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

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Accepted 27 December 2008
Journal of Cell Science 122, 1220-1228 Published by The Company of Biologists 2009
doi:10.1242/jcs.041640

Summary

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. In DT40 cells, stimulation of BCRs with anti-IgM antibodies induced Ca^{2+} release and subsequent Ca^{2+} entry in the presence of 0.3 μM La^{3+}, a condition in which CCE is completely blocked. This phenomenon was not observed in inositol 1,4,5-trisphosphate receptor-deficient DT40 (IP3R-KO) cells. However, in response to thapsigargin pretreatment, BCR stimulation induced La^{3+}-resistant Ca^{2+} entry into both wild-type and IP3R-KO cells. These results indicate that BCR stimulation alone does not activate Ca^{2+} entry, whereas BCR stimulation and depleted Ca^{2+} stores (either due to IP3R-mediated Ca^{2+} release or Ca^{2+} uptake inhibition) work in concert to activate La^{3+}-resistant Ca^{2+} entry. This Ca^{2+} entry was inhibited by genistein. In addition, BCR-mediated Ca^{2+} entry was completely abolished in Stim1-deficient DT40 cells and was restored by overexpression of YFP-Stim1, but was unaffected by double knockdown of Orai1 and Orai2. These results demonstrate a unique non-CCE pathway, in which Ca^{2+} entry depends on Stim1- and BCR-mediated activation of tyrosine kinases.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/8/1220/DC1

Key words: Ca^{2+} entry, Ca^{2+} store, Stim1, B-cell receptor, Inositol 1,4,5-trisphosphate receptor, DT40 cells

Introduction

Elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key signal that regulates a variety of physiological processes (Berridge, 1993; Berridge et al., 2000). This Ca^{2+} signal can be derived from internal stores or from the extracellular space. Phospholipase C (PLC)-dependent Ca^{2+} release from the endoplasmic reticulum (ER) and subsequent Ca^{2+} entry from the extracellular space, represent a universal mechanism of receptor-induced Ca^{2+} signaling in non-excitable cells (Barritt, 1999; Berridge et al., 2003; Clapham, 1995). Receptor-mediated PLC activation occurs through distinct coupling mechanisms: G-protein-coupled receptors activate PLCβ isoforms, whereas receptor and non-receptor tyrosine kinases activate PLCγ isoforms (Berridge et al., 2000; Berridge et al., 2003; Rhee, 2001). These PLCs cleave phosphatidylinositol-4,5-bisphosphate into inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3 or IP_3] and diacylglycerol (DAG). Subsequently, Ins(1,4,5)P_3 binds to the Ins(1,4,5)P_3 receptor (IP3R; official protein symbol ITPR1), an ER membrane channel, allowing Ca^{2+} release from ER stores to increase $[Ca^{2+}]_i$, which, in turn, stimulates external Ca^{2+} entry through plasma membrane channels.

There are several different types of Ca^{2+} channel and several different mechanisms controlling Ca^{2+} entry in response to intracellular messengers and depletion of intracellular stores. Depletion of intracellular Ca^{2+} stores induces Ca^{2+} entry across the plasma membrane, a phenomenon referred to as capacitative or store-operated Ca^{2+} entry (CCE or SOC) (Parekh and Putney, 2005; Putney, 2007; Venkatachalam et al., 2002). CCE is activated solely by the depletion of Ca^{2+} stores, which can be induced in a PLC-independent manner by the ER Ca^{2+}-pump blocker thapsigargin (ThG) or the Ca^{2+} ionophore ionomycin (Parekh and Putney, 2005; Putney, 2007). This characteristic is apparently ubiquitous in non-excitable cells. Thus, IP3R-mediated Ca^{2+} release from intracellular stores indirectly contributes to CCE activation by physiological stimuli. In addition to CCE, physiological stimuli can evoke Ca^{2+} entry via a variety of Ca^{2+}-permeable channels that are activated by intracellular messengers such as DAG.

Over the past 20 years, special attention has been paid to the precise nature of CCE and its regulatory mechanisms. Recently, the proteins Stim1 and Orai1 have emerged as candidate components mediating CCE (Feske et al., 2006; Liou et al., 2005; Putney, 2007; Roos et al., 2005). Specifically, Stim1 responds to the depletion of Ca^{2+} stores, activating CCE via an interaction with Orai1 (official symbol CRACM1), a component of the calcium-release-activated calcium (CRAC) channels found in the plasma membrane (Hewavitharana et al., 2007; Liou et al., 2005; Putney, 2007; Vig et al., 2006; Zhang et al., 2005). Although it is clear that Stim1 moves close to the plasma membrane in response to store depletion and that it activates CCE via Orai1, the mechanisms by which this activation occurs remain unclear. In addition, several studies have suggested that Stim1 is involved in Ca^{2+} entry via other channels [e.g. TRPCs and arachidonic-acid-dependent Ca^{2+}-selective (ARC) channels] (Huang et al., 2006; López et al., 2006; Mignen et al.,

2007; Ong et al., 2007; Yuan et al., 2007). These Ca^{2+}-entry mechanisms are sometimes referred to as 'non-CCE mechanisms' or 'tissue-specific SOC subtypes' (Venkatachalam et al., 2002).

Previous studies have suggested that IP3Rs are Ca^{2+}-release channels located in the ER membranes. There is additional evidence to suggest that these channels are also located in the plasma membrane, where they function as Ca^{2+}-entry channels (Dellis et al., 2006; Khan et al., 1992; Kuno and Gardner, 1987; Tanimura et al., 2000). Our previous studies have shown that anti-IgM antibody (anti-IgM) stimulation of B-cell receptors (BCRs) can induce Ca^{2+} release and La^{3+}-resistant Ca^{2+} entry in DT40 cells, a chicken B-cell line (Morita et al., 2004). However, BCR stimulation did not induce Ca^{2+} release or entry in mutant DT40 cells lacking all three IP3R isoforms (IP3R-KO DT40 cells) (Morita et al., 2004; Patterson et al., 2002; Sugawara et al., 1997; van Rossum et al., 2004; Vazquez et al., 2002; Venkatachalam et al., 2001). Based on these results, we speculated that IP3Rs are involved in BCR-mediated entry of Ca^{2+} into DT40 cells.

In the present study, we examined the role of IP3Rs 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 might be involved in the regulation of multiple Ca^{2+}-entry pathways.

Results

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

Previous studies have shown that DT40 cells express the α-IgM isotype BCR, which mediates an increase in $[Ca^{2+}]_i$ through the activation of PLCγ2 (Kurosaki et al., 2000). In the absence of extracellular Ca^{2+}, anti-IgM-mediated BCR activation induced oscillatory Ca^{2+} responses, which ceased within 10 minutes in most wild-type (WT) DT40 cells (Fig. 1A). Following a second application of anti-IgM, an additional (albeit small) release of Ca^{2+} was observed. However, this response was minimal by the third application of anti-IgM. Restoration of extracellular Ca^{2+} and the presence of anti-IgM dramatically increased $[Ca^{2+}]_i$, as a result of Ca^{2+} entry from the extracellular space. This effect occurred even in the presence of La^{3+} (Fig. 1A,B). As shown in Fig. 1C, ThG induced a transient increase in $[Ca^{2+}]_i$ upon leakage from Ca^{2+} stores.

