentrations (≤3 ng/ml) of antigen induced no substantial $[Ca^{2+}]_i$ increase in the FFF ITAM-expressing cells (Fig. 6B). At an optimal concentration (30 ng/ml), antigen caused significant $[Ca^{2+}]_i$ increase in the FFF ITAM-expressing cells, which was almost comparable to that seen in the YYY ITAM-expressing

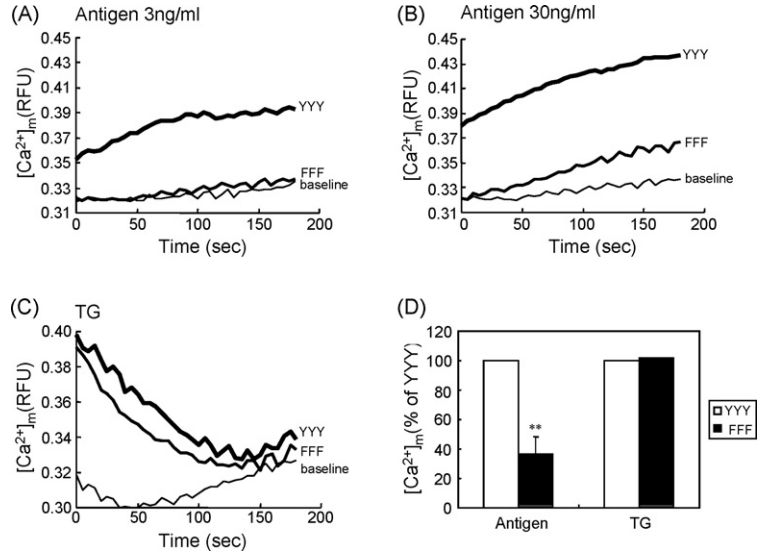


**Fig. 5 – FcεRIβ ITAM is essential for antigen-induced $[Ca^{2+}]_m$ uptake. The YYY ITAM-expressing BMMCs (YYY) and the FFF ITAM-expressing cells (FFF) were loaded with nonfluorescent dihydrorhod-2/AM as described above. The cells were stimulated 3 ng/ml (A) or 30 ng/ml TNP-BSA (B) or 1 μM TG (C), and then fluorescence was measured in a microplate fluorescence reader. Results are expressed in RFU, and are representative of three independent experiments. (D) Statistical analysis data of the maximal $[Ca^{2+}]_m$. The data represent the mean ± S.E.M. of three independent experiments.**

cells stimulated with a lower concentration of antigen (Fig. 6B). Furthermore, compared to the YYY ITAM-expressing cells, the FFF ITAM-expressing cells display a significant delay in the initiation of calcium response, as reported previously [28]. In response to TG, by contrast, these two cell types showed a comparable calcium response (data not shown). We also found that calcium responses of these two cell types were distinct in terms of their pharmacological properties. In the YYY ITAM-expressing cells nifedipine, a dihydropyridine receptor antagonist, profoundly decreased the antigen-induced $[Ca^{2+}]_i$ increase (Fig. 6C). In contrast, nifedipine displayed no effect on the $[Ca^{2+}]_i$ increase in the FFF ITAM-expressing cells (Fig. 6D). On the other hand, SK&F96365, a SOCE antagonist, blocked calcium response regardless of the type of ITAM expressed (Fig. 6C and D).

### 3.6. *FcεRI activation but not TG can stimulate a store-independent calcium entry that is sensitive to nifedipine and wortmannin but not to SOCE antagonists*

