PROCEEDING

A novel Stim1-dependent, non-capacitative Ca^{2+} entry pathway is activated by B cell receptor stimulation and depletion of Ca^{2+} stores

Takao Morita[1], Akihiko Tanimura[1], Yoshihiro Baba[2], Tomohiro Kurosaki[2], and Yosuke Tojyo[1]

[1]*Department of Pharmacology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan ; and* [2]*Laboratory for Lymphocyte Differentiation, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan*

Abstract : In most non-excitable cells, the depletion of intracellular Ca^{2+} stores activates capacitative Ca^{2+} entry (CCE), which is a Ca^{2+}-selective and La^{3+}-sensitive entry pathway. Here, we report a novel mechanism of La^{3+}-resistant Ca^{2+} entry that is synergistically regulated by B cell receptor (BCR) stimulation and Ca^{2+} store depletion (B-SOC). In the wild-type (WT) DT40 cells, BCR stimulation with anti-IgM antibodies induced Ca^{2+} release and subsequent Ca^{2+} entry in the presence of 0.3 μM La^{3+} which blocks CCE completely. In the inositol 1,4,5-trisphosphate receptor-deficient (IP_3R-KO) cells, BCR stimulation elicited neither Ca^{2+} release nor Ca^{2+} entry. However, under pretreatment of thapsigargin (ThG), BCR stimulation induced La^{3+}-resistant Ca^{2+} entry into both WT and IP_3R-KO cells. These results indicate that BCR stimulation and Ca^{2+} store depletion work in concert to activate the La^{3+}-resistant Ca^{2+} entry pathway. B-SOC was inhibited by tyrosine kinase inhibitor, genistein. In addition, B-SOC was completely abolished in Stim1-deficient cells and was restored by overexpression of yellow fluorescent protein (YFP)-tagged Stim1, but was unaffected by double knockdown of Orai1/Orai2. These results demonstrate a unique non-CCE pathway, in which Ca^{2+} entry depends on Stim1 and tyrosine kinase activation. It is likely that similar regulation of Ca^{2+} entry occurs in other cell types including salivary gland cells. J. Med. Invest. 56 Suppl. : 383-387, December, 2009

Keywords **:** *Ca^{2+} entry, Ca^{2+} store, Stim1, B cell receptor, tyrosine kinase*

INTRODUCTION

Elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key signal that regulates a variety of physiological processes (1, 2). This Ca^{2+} signal can be derived from internal stores or from the extracellular space. Receptor-mediated phospholipase C (PLC) activation generates inositol 1,4,5-trisphosphate (IP_3) and induces Ca^{2+} release from endoplasmic reticulum (ER) stores through the IP_3 receptor (IP_3R). Depletion of intracellular Ca^{2+} stores induces Ca^{2+} entry across the plasma membrane (PM), a phenomenon referred to as capacitative or store-operated Ca^{2+} entry (CCE or SOC, ref. 3, 4). CCE is activated solely via the depletion of Ca^{2+} stores, which can be induced in a PLC-independent manner

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Address correspondence and reprint requests to Akihiko Tanimura, Department of Pharmacology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan and Fax : +81-133-23-1399.

by the ER Ca^{2+} pump blocker thapsigargin (ThG) or the Ca^{2+} ionophore ionomycin (3, 4). Thus, IP_3R-mediated Ca^{2+} release from intracellular stores indirectly contributes to CCE activation by physiological stimuli.

Recently, the proteins Stim1 and Orai1 have emerged as candidate components mediating CCE (4-7). Specifically, Stim1 responds to the depletion of Ca^{2+} stores, activating CCE via an interaction with Orai1 (also called CRACM1), a component of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels found in the PM (4, 6, 8, 9). Although it is clear that Stim1 moves close to the PM in response to store depletion and that it activates CCE via Orai1, the mechanisms by which this activation occurs remain unclear.

In the present study, we examined the role of IP_3Rs in BCR-mediated Ca^{2+} entry and demonstrate that these molecules contribute indirectly to La^{3+}-resistant Ca^{2+} entry by depleting Ca^{2+} stores. In addition, our findings reveal that BCR stimulation and Ca^{2+} depletion synergistically activate a novel La^{3+}-resistant Ca^{2+} entry pathway in a Stim1-dependent manner. Our results suggest that, in addition to CCE, Stim1 may be involved in the regulation of multiple Ca^{2+} entry pathways.