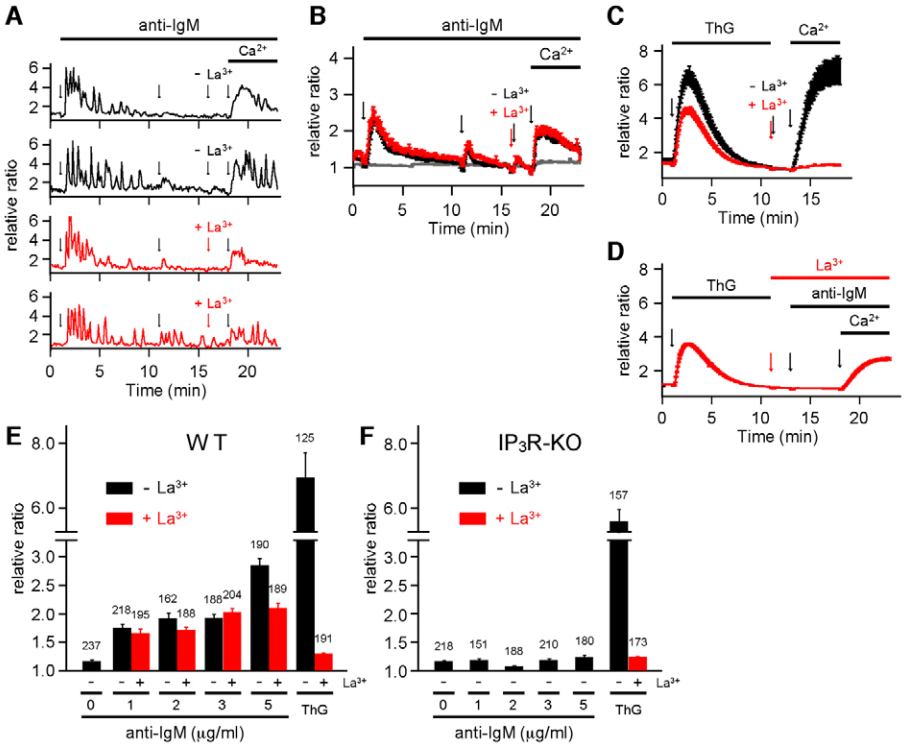

Fig. 1. BCR-mediated La^{3+}-resistant Ca^{2+} entry into DT40 cells. (A) WT DT40 cells were stimulated with 3 μg/ml anti-IgM in a nominally Ca^{2+}-free medium, followed by the addition of 1.3 mM Ca^{2+} in the absence (black) or presence (red) of 0.3 μM La^{3+}. Typical Ca^{2+} responses in single DT40 cells in the absence (black) and presence (red) of La^{3+} are shown. Fluorescence ratios (340 nm/380 nm) were normalized to the ratio obtained just before addition of Ca^{2+} (relative ratios). Horizontal bars indicate the presence of anti-IgM and Ca^{2+}. Time points for the addition of reagents by exchanging medium are indicated by the arrows. The red arrow indicates the time of La^{3+} addition. (B) Normalized fluorescence ratios were collected at 5 second intervals from cells examined in the absence (black) or presence (red) of La^{3+}. The gray trace indicates the normalized fluorescence ratio in the absence of La^{3+} and anti-IgM stimulation. Traces shown are the means ± s.e.m. of 187 and 204 cells in the absence or presence of La^{3+}, respectively, and 237 unstimulated cells. Horizontal bars indicate the presence of anti-IgM and Ca^{2+}. (C) 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+}. Traces are shown as the means ± s.e.m. of 125 and 191 cells in the absence or presence of La^{3+}, respectively. Horizontal bars indicate the presence of ThG and Ca^{2+}. (D) Anti-IgM (2 μg/ml) was added before the addition of Ca^{2+} in the presence La^{3+}. The trace shown is the mean ± s.e.m. of 234 cells. Horizontal bars indicate the presence of ThG, anti-IgM, La^{3+} and Ca^{2+}. (E,F) Effects of La^{3+} on BCR- or ThG-induced Ca^{2+} entry into WT (E) and IP3R-KO (F) DT40 cells. Maximal increases in fluorescence ratios following the addition of Ca^{2+} to cells treated with 0-5 μg/ml anti-IgM or 1 μM ThG in the absence (black columns) or presence (red columns) of La^{3+}. Anti-IgM, ThG, La^{3+}, and Ca^{2+} were applied at time points depicted in B or C. The total number of cells examined is shown above each column.

ThG treatment depleted intracellular Ca^{2+} 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. 1C); however, the addition of anti-IgM after ThG-induced store depletion elicited La^{3+}-resistant Ca^{2+} entry (Fig. 1D). 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. 1B-D). Thus, BCR stimulation appears to induce Ca^{2+} entry via a CCE-independent pathway.

We further examined the effects of various concentrations of anti-IgM and ThG on Ca^{2+} entry, in the presence or absence of 0.3 μM La^{3+} in DT40 cells (Fig. 1E). When DT40 cells were stimulated with 1-3 μg/ml anti-IgM, comparable levels of Ca^{2+} entry were observed in the absence or presence of La^{3+}. In the absence of La^{3+}, an approximately twofold greater level of Ca^{2+} entry was observed following stimulation with 5 μg/ml anti-IgM than with 3 μg/ml anti-IgM. However, the presence of 0.3 μM La^{3+} reduced Ca^{2+} entry by ~60%, to a level comparable to that observed with 3 μg/ml anti-IgM. Moreover, the presence of 0.3 μM La^{3+} reduced ThG-induced Ca^{2+} entry to 5.1% of the level measured in the absence of La^{3+}. These results suggest that, at relatively low concentrations of anti-IgM (1-3 μg/ml), La^{3+}-resistant Ca^{2+} entry dominates over CCE in BCR-mediated Ca^{2+} entry and that the contribution of CCE becomes more significant during strong BCR stimulation, which is probably due to pronounced depletion of the Ca^{2+} stores.

We previously reported that BCR-mediated Ca^{2+} release and subsequent Ca^{2+} entry were abolished completely in IP3R-KO DT40 cells (Morita et al., 2004). Indeed, no increase in $[Ca^{2+}]_i$ occurred following stimulation of IP3R-KO DT40 cells with anti-IgM (data not shown). In addition, Ca^{2+} entry was not induced by BCR stimulation with any concentration of anti-IgM in IP3R-KO DT40 cells (Fig. 1F). These results suggest that BCR stimulation alone is not sufficient to activate Ca^{2+} entry and IP3Rs are involved in the BCR-mediated Ca^{2+} entry.