The above data suggest that the FcεRIβ ITAM is essential for a DHP-sensitive calcium influx. To exclude the possibility that this is an aberrant special case among mast cells expressing the reconstituted FcεRI, we sought to determine whether such a DHP-sensitive calcium channel operates under physiological conditions. Because other DHP-sensitive calcium channels, such as L-type calcium channels (LTCCs), are known to be activated independently of store emptying, this channel too can be activated in a store-independent manner. To test this, RBL-2H3 cells suspended in nominally calcium-free medium were treated with TG in order to deplete internal calcium stores, washed extensively, and then transferred to calcium-containing medium. Following TG application, $[Ca^{2+}]_i$ increased rapidly (reaching its peak within 30 s), then returned to resting levels at 5 min, indicating the depletion of intracellular calcium stores. As expected, even when $Ca^{2+}$ was replenished, TG could no longer induce any substantial calcium influx in the store-depleted cells (Fig. 7A). In contrast, antigen could still induce a calcium influx in a dose-dependent manner when calcium was reintroduced (Fig. 7A), indicating the occurrence of non-SOCE. This non-SOCE was inhibited by nifedipine but not affected by SK&F96365 or 2-APB (Fig. 7B). Essentially similar results were obtained with BMMCs, too (data not shown). Because antigen-induced $[Ca^{2+}]_m$ uptake and TG-induced $[Ca^{2+}]_m$ uptake are distinct in terms of the dependence on PI3K activity, we also examined the role of PI-3K activity. Wortmannin at concentrations of ≥30 nM dose-dependently inhibited the non-SOCE (Fig. 7C). On the other hand, both SK&F96365 and 2-APB significantly inhibited SOCE, while neither nifedipine nor wortmannin up to 100 nM reduced it (Fig. 7E, F and G). Fig. 7G shows statistical analysis data. The data indicate that both nifedipine and wortmannin blocks the non-SOCE selectively. Thus, FcεRI stimulation can activate not only SOCE but also non-SOCE influx via a DHP-sensitive calcium channel by separate signaling pathways, and that FcεRIβ and PI-3K activity play a selective role in the non-SOCE (Fig. 8).

## 4. Discussion

The data presented in this paper clearly demonstrate that FcεRI activation results in increased $[Ca^{2+}]_m$ uptake, and that this response is distinct from that in resting cells in terms of the dependences on the PTP and the mNCX. It has been widely accepted that mitochondria take up calcium through the $\Delta\Psi_m$-dependent calcium uniporter, while the accumulated $[Ca^{2+}]_m$ is released slowly, mainly through the mNCX [20]. Our data

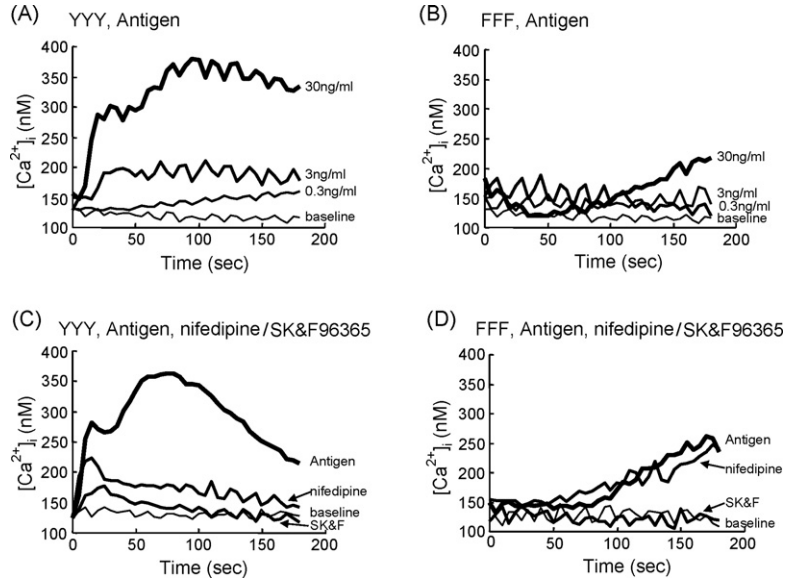


**Fig. 6 – The FcεRIβ ITAM is essential for an immediate nifedipine-sensitive calcium influx. The YYY ITAM-expressing BMMCs (YYY, A) and the FFF ITAM-expressing cells (FFF, B) ($1 \times 10^6$ ml$^{-1}$) were incubated with 4 μM Fluo3/AM at 37 °C for 30 min, washed with HBSS and then resuspended in calcium-containing medium. The Fluo-3-loaded cells were stimulated with TNP-BSA at the indicated concentrations and then fluorescence was monitored in a microplate fluorescence reader. (C and D) The Fluo3/AM-loaded YYY (C) and FFF cells (D) were treated with 1 μM nifedipine or 100 μM SK&F96365 (SK&F) and immediately stimulated with 30 ng/ml TNP-BSA, and then fluorescence was measured. Results are expressed in $[Ca^{2+}]_i$ and are representative of two to four independent experiments.**