RESULTS

BCR-mediated La^{3+}-resistant Ca^{2+} entry into DT40 cells

In the absence of extracellular Ca^{2+}, anti-IgM-mediated BCR activation induced Ca^{2+} responses in most wild-type (WT) DT40 cells. Restoration of extracellular Ca^{2+} and the presence of anti-IgM dramatically increased $[Ca^{2+}]_i$, due to Ca^{2+} entry from the extracellular space. This effect occurred even in the presence of 0.3 μM La^{3+} (Fig. 1A). As shown in Fig. 1B, ThG treatment depleted intracellular Ca^{2+}

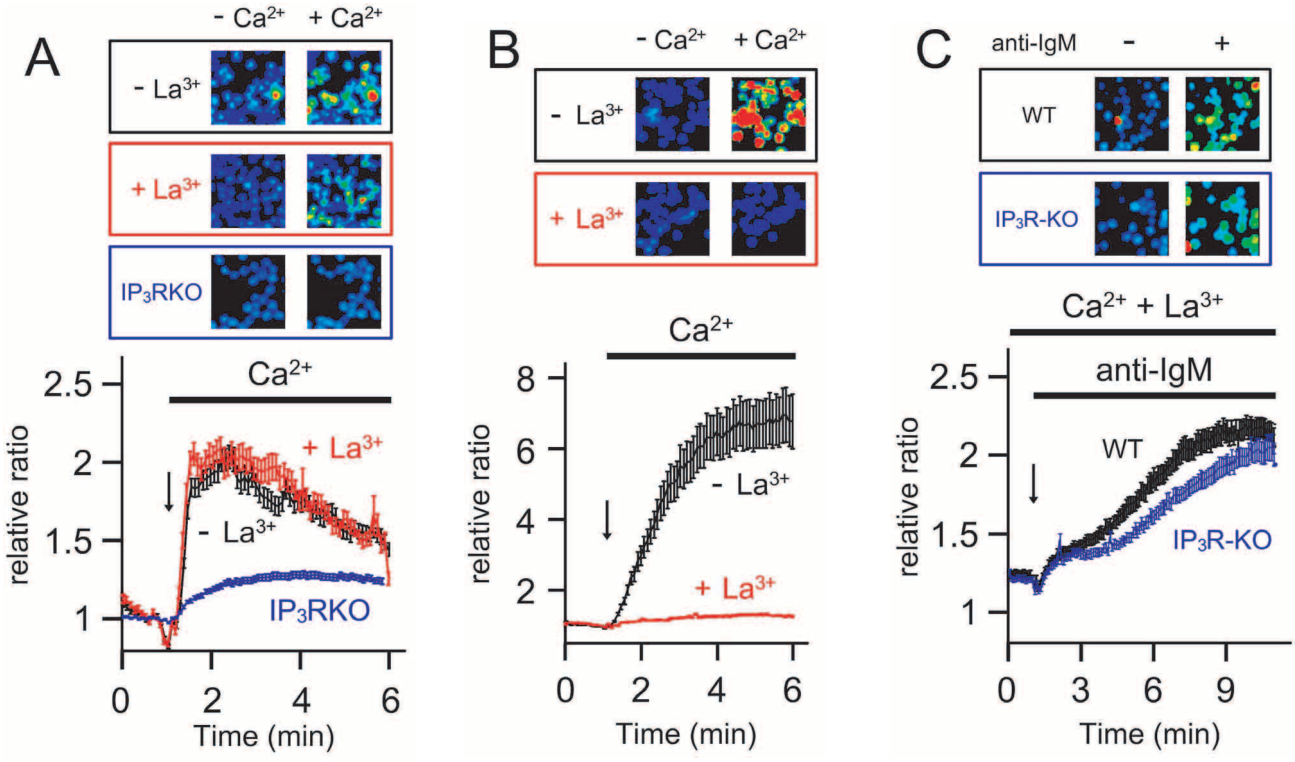

Figure 1. BCR-mediated La^{3+}-resistant Ca^{2+} entry into DT40 cells.
To monitor $[Ca^{2+}]_i$, cells were attached to a small recording chamber coated with 0.5 mg/ml poly-L-lysine and Cellmatrix (diluted 1 : 10). Attached cells were incubated in culture medium for 1 h at 37°C and loaded with 2 μM fura-2/AM. All experiments were performed in PO_4^{3-}-, SO_4^{2-}-, CO_3^{2-}-free HBSS-H at room temperature.
A : WT (black and red) or IP_3R-KO (blue) DT40 cells were stimulated with 3 μg/ml anti-IgM in a nominally Ca^{2+}-free medium, followed by the addition of 1.3 mM Ca^{2+} in the absence (black and blue) or presence (red) of 0.3 μM La^{3+}. Fluorescence ratios (340 nm/380 nm) were normalized to the ratio obtained just prior to the addition of Ca^{2+} (relative ratios). Upper : pseudocolor images of fura-2 ratios. Lower : normalized fluorescence ratio. Traces are the means± s.e.m.
B : WT cells were treated with 1 μM ThG in nominally Ca^{2+}-free medium, followed by the addition of Ca^{2+} in the absence (black) or presence (red) of La^{3+}.
C : WT (black) or IP_3R-KO (blue) cells were pretreated for 10 min with 1 μM ThG in nominally Ca^{2+}-free medium and then 2 μg/ml anti-IgM was added in the presence of La^{3+} and Ca^{2+}. Modified from ref. 10.