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. After depletion of the Ca^{2+} store with ThG, La^{3+} and Ca^{2+} were added to the cells, followed by BCR stimulation with anti-IgM (Fig. 2). In ThG-treated WT DT40 cells, anti-IgM induced an increase in $[Ca^{2+}]_i$ in the presence of 0.3 μM La^{3+} (Fig. 2A). This Ca^{2+} increase reached a plateau within 5 to 10 minutes. Unexpectedly, comparable BCR-mediated La^{3+}-resistant Ca^{2+} entry was observed in ThG-treated IP3R-KO DT40 cells (Fig. 2B,C). These results indicate that IP3Rs are not required for BCR-mediated Ca^{2+} entry into ThG-treated DT40 cells.

We then examined La^{3+}-resistant Ca^{2+} entry in the presence of various concentrations of anti-IgM in ThG-treated WT (Fig. 2C, black bars) and IP3R-KO (Fig. 2C, white bars) DT40 cells (Fig. 2C). In WT DT40 cells, 2 μg/ml anti-IgM induced a level of La^{3+}-resistant Ca^{2+} entry comparable with the maximum observed for La^{3+}-resistant entry in ThG-untreated cells (Fig. 1E; Fig. 2C). No further increases in La^{3+}-resistant Ca^{2+} entry were induced by treatment with 3 μg/ml or 5 μg/ml anti-IgM (Fig. 1E; Fig. 2C). These results suggest that weak BCR stimulation (2-3 μg/ml anti-IgM) is sufficient for maximal activation of La^{3+}-resistant Ca^{2+} entry. Similar results were observed in IP3R-KO DT40 cells, although the La^{3+}-resistant Ca^{2+} entry elicited by 3 μg/ml or 5 μg/ml anti-IgM was slightly less than that in WT DT40 cells.

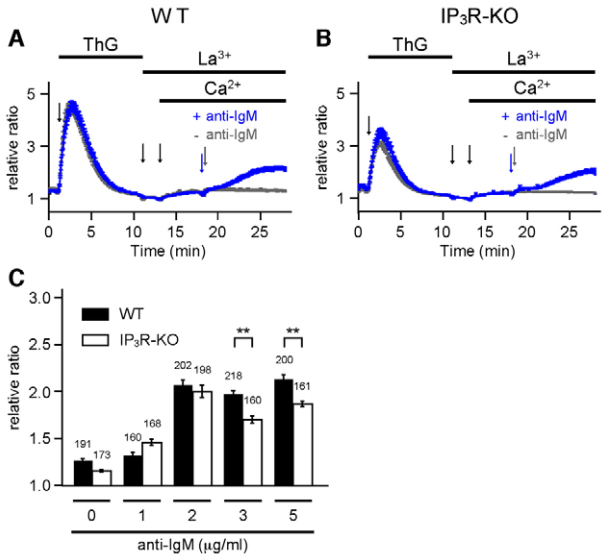

Fig. 2. BCR-mediated La^{3+}-resistant Ca^{2+} entry after store depletion in WT and IP3R-KO DT40 cells. (A,B) WT (A) and IP3R-KO (B) DT40 cells were pretreated for 10 minutes with 1 μM ThG in nominally Ca^{2+}-free medium, after which time La^{3+}, Ca^{2+}, and either 2 μg/ml anti-IgM (blue) or vehicle (gray) were added. Traces shown are the means ± s.e.m. of 191 and 202 cells in the absence or presence of anti-IgM in WT cells, and 173 and 198 cells in the absence or presence of anti-IgM in IP3R-KO cells, respectively. The horizontal bars in A and B indicate the presence of ThG, La^{3+} and Ca^{2+}. Time points for the addition of reagents by exchanging medium are indicated by arrows. Anti-IgM was added at the time indicated by the blue arrow. (C) La^{3+}-resistant Ca^{2+} entry in the presence of increasing anti-IgM stimulation of ThG-pretreated WT (black) and IP3R-KO (white) cells. Increases in fluorescence ratios at 10 minutes after the addition of various concentrations of anti-IgM (0 μg/ml to 5 μg/ml) to ThG-pretreated cells in the presence of La^{3+} and Ca^{2+}. Data shown are means ± s.e.m. The total number of cells examined is shown above each column. $^{**}P<0.01$.

Unlike results in cells not treated with ThG, BCR stimulation following store depletion induced La^{3+}-resistant Ca^{2+} entry in both IP3R-KO and WT DT40 cells. These results indicate that ThG-induced depletion of Ca^{2+} stores can compensate for the absence of IP3Rs, which mediate Ins(1,4,5)P_3-induced release of stored Ca^{2+}. Indeed, stimulation with 3 μg/ml anti-IgM caused a partial depletion of stored Ca^{2+} (data not shown). We hypothesize, therefore, that BCR-mediated Ins(1,4,5)P_3 production and subsequent Ca^{2+} release through IP3Rs contributes to the activation of BCR-mediated La^{3+}-resistant Ca^{2+} entry via the (partial) depletion of stored Ca^{2+}. 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).

La^{3+}-resistant Ca^{2+} entry requires BCR-mediated activation of tyrosine kinase but not activation of PLC

We examined the mechanisms that occur downstream of BCR stimulation using a tyrosine kinase inhibitor (genistein), a PLC inhibitor (U-73122), and a Ser/Thr kinase inhibitor (staurosporine) (Fig. 3). Anti-IgM stimulation of DT40 cells induced Ca^{2+} release via a non-receptor tyrosine kinase (RTK)-linked cascade, generating Ins(1,4,5)P_3 and DAG through activation of PLCγ2. BCR-mediated Ca^{2+} release was reduced following treatment with 50 μM genistein or 2 μM U-73122 (supplementary material Fig. S1), confirming that these reagents inhibited BCR-mediated tyrosine kinase activity