indicate that while the FcεRI-stimulated $[Ca^{2+}]_m$ uptake is mediated by the calcium uniporter, the $[Ca^{2+}]_m$ efflux is mediated by the PTP rather than the mNCX, because pharmacological PTP modulators such as bongkrekic acid and atractyloside can affect the $[Ca^{2+}]_m$ uptake, while CGP-37157, a selective inhibitor of mNCX, cannot. This is consistent with our previous study demonstrating that the PTP plays a key role in the regulation of $[Ca^{2+}]_m$ homeostasis and calcium signaling in mast cells upon FcεRI stimulation [26]. Bongkrekic acid can inhibit adenine nucleotide translocase (ANT), a PTP component, and stabilizes it on the matrix side, thereby closing the PTP, while atractyloside stabilizes ANT on the cytoplasm side, thereby opening the PTP [36,37]. Thus, our data suggest that FcεRI activation can switch the mode of $[Ca^{2+}]_m$ homeostasis to one more closely associated with ANT. It is noteworthy that ANT has three cysteine residues whose reversible oxidation is critical for the PTP open-closed transitions and the $[Ca^{2+}]_m$ efflux [38,39]. As a result, the PTP open-closed transitions are controlled reversibly by ANT. Such a transient activation of PTP might cause a minimal damage to mitochondrial integrity, thereby regulating $[Ca^{2+}]_m$ homeostasis without inducing cell death. Consistent with this view, in non-neuronal cells, the PTP has been implicated as a fast calcium-release mechanism in physiological calcium signaling. On the other hand, a sustained and/or irreversible PTP opening in an ANT-independent manner, may lead to a disruption of mitochondrial $[Ca^{2+}]_m$ homeostasis and integrity. We have recently found that TG-induced $[Ca^{2+}]_m$ efflux is not mediated by the PTP, and that TG displays strong pro-apoptotic activity in these mast cells [40], while FcεRI activation causes cell death only under limited conditions, being consistent with earlier reports [41–44]. Thus, the switching of $[Ca^{2+}]_m$ homeostasis to one more closely associated with ANT might be involved in mast cell survival following cell activation. Further studies attempting to establish this idea are underway.

The next question to be addressed is how FcεRI regulates $[Ca^{2+}]_m$ homeostasis. The SERCA blocker TG can serve as a powerful tool for analyzing the role of the FcεRI-specific signal transduction pathway, because TG can activate mast cells by bypassing signals proximal to FcεRI aggregation [32]. We found that TG could induce $[Ca^{2+}]_m$ uptake only transiently and insensitive to PTP modulators. The data suggest that the proximal signal(s) bypassed by TG may be essential for prolongation of the response and PTP sensitivity. Pharmacological studies revealed the critical role of src family kinase and PI3K activity in such prolongation. Because the FcεRIβ ITAM serves as an important site of interaction with Lyn for signal transduction [4], we tested whether the FcεRIβ ITAM was involved in the prolonged $[Ca^{2+}]_m$ uptake. Analyses employing BMMCs lacking tyrosine residues of the FcεRIβ ITAM clearly demonstrated that the FcεRIβ ITAM is essential for antigen-induced $[Ca^{2+}]_m$ uptake specifically. The dysfunction of the FcεRIβ ITAM has been shown to result in the impairment of the physical interaction between Lyn and the FcεRIβ via its ITAM and downstream signaling pathways including activation of PI-3K [28]. In accordance with this, our pharmacological data indicate that PI-3K activity is essential for antigen-induced $[Ca^{2+}]_m$ uptake. On the other hand, TG, which evokes calcium signaling independently of the FcεRIβ, induces $[Ca^{2+}]_m$ uptake in a PI-3K-independent manner. Indeed, the lack of the FcεRIβ ITAM had a minimal effect on