stores and resulted in CCE following the addition of extracellular Ca^{2+}. We did not detect ThG-induced CCE in the presence of 0.3 μM La^{3+} (Fig. 1B). These results indicate that, unlike ThG treatment alone, BCR-stimulation activates a Ca^{2+} entry pathway that is not inhibited by 0.3 μM La^{3+} (Fig. 1A, B). Thus, BCR stimulation appears to induce Ca^{2+} entry via a CCE-independent pathway.

La^{3+}-resistant Ca^{2+} entry requires depletion of Ca^{2+} stores and BCR stimulation

Next, we examined BCR-mediated La^{3+}-resistant Ca^{2+} entry using DT40 cells treated with ThG. In inositol 1,4,5-trisphosphate receptor-deficient (IP_3R-KO) cells, BCR stimulation elicited neither Ca^{2+} release nor Ca^{2+} entry (Fig. 1A). After depletion of Ca^{2+} with ThG, La^{3+} and Ca^{2+} were added to the cells, followed by BCR stimulation with anti-IgM. BCR-mediated La^{3+}-resistant Ca^{2+} entry was observed in ThG-treated IP_3R-KO DT40 cells as well as in WT cells (Fig. 1C). Our results suggest that the La^{3+}-resistant Ca^{2+} entry pathway is activated by an interaction between BCR stimulation and Ca^{2+} store depletion, and we propose to name this putative Ca^{2+} entry pathway 'BCR-mediated store-operated Ca^{2+} entry' (B-SOC).

B-SOC requires BCR-mediated activation of tyrosine kinase

We examined the mechanisms that occur downstream of BCR stimulation using a tyrosine kinase inhibitor, genistein. In both WT and IP_3R-KO ThG-treated DT40 cells, BCR-mediated La^{3+}-resistant Ca^{2+} entry was diminished by 50 μM genistein, indicating that B-SOC requires BCR-mediated activation of tyrosine kinase activity (10).

Role of Stim1 in B-SOC

Recent reports have demonstrated that the ER-resident protein Stim1 plays an essential role in SOC (4, 6, 7). We used Stim1-deficient (Stim1-KO) DT40 cells to examine whether Stim1 plays a role in BCR-mediated La^{3+}-resistant Ca^{2+} entry (11). In Stim1-KO DT40 cells, B-SOC was completely abolished (Fig. 2A). In addition, La^{3+}-resistant Ca^{2+} entry was completely restored by overexpression of