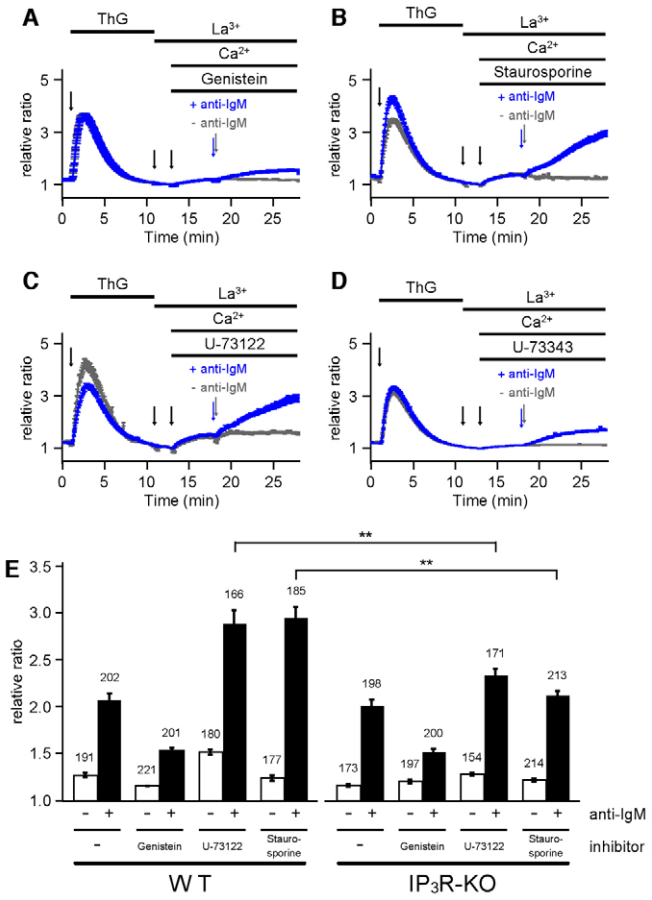

Fig. 3. Effects of genistein, U-73122, and staurosporine on BCR-mediated Ca^{2+} entry following ThG pretreatment. (A-D) WT DT40 cells were treated with ThG in nominally Ca^{2+}-free medium. Subsequently, cells were treated with 50 µM genistein (A), 100 nM staurosporine (B), 2 µM U-73122 (C) or 2 µM U-73343 (D) followed by addition of La^{3+} and Ca^{2+} and stimulation with anti-IgM (blue) or vehicle (gray). Traces shown are the means ± s.e.m. of 221 and 201 cells in the absence or presence of anti-IgM in A; 177 and 185 cells in the absence or presence of anti-IgM in B; 180 and 166 cells in the absence or presence of anti-IgM in C; and 188 and 218 cells in the absence or presence of anti-IgM in D, respectively. The horizontal bars indicate the presence of inhibitors, ThG, La^{3+} and Ca^{2+}. Time points for the addition of reagents by exchanging medium are indicated by the arrows. Anti-IgM was added at the time indicated by the blue arrow. (E) Effects of various inhibitors on BCR-mediated La^{3+}-resistant Ca^{2+} entry in ThG-pretreated WT and IP3R-KO DT40 cells. Increases in fluorescence ratios of the ThG-pretreated cells were obtained 10 minutes after the addition of 2 µg/ml anti-IgM (black) or vehicle (white) in the presence of La^{3+}, Ca^{2+}, and each inhibitor. The total number of cells examined is shown above each column. $^{**}P<0.01$.

and PLC activity, respectively. In both WT and IP3R-KO ThG-treated DT40 cells, BCR-mediated La^{3+}-resistant Ca^{2+} entry was diminished by 50 µM genistein (Fig. 3A,E), indicating that B-SOC requires BCR-mediated activation of tyrosine kinase activity. By contrast, 100 nM staurosporine or 2 µM U-73122 (Fig. 3B,C,E) enhanced the BCR-mediated La^{3+}-resistant Ca^{2+} entry in WT DT40 cells, but had no effect in IP3R-KO DT40 cells. An inactive analogue of U-73122, U-73343 (2 µM), did not stimulate or inhibit B-SOC (Fig. 3D). These observations are consistent to the hypothesis that B-SOC does not require Ins(1,4,5)P_3. The enhancement of B-SOC in response to staurosporine or U73122 in WT DT40 cells may suggest the presence of IP3R-dependent modulations of B-SOC through PLC and/or protein kinase C, but we did not examine these effects in further detail.

PLCβ is expressed in DT40 cells and it is activated by protease-activated receptor 2 (PAR2), a G-protein-coupled receptor; thus, trypsin induces Ins(1,4,5)P_3-mediated Ca^{2+} responses in this cell line (Lievremont et al., 2005; Morita et al., 2004). In fact, although stimulation with 100 U/ml trypsin in WT cells induced Ca^{2+} responses, we did not observe induction of La^{3+}-resistant Ca^{2+} entry in ThG-treated DT40 cells following stimulation with 100 U/ml trypsin (supplementary material Fig. S2). These results provide additional support for the requirement of BCR stimulation rather than PLC activity in B-SOC.

Role of Stim1 in BCR-induced Ca^{2+} entry

Recent reports have demonstrated that the ER-resident protein Stim1 plays an essential role in SOC (Liou et al., 2005; Putney, 2007; Roos et al., 2005). In particular, Stim1 appears to function as a sensor of ER luminal Ca^{2+} levels, transducing information directly to plasma membrane SOC channels via redistribution within the ER. As Stim1 is known to both relocate to regions underlying the plasma membrane following depletion of stored Ca^{2+} and to activate plasma membrane Ca^{2+} channels, we used Stim1-deficient (Stim1-KO) DT40 cells to examine whether Stim1 plays a role in BCR-mediated La^{3+}-resistant Ca^{2+} entry (Baba et al., 2006). In Stim1-KO DT40 cells, BCR stimulation (2 µg/ml anti-IgM) induced oscillatory Ca^{2+} responses, but subsequent Ca^{2+} entry was completely abolished (Fig. 4A). In addition, La^{3+}-resistant Ca^{2+} entry was completely restored by overexpression of yellow fluorescent protein (YFP)-tagged Stim1 (YFP-Stim1) (Fig. 4A).

We also examined BCR-mediated Ca^{2+} entry in ThG-treated Stim1-KO DT40 cells. Following ThG-induced store depletion, BCR-mediated La^{3+}-resistant Ca^{2+} entry was not detected in Stim1-KO DT40 cells (Fig. 4B). In YFP-Stim1-expressing DT40 cells, a small but significant La^{3+}-resistant Ca^{2+} entry was observed after ThG-treatment, and subsequent BCR stimulation induced additional La^{3+}-resistant Ca^{2+} entry (Fig. 4B). These experiments clearly demonstrate that Stim1 has an essential role in BCR-mediated La^{3+}-resistant Ca^{2+} entry. The results shown in Fig. 4C confirm that CCE was abolished completely in Stim1-KO DT40 cells and was restored by overexpression of YFP-Stim1. Indeed, the overexpression of YFP-Stim1 enhanced ThG-induced Ca^{2+} entry. Although the ThG-induced Ca^{2+} entry was reduced to 15.2±3.7% by 0.3 µM La^{3+}, we still observed significant La^{3+}-resistant Ca^{2+} entry upon the addition of Ca^{2+}. This Ca^{2+} entry was not blocked by 1.0 µM La^{3+} or 1.0 µM Gd^{3+} (supplementary material Fig. S3). Although these results suggest that there is some BCR-independent La^{3+}-resistant Ca^{2+} entry in YFP-Stim1-overexpressing cells, we did not study this pathway in further detail.