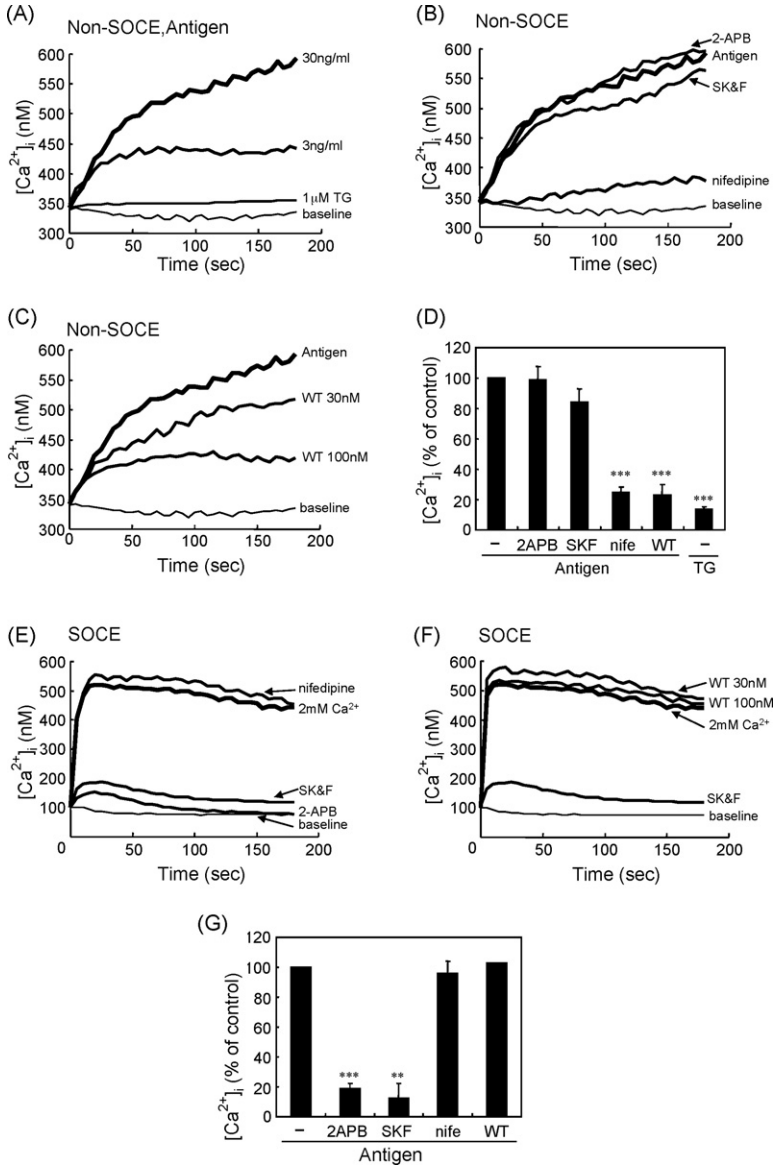


**Fig. 7 – Both nifedipine and wortmannin inhibit non-SOCE influx but not SOCE. (A) The Fluo3-loaded RBL-2H3 cells ($1 \times 10^6$ ml$^{-1}$) were suspended in nominally calcium-free medium and treated with 1 μM TG for 10 min in order to deplete intracellular calcium stores. After extensive washing, the cells were resuspended in calcium-containing medium and then stimulated with 1 μM TG or TNP-BSA at the indicated concentrations. (B and C) The store-depleted cells were treated with 10 μM 2-APB, 100 μM SK&F96365 (SK&F), 1 μM nifedipine (B) or wortmannin at the indicated concentrations (WT, C) and immediately stimulated with 30 ng/ml TNP-BSA. (E and F) The Fluo3-loaded RBL-2H3 cells ($1 \times 10^6$ ml$^{-1}$) were treated as described above to deplete intracellular calcium stores. The cells were replenished with 2 mM calcium in the presence or absence of 10 μM 2-APB, 100 μM SK&F96365 (SK&F), 1 μM nifedipine (E) or wortmannin at the indicated concentrations (WT, F). Results are expressed in $[Ca^{2+}]_i$ and are representative of four independent experiments. (D and G) Statistical analysis data of the maximal $[Ca^{2+}]_i$. The data represent the mean ± S.E.M.**

TG-induced $[Ca^{2+}]_m$ uptake. Collectively, these data suggest that $[Ca^{2+}]_m$ uptake occurs through at least two distinct signaling pathways, dependent or independent of PI-3K activity, and suggest that FcεRIβ ITAM mediates the PI-3K-dependent pathway selectively.

It has recently been demonstrated that mitochondria contribute to the dynamics of spatiotemporal calcium signaling by modulating SOCE (for a review see [45]). We demonstrate, consistently with this view, that impairment of the $[Ca^{2+}]_m$ uptake by the dysfunction of the FcεRIβ ITAM is accompanied by reduced calcium influx. A similar reduced calcium influx in FFF ITAM-expressing cells compared to YYY ITAM-expressing cells has been reported previously [28]. Moreover, we have found that the effect of the ITAM mutation on antigen-induced calcium influx depends on the intensity of FcεRI stimulation: upon weak stimulation, calcium responses were entirely abolished, while upon strong stimulation, an immediate calcium response was abrogated preferentially.