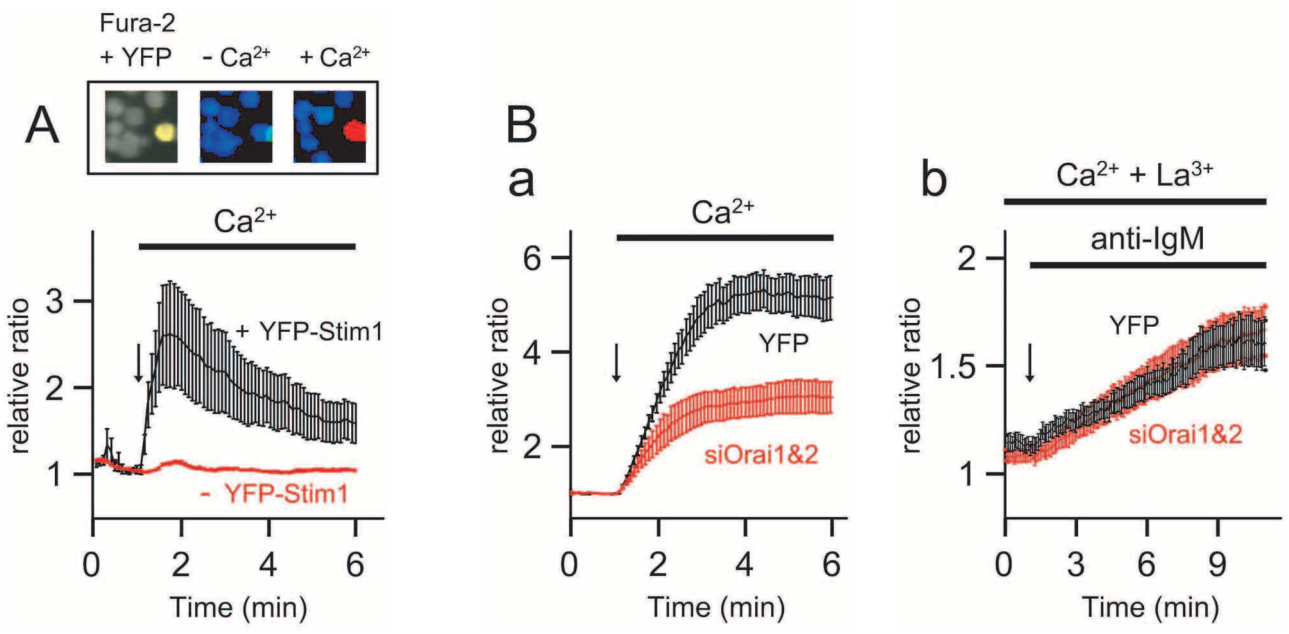

Figure 2. Involvement of Stim1 but not Orai1/2 in B-SOC.
A : Involvement of Stim1 in B-SOC
Stim1-KO cells were transfected with the YFP-Stim1 plasmid and Ca^{2+} responses in YFP-positive (black) and YFP-negative (red) cells were examined. Cells were stimulated with 2 μg/ml anti-IgM in nominally Ca^{2+}-free medium, followed by the addition of La^{3+} and Ca^{2+}. Upper : Fura-2 and YFP fluorescence or pseudocolor images of fura-2 ratios. Lower : normalized fluorescence ratio. Traces are the means± s.e.m.
B : Effect of Orai1 and Orai2 siRNAs (siOrai1 and siOrai2) on CCE and B-SOC.
WT cells were co-transfected with the YFP plasmid and siOrai1 and siOrai2.
(a) Cells were treated with ThG in nominally Ca^{2+}-free medium and then Ca^{2+} was added. Black : YFP-transfected cells ; Red : YFP-positive siOrai1- and siOrai2-transfected cells.
(b) Cells were treated with ThG in nominally Ca^{2+}-free medium and then 3 μg/ml anti-IgM was added in the presence of La^{3+} and Ca^{2+}. Black : YFP-transfected cells ; Red : YFP-positive siOrai1- and siOrai2-transfected cells. Modified from ref. 10.

yellow fluorescent protein (YFP)-tagged Stim1 (YFP-Stim1 ; Fig. 2A). Similarly, BCR-mediated Ca^{2+} entry after ThG treatment was not detected in Stim1-KO DT40 cells but restored in YFP-Stim1-expressing DT40 cells (10). These experiments clearly demonstrate that Stim1 plays an essential role in BCR-mediated La^{3+}-resistant Ca^{2+} entry.

Orai1 and Orai2 do not play a role in B-SOC

We next examined the effects of Orai1 and Orai2 knockdown on CCE and B-SOC in WT DT40 cells. Co-transfection with Orai1 and Orai2 siRNAs and an YFP plasmid as a transfection marker reduced ThG-induced Ca^{2+} entry (Fig. 2Ba). In contrast, BCR-mediated Ca^{2+} entry after ThG-treatment was not altered by the co-transfection of Orai1 and Orai2 siRNAs (Fig. 2Bb). Together with our finding that B-SOC is not inhibited by the CCE blocker La^{3+}, these results provide strong evidence that Stim1 performs an essential role in B-SOC via an Orai-independent pathway.

DISCUSSION

Here, we have described a novel La^{3+}-resistant Ca^{2+} entry pathway that is regulated in concert with depletion of Ca^{2+} stores via Stim1- and BCR-mediated activation of tyrosine kinases. We propose calling this novel entry mechanism 'BCR-mediated and store-operated Ca^{2+} entry' (B-SOC). A similar La^{3+}-resistant Ca^{2+} entry was observed in Jurkat T cells, where ThG-induced Ca^{2+} entry was completely blocked by 1 μM La^{3+}, and subsequent activation of T-cell receptors by anti-CD3 antibodies (3 μg/ml) induced significant Ca^{2+} entry (10).

We found that BCR-mediated Ca^{2+} entry was completely abolished in Stim1-KO DT40 cells and was restored by overexpression of YFP-Stim1. Treatment with the mixture of Orai1 and Orai2 siRNAs did not significantly decrease B-SOC. In addition, total internal reflection fluorescence (TIRF) imaging revealed that partial relocations of YFP-Stim1 were induced by weak BCR stimulation (10). These results suggest that Stim1 has an essential role in the regulation of B-SOC through Orai1-independent pathway. The B-SOC pathway is likely to be the principal route of Ca^{2+} entry, particularly in the presence of weak BCR stimulation. It is likely that similar regulation of Ca^{2+} entry occurs via Stim1-dependent and tyrosine kinase-mediated responses of other cell types including salivary gland cells. Thus, the physiological roles and mechanisms of B-SOC regulation, including channel properties and molecular interactions, should be explored in future studies.

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