Orai1 and Orai2 do not have a role in BCR-induced Ca^{2+} entry

We next examined the effects of RNA interference on CCE and B-SOC in WT DT40 cells. Cotransfection of Orai1 and/or Orai2 siRNAs with an EYFP plasmid enabled us to identify transfected cells and, thus, measure the effect of Orai1 and/or Orai2 knockdown on CCE and B-SOC. Transfection with chicken *Orai1* siRNA did not significantly affect ThG-induced Ca^{2+} entry, whereas cotransfection with *Orai1* and *Orai2* siRNAs reduced ThG-induced Ca^{2+} entry to 44.7±7.6% of that of untransfected DT40 cells (Fig. 5A,D). These experiments indicate that both Orai1 and Orai2 are involved in CCE in DT40 cells. By contrast, BCR-mediated Ca^{2+}

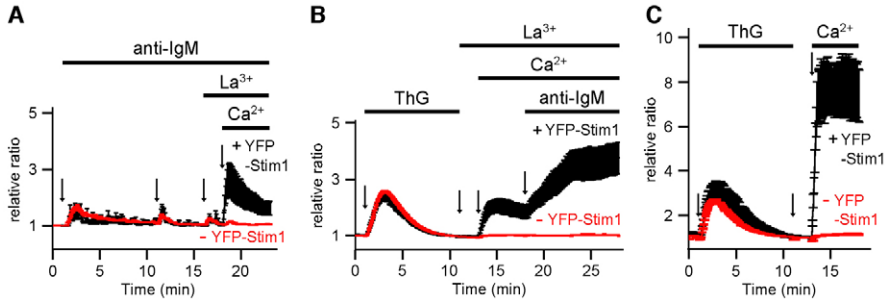

Fig. 4. BCR-mediated Ca^{2+} entry in Stim1-KO cells. Stim1-KO cells were transfected with the YFP-Stim1 expression vector and Ca^{2+} responses in YFP-positive (black) and YFP-negative (red) cells were examined. (A) Cells were stimulated with 2 μg/ml anti-IgM in nominally Ca^{2+}-free medium, followed by the addition of La^{3+} and Ca^{2+}. (B,C) Cells were treated with ThG in nominally Ca^{2+}-free medium, followed by the addition of La^{3+}, Ca^{2+} and 2 μg/ml anti-IgM (B) or Ca^{2+} (C). Traces shown are the means ± s.e.m. of normalized fluorescence ratios from Stim1-KO (YFP-negative; red) and YFP-Stim1 overexpressing Stim1-KO (YFP-positive; black) DT40 cells (17 and 217 YFP-positive and -negative cells, respectively, in A; 17 and 229 YFP-positive and -negative cells, respectively, in B; and 7 and 227 YFP-positive and -negative cells, respectively, in C). Horizontal bars indicate the presence of ThG, anti-IgM, La^{3+} and Ca^{2+}. Time points for the addition of reagents are indicated by the arrows.

entry after ThG treatment was not altered by cotransfection with *Orai1* and *Orai2* siRNAs (Fig. 5B-D).

Effects of 2-APB and SKF96365 on CCE and B-SOC

To further assess the pharmacological features of B-SOC, we examined the effects of the CCE inhibitors 2-APB and SKF96365. Pretreatment with 75 μM 2-APB decreased ThG-induced CCE to 13.6±1.2%, whereas it reduced B-SOC only to 64.1±3.0% (supplementary material Fig. S4). CCE and B-SOC were inhibited by 10 μM SKF96365 to 29.1±1.2% and 40.9±2.3%, respectively (supplementary material Fig. S4). These results suggest that CCE and B-SOC possess different sensitivities to 2-APB and SKF96365. However, these reagents are not sufficiently specific to further analyze functional components or channel properties of B-SOC.

Effects of BCR stimulation on Stim1 and Ca^{2+} stores

Previous studies have demonstrated that Stim1 forms a comet-like structure and travels on the ER membrane when the Ca^{2+} store is filled (Baba et al., 2006; Grigoriev et al., 2008; Smyth et al., 2008). Depletion of Ca^{2+} leads to oligomerization of Stim1 and relocalization of this protein into immobile puncta (Liou et al., 2007; Stathopulos et al., 2006; Wu et al., 2006). To further investigate the involvement of Stim1 in BCR-mediated Ca^{2+} entry, we examined the effects of anti-IgM stimulation on Stim1 function. Several comet-like structures of YFP-Stim1 were observed in unstimulated DT40 cells. These structures were mostly mobile and continuously appeared and disappeared (Fig. 6A). Stimulation with anti-IgM (3 μg/ml) reduced the mobility of the comet-like structures and resulted in the appearance of a small number of immobile puncta (Fig. 6B) and an increased intensity of total internal reflection fluorescence (TIRF) (Fig. 6D). Subsequent addition of ThG induced the relocalization of YFP-Stim1 into the immobile puncta (Fig. 6C). Quantitative analysis of TIRF fluorescent intensity indicated that the effects of BCR stimulation on the relocalization of YFP-Stim1 was 31.7±10.9% of that of complete store depletion by ThG (Fig. 6D). This partial relocalization of YFP-Stim1 by the weak stimulation of BCR with 3 μg/ml anti-IgM was associated with partial depletion of stored Ca^{2+} (data not shown). These results suggest that, in combination with BCR stimulation, the partial relocalization of Stim1 is sufficient to activate La^{3+}-resistant Ca^{2+} entry, but not sufficient to activate CCE.

Discussion

Here, we have described a novel La^{3+}-resistant Ca^{2+}-entry mechanism that is synergistically activated by BCR stimulation and depletion of Ca^{2+} stores. In marked contrast to ThG-induced Ca^{2+} entry (CCE), the novel pathway described in this study is not inhibited by 0.3 μM La^{3+}. Additionally, BCR-mediated Ca^{2+} release and Ca^{2+} entry was completely abolished in IP3R-KO DT40 cells. However, after depletion of Ca^{2+} stores by ThG, the subsequent stimulation of BCR induced similar levels of La^{3+}-resistant Ca^{2+} entry into IP3R-KO and WT DT40 cells. These results demonstrate that BCR stimulation without Ca^{2+} release does not promote Ca^{2+} entry and that BCR stimulation and depletion of Ca^{2+} stores work together to activate La^{3+}-resistant Ca^{2+} entry. To our knowledge, this is the first report of activation of non-CCE via the synergistic interaction of these pathways. We propose naming 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 (TCRs) by anti-CD3 antibodies (3 μg/ml) induced significant Ca^{2+} entry (supplementary material Fig. S5). Thus, BCR- or TCR-mediated La^{3+}-resistant Ca^{2+}-entry pathways might be a common phenomenon in immune cells.