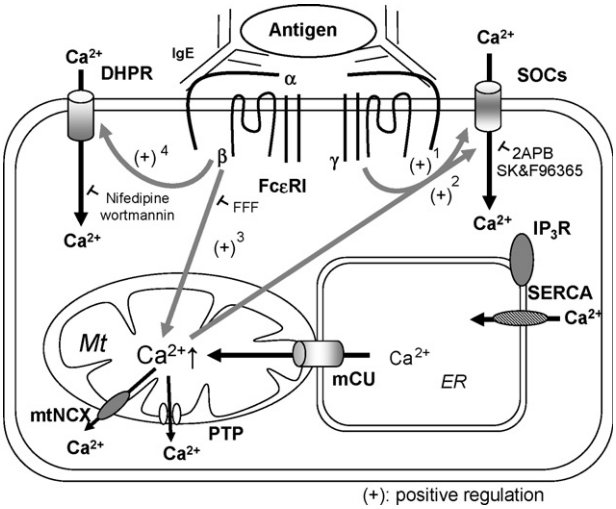


**Fig. 8 – Model for FcεRI-triggered calcium signaling in mast cells. Cross-linking of FcεRI on mast cells results in aggregation of the receptor and initiates a cascade of intracellular events leading to calcium influx. In the traditional view, the main pathway is activation of the FcεRIγ-Syk-PLCγ-$IP_3$ pathway, which causes depletion of intracellular calcium stores and calcium entering through SOCs (1). A growing body of evidence indicates that mitochondria play a key role in activating SOCs distal to calcium store emptying (2). The current study demonstrates that FcεRI can control mitochondrial calcium uptake via the β chain ITAM (3) and another calcium influx pathway via a DHPR (4). This pathway appears to contribute to mitochondrial calcium homeostasis.**

Our data demonstrate that the immediate calcium response regulated by the FcεRIβ ITAM is sensitive to the DHPR antagonist nifedipine but not to the SOCE antagonist SK&F96365. Moreover, FcεRI stimulation activates non-SOCE sensitive to nifedipine but not to SOCE antagonists among all types of mast cells tested. Taken together, the data suggest the occurrence of a DHPR-mediated calcium influx in mast cells. The DHPR is well known originally in excitable cells as the $\alpha_1$ subunit of LTCCs [46,47]. Indeed, in several immune cells such as B and T cells, antigen receptor stimulation evokes a calcium influx via a DHP-sensitive calcium channels; this plays a role in B and T cell functions including interleukin-4 production [48–50]. If we draw an analogy to TCR and BCR, it is possible that FcεRI stimulation also evokes a similar DHPR-mediated calcium influx in mast cells, an influx under the control of the mitochondria. Further investigations to characterize such a DHP-sensitive calcium channel are underway in our laboratory.

It is now widely accepted that the FcεRIβ acts as an important signal modulator in mast cells. The FcεRIβ was initially believed to act as an amplifier, because it has been demonstrated in humans that the FcεRIβ can amplify early activation signals through the FcεRIγ and allergic reactions [51]. The emerging view is that the FcεRIβ is a critical signal modulator that regulates activation signals both positively and negatively [28,35]. Moreover, it is known that the modulatory functions of the FcεRIβ strictly depend on the intensity of FcεRI stimulation. Like the effect on $[Ca^{2+}]_m$ uptake discovered in the present study, the FcεRIβ acts as an amplifier of activation signals induced by weak stimulation, while it is shown to dampen those induced by strong stimulation. Therefore, our findings lead us to the idea that the FcεRIβ might elicit a modulatory function by affecting the mitochondrial calcium homeostasis. We are now attempting to examine this hypothesis further.

In conclusion, we demonstrate that FcεRI regulates $[Ca^{2+}]_m$ uptake via the FcεRIβ ITAM and that this plays a key role in regulating non-SOCE via a DHP-sensitive calcium channel in mast cells. These findings may uncover as yet unrecognized roles of mitochondria and non-SOCE in regulating calcium signaling.

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