Perhaps equally important are our findings regarding the involvement of Stim1 in B-SOC. We found that BCR-mediated Ca^{2+} entry was completely abolished in Stim1-KO DT40 cells and was restored by overexpression of YFP-Stim1. These data clearly demonstrate that Stim1 has an essential role in B-SOC. In addition, cotransfection with *Orai1* and *Orai2* siRNAs reduced CCE, but had no effect on B-SOC. 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. Furthermore, our data suggest that the amount of stored Ca^{2+} can regulate different Ca^{2+}-entry pathways. For example, B-SOC was fully activated by relatively small decreases in stored Ca^{2+} (in conjunction with BCR stimulation), whereas CCE required more extreme Ca^{2+} depletion. Indeed, Ca^{2+} entry elicited by 3 μg/ml anti-IgM was entirely resistant to La^{3+}. However, at high concentrations of anti-IgM (5 μg/ml), Ca^{2+} entry exhibited both La^{3+}-insensitive and La^{3+}-sensitive components. These results suggest that B-SOC dominates over CCE in response to weak BCR stimulation, and that the contribution of CCE

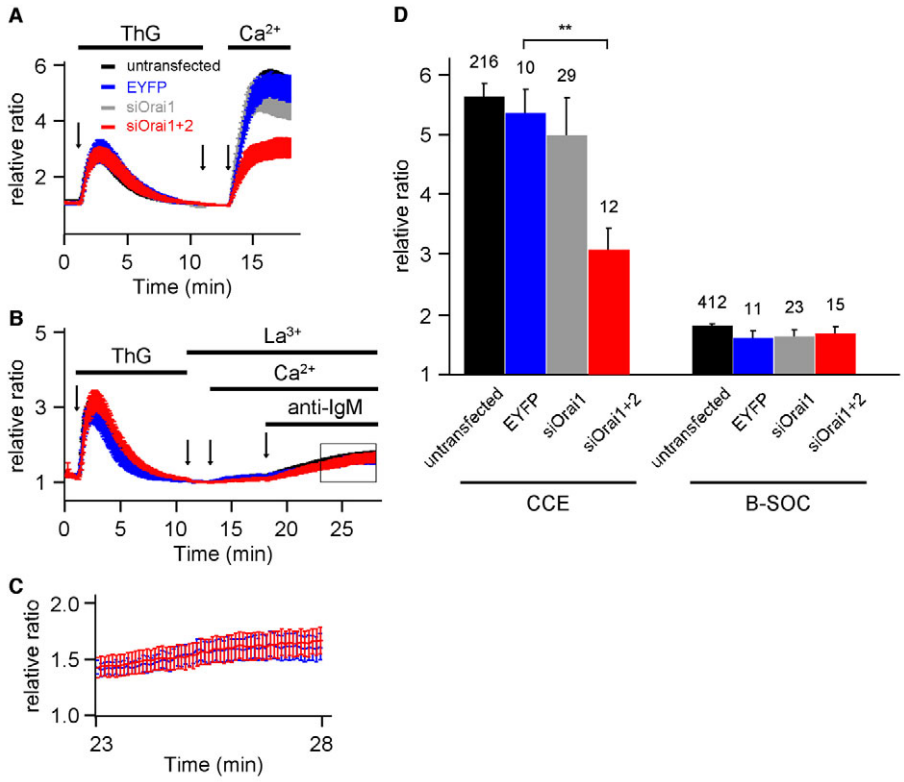

Fig. 5. Effect of Orai1 siRNA (siOrai1) and Orai2 siRNA (siOrai2) on CCE and BCR-mediated Ca^{2+} entry following treatment with ThG. (A) WT DT40 cells were cotransfected with the EYFP expression vector and siOrai1 and/or siOrai2. Cells were treated with ThG in nominally Ca^{2+}-free medium and then Ca^{2+} was added. Traces shown are the means ± s.e.m. of normalized fluorescence ratios from YFP-positive siOrai1-transfected cells (gray), YFP-positive siOrai1- and siOrai2-transfected cells (red), YFP-transfected cells (blue) or untransfected cells (black). The horizontal bars indicate the presence of ThG and Ca^{2+}. Arrows indicate the times of addition of reagents by exchanging medium. Results were obtained from 4-12 independent experiments, and the total number of cells examined is shown above each column in D. (B) WT DT40 cells were cotransfected with the EYFP expression vector and siOrai1 and/or siOrai2. 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+}. Traces shown are the means ± s.e.m. of normalized fluorescence ratios from YFP-positive siOrai1- and siOrai2-transfected cells (red), YFP-transfected cells (blue) or untransfected cells (black). The horizontal bars indicate the presence of anti-IgM, ThG, La^{3+} and Ca^{2+}. Results were obtained from 8-10 independent experiments, and the total number of cells examined is shown above each column in panel D. (C) Responses in YFP-positive siOrai1- and siOrai2-transfected cells (red) or YFP-transfected cells (blue) using an expanded scale for the boxed region in B. (D) Effect of siOrai1 and/or siOrai2 on CCE or B-SOC in WT DT40 cells. Increased fluorescence ratios were induced by the addition of Ca^{2+} to ThG-pretreated WT DT40 cells (CCE) or by the addition of 3 μg/ml anti-IgM in the presence of La^{3+} and Ca^{2+} to ThG-pretreated (B-SOC) WT DT40 cells. Maximal increases in fluorescence ratios following the addition of Ca^{2+} to 1 μM ThG-treated cells (CCE), or the fluorescence ratios 10 minutes after addition of anti-IgM to ThG-pretreated cells (B-SOC) in the presence of La^{3+} and Ca^{2+}, are shown. Black, YFP-negative cells; blue, YFP-positive cells in samples transfected only with YFP; gray, YFP-positive cells in siOrai1-transfected samples; red, YFP-positive cells in siOrai1 and siOrai2 transfected samples. The total number of cells examined is shown above each column. $^{**}P<0.01$.

becomes more significant during strong BCR stimulation, probably because of pronounced depletion of stored Ca^{2+}. Based on the hypothesis that B-SOC is sensitive to small decreases in stored Ca^{2+}, we suggest that B-SOC serves to regulate the level of stored Ca^{2+} during weak immune responses.

Recent studies have indicated that Stim1 displays comet-like behavior, in which Stim1 travels on the ER membrane while Ca^{2+} stores are filled, and relocates to regions underlying the plasma membrane where it activates CCE following the depletion of stored Ca^{2+} (Baba et al., 2006; Grigoriev et al., 2008; Liou et al., 2007; Smyth et al., 2008; Stathopulos et al., 2006; Wu et al., 2006). Here, we show that the partial depletion of stored Ca^{2+} by 3 μg/ml anti-IgM induces a partial relocation of Stim1. These results confirm that the mobility of Stim1 is altered by weak BCR stimulation, although the extent of relocation is much smaller than that induced by complete depletion of the Ca^{2+} stores (by ThG). Although the requirement of Stim1 relocation for B-SOC has not been clarified, these results suggest that B-SOC is more sensitive to Stim1 than CCE.

The molecular basis of the unique activation of B-SOC remains largely unknown. Previous studies have shown that Stim1 and Orai1 are essential components of CCE (Feske et al., 2006; Liou et al., 2005; Roos et al., 2005; Vig et al., 2006). Stim1 activates CCE via interactions with Orai1, which is thought to be a La^{3+}-sensitive CRAC channel. Three closely related Orai genes (*ORAI1*, *ORAI2* and *ORAI3*) exist in mammalian cells, whereas two genes (*ORAI1* and *ORAI2*), which both form Orai channels sensitive to La^{3+}, exist in chicken cells. Double knockdown of Orai1 and Orai2 reduced CCE but had no effect on B-SOC. Several studies have suggested that Stim1 exerts a direct or indirect effect on the regulation of TRPC channels (Huang et al., 2006; López et al., 2006; Ong et al., 2007; Yuan et al., 2007). However, low concentrations of La^{3+} and Gd^{3+} inhibit TRPC1 (Beech et al., 2003), and activate TRPC4 and TRPC5 (Plant and Schaefer, 2003). Furthermore, TRPC3, TRPC6 and TRPC7 are activated by PLC and DAG (Trebak et al., 2003) and IP3Rs are reported to be involved in the activation of TRPC7 (Vazquez et al., 2006). Based on these results, the involvement of these TRPC and Orai channels

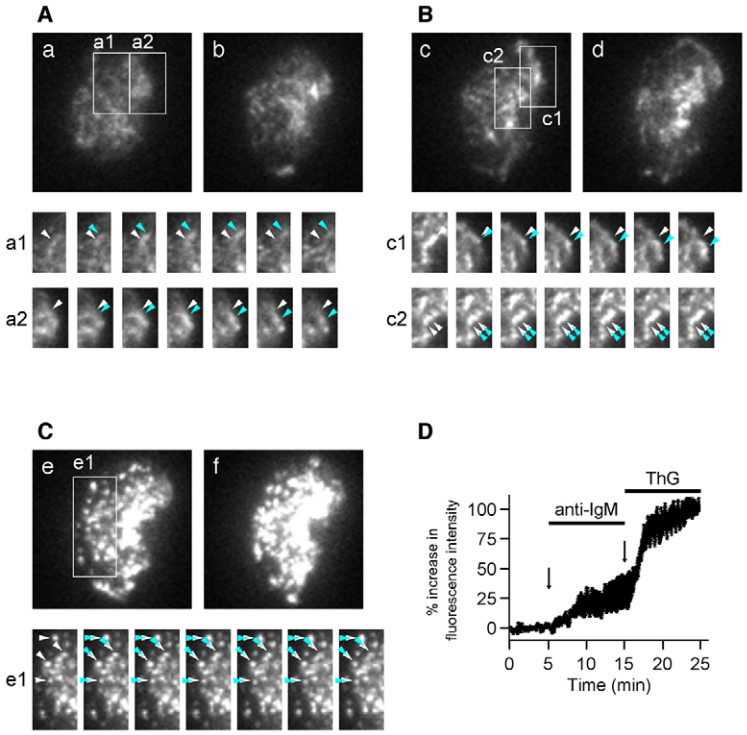

Fig. 6. Dynamic behavior of YFP-Stim1 in DT40 cells. (A-C) Stim1-KO cells were transfected with the YFP-Stim1 expression vector. YFP-Stim1-expressing Stim1-KO cells were incubated in nominally Ca^{2+}-free medium for 5 minutes (A) and stimulated with 3 μg/ml anti-IgM in Ca^{2+}-free medium for 10 minutes (B), followed by addition of ThG and incubation for 5 minutes (C). YFP signals of Stim1 near the plasma membrane were monitored by TIRF microscopy. Panels a-f are TIRF images at the beginning (a) and 5 minutes after (b) incubation in nominally Ca^{2+}-free medium, 5 minutes (c) and 10 minutes (d) after the addition of anti-IgM stimulation and 3 minutes (e) and 5 minutes (f) after the addition of ThG. Small TIRF images (a1, a2, c1, c2 and e1) were obtained at 5 second intervals in the areas indicated in panels a, c and e, respectively. Images in a1 and a2 were obtained approximately 3 minutes after incubation in Ca^{2+}-free medium. Images c1 and c2 were obtained approximately 8 minutes after stimulation with anti-IgM. Image e1 was obtained approximately 1.5 minutes after the addition of ThG. The movement of YFP-Stim1 from the white to blue arrowheads is shown on each image. (D) Time-dependent changes in TIRF fluorescence intensity in YFP-Stim1-expressing cells. The trace shown is the mean ± s.e.m. of percentage change in fluorescent intensity, where values at each time point (1 second intervals) were normalized by the average TIRF fluorescence intensity measured for 5 minutes before the addition of anti-IgM and the intensity at 10 minutes after the addition of ThG as the minimum and maximum, respectively. The results are the mean ± s.e.m. of four cells from independent experiments. The horizontal bars indicate the presence of anti-IgM and ThG. The arrows indicate the times of medium exchange.

in B-SOC is less likely. However, various endogenous TRPC molecules assemble and form heteromultimeric channels and the combinatorial assembly of TRPC proteins might give rise to channels with different properties. Therefore, it is difficult to predict candidate molecules that might be involved in B-SOC. Electrophysiological characterization and functional screening approaches (such as random mutagenesis) will be required to explore the mechanisms underlying B-SOC.

In DT40 cells, BCR stimulation leads to activation of non-receptor tyrosine kinases and PLCγ2 (Kurosaki et al., 2000). Since B-SOC is inhibited by genistein, it is likely that BCR-dependent activation of tyrosine kinases is involved in B-SOC. U-73122 failed to suppress B-SOC, and PLC-β activation via PAR2 stimulation failed to induce La^{3+}-resistant Ca^{2+} entry after store depletion. Thus, it is unlikely that PLC activity is required for B-SOC. Stimulation of BCR activates several tyrosine kinases, including Lyn, Syk and Btk (Kurosaki et al., 2000). However, the mechanisms underlying B-SOC regulation remain unknown.

Previous studies have suggested that IP3Rs have a number of regulatory roles in Ca^{2+} entry (Berridge, 1993; Berridge, 1997; Putney et al., 2001) and we previously described La^{3+}-resistant BCR-mediated Ca^{2+} entry in DT40 cells (Morita et al., 2004). As this non-CCE pathway was completely attenuated in IP3R-KO cells, IP3Rs might function as plasma membrane Ca^{2+} entry channels or might indirectly activate Ca^{2+} entry. Indeed, electrophysiological experiments have shown that functional IP3Rs are expressed at the plasma membrane and that they contribute to Ca^{2+} entry, although their numbers appear to be very low (approximately two per cell) (Dellis et al., 2006). Here, we confirmed the lack of BCR-induced Ca^{2+} entry into IP3R-KO DT40 cells and found that Ca^{2+} depletion restores BCR-mediated La^{3+}-resistant Ca^{2+} entry into IP3R-KO cells. Thus, we conclude that IP3Rs contribute indirectly to B-SOC via the depletion of stored Ca^{2+}. Although we cannot exclude the possibility that IP3Rs have a role in Ca^{2+} entry, our results indicate that neither IP3Rs nor Ins(1,4,5)P_3 production have crucial roles in La^{3+}-resistant Ca^{2+} entry following BCR stimulation in DT40 cells. Although it is clear from our data using IP3R-KO cells that IP3Rs are not essential in B-SOC, treatment with U-73122 or staurosporine enhanced La^{3+}-resistant Ca^{2+} entry in WT cells, but not in IP3R-KO cells. Thus, we cannot exclude the possible involvement of IP3Rs in regulation of B-SOC and/or other types of La^{3+}-resistant Ca^{2+} entry.

Here, we have described B-SOC, a unique Ca^{2+}-entry pathway that is regulated in concert with depletion of Ca^{2+} stores via Stim1- and BCR-mediated activation of tyrosine kinases. The B-SOC pathway is likely to be the principal route of Ca^{2+} entry, particularly in the presence of weak BCR stimulation (at least in DT40 cells and Jurkat cells). Similar regulation of Ca^{2+} entry probably occurs via the tyrosine kinase-mediated responses of other immune cells, as well as other cell types. Thus, the physiological roles and mechanisms of B-SOC regulation, including channel properties and molecular interactions, should be explored in future studies.

Materials and Methods

Materials and media

An anti-chicken IgM antibody (supernatant, M-4 clone) was obtained from Southern Biotechnology Associates (Birmingham, AL), fura-2/AM was obtained from Dojin Chemicals (Kumamoto, Japan), poly-L-lysine was obtained from Sigma (St Louis, MO) and Cellmatrix I-C was obtained from Nitta Gelatin (Osaka, Japan). The anti-CD3 mouse monoclonal antibody (UCHT1) was obtained from Calbiochem (San Diego, CA). The plasmid encoding the enhanced yellow fluorescent protein (EYFP)-tagged Stim1 was the kind gift of Tobias Meyer (Department of Molecular Pharmacology, Stanford University, CA) (Liou et al., 2005).

Modified Hanks' balanced salt solution buffered with HEPES (HBSS-H) contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 4.2 mM $NaHCO_3$ and 20 mM HEPES-NaOH (pH 7.4). Nominally Ca^{2+}-free HBSS-H was identical in composition to HBSS-H, except for the omission of $CaCl_2$. The PO_4^{3-}, SO_4^{2-}, CO_3^{2-}-free HBSS-H medium contained 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.81 mM $MgCl_2$, 5.6 mM glucose and 20 mM HEPES-NaOH (pH 7.4).

Cell culture and transfection

The DT40 chicken B-cell line, the triple IP3R-deficient cell line (IP3R-KO DT40 cells), and the Stim1-deficient cell line (Stim1-KO DT40 cells) were cultured in RPMI 1640 (Sigma) and supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD), 1% chicken serum (Gibco BRL), 4 mM glutamine (Gibco BRL), 50 μM 2-mercaptoethanol (Nakarai Chemicals, Kyoto, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Jurkat T cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transient transfections with the YFP-Stim1 and EYFP plasmid (Clontech, Mountain View, CA) were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and 1.6 μg/ml plasmid, according to the manufacturer's instructions, and incubated for 12-18 hours.

Imaging of $[Ca^{2+}]_i$

To monitor $[Ca^{2+}]_i$, cells were attached to a small recording chamber that consisted of a 10 mm ring and 22 mm glass coverslips coated with 0.5 mg/ml poly-L-lysine and Cellmatrix (diluted 1:10). Attached cells were incubated in culture medium for 1 hour at 37°C, loaded with 2 μM Fura-2 AM in HBSS-H containing 1% BSA and incubated for 30 minutes at room temperature. Fura-2-loaded cells were washed with HBSS-H and rested for at least 30 minutes prior to Ca^{2+} measurements. All experiments were performed in PO_4^{3-}, SO_4^{2-}, CO_3^{2-}-free HBSS-H at room temperature. For monitoring Fura-2 fluorescence, cells were alternately excited at 340 nm and 380 nm, with emission signals recorded at 500 nm to 530 nm. YFP fluorescence images were captured using an excitation wavelength of 490 nm and emission wavelengths of 500 nm to 530 nm using the ARGUS-HiSCA imaging system (Hamamatsu Photonics, Shizuoka, Japan) attached to an inverted fluorescence microscope, equipped with a Nikon Fluor ×40 objective.

Total internal reflection fluorescence (TIRF) microscopy imaging

The molecular dynamics of YFP-Stim1 were examined via TIRF microscopy. The YFP-Stim1-expressing Stim1-KO cells were attached to poly-L-lysine and Cellmatrix-coated chambers and were incubated in culture medium for 1 hour at 37°C. Cells were maintained in HBSS-H at room temperature, then treated in nominally Ca^{2+}-free and PO_4^{3-}, SO_4^{2-}, CO_3^{2-}-free HBSS-H medium for 5 minutes before BCR stimulation. TIRF analysis was performed using a White TIRF system (Nikon, Tokyo, Japan), which consisted of a TIRF illumination system attached to an inverted fluorescence microscope (Nikon TE2000) that was equipped with a Nikon Apo Fluor ×60 oil immersion objective (NA 1.45) and a perfect focus system. Fluorescence of YFP-Stim1 was monitored by excitation at 480 nm and emission at 535 nm and images were captured using an EM-CCD camera. The AQUACOSMOS 2.6 software (Hamamatsu photonics, Shizuoka, Japan) was used to control the TIRF system and acquire data. All experiments were performed at room temperature.

RNA interference using *Orai1* and *Orai2* siRNAs

We designed siRNAs against chicken genes encoding Orai1 and/or Orai2 (*Orai1* siRNA and *Orai2* siRNA, respectively) as follows: *Orai1* siRNA (sense, 5′-CCC-UUUGGACUGAUUUUCAUUGUCU-3′ and antisense, 5′-AGACAAUGAAAAU-CAGUCCAAAGGG-3′); Orai2 siRNA (sense, 5′-UGGUGCUGCUGUGCUG-GAUCAAAUU-3′ and antisense, 5′-AAUUUGAUCCAGCACAGCAGCACCA-3′). These siRNA constructs were obtained from Invitrogen (Stealth RNAi). The siRNAs (50 nM each) and transfection marker (1.6 μg/ml EYFP plasmid) were cotransfected using LipofectAMINE 2000. After 24-48 hours of incubation, cells were harvested and attached to recording chambers for Ca^{2+} measurements, as described above.

Statistical analysis

Results are presented as means ± s.e.m. of all cells examined in at least three independent experiments. Statistical significance was assessed using Student's *t*-test and considered significant at $^{**}P<0.01$.

The authors would like to thank James Putney for his critical comments and suggestions. This study was supported in part by Grants-in-Aid for Scientific Research (19592154 to T.M., 14571770 to A.T. and 15591975 to Y.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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