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Negative Control of Store-Operated Ca\(^{2+}\) Influx by B Cell Receptor Cross-Linking

Akiko Hashimoto,* Kenzo Hirose,* Tomohiro Kuroasaki,† and Masamitsu Iino*§

An increase in the intracellular Ca\(^{2+}\) concentration by B cell receptor (BCR) cross-linking plays important roles in the regulation of B cell functions. [Ca\(^{2+}\)]\(_i\) is regulated by Ca\(^{2+}\) release from the Ca\(^{2+}\) store as well as store-operated Ca\(^{2+}\) influx (SOC). Protein tyrosine kinases downstream of BCR cross-linking were shown to regulate the mechanism for Ca\(^{2+}\) release. However, it remains elusive whether BCR cross-linking regulates SOC or not. In this study, we examined the effect of BCR cross-linking on thapsigargin; WT, wild type.

In n B lymphocytes, B cell receptor (BCR) cross-linking activates a series of protein tyrosine kinases (PTKs) (1, 2). Several of these PTKs were shown to be involved in the activation of phospholipase C \(_\gamma\) (PLC\(_\gamma\)), which catalyzes the production of inositol 1,4,5-trisphosphate (IP\(_3\)). IP\(_3\) mobilizes the intracellular Ca\(^{2+}\) stores via the IP\(_3\)R, and generates a phasic increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) (3). The resulting decrease in the Ca\(^{2+}\) content within the Ca\(^{2+}\) stores triggers store-operated Ca\(^{2+}\) influx (SOC), which is required for the tonic phase of increase in [Ca\(^{2+}\)]\(_i\) (4, 5). The increase in [Ca\(^{2+}\)]\(_i\) (4), activates transcription factors resulting in differentiation, proliferation, or death of cells (6).

Among PTKs, Syk and Bruton’s tyrosine kinase (Btk) are critical for BCR-mediated positive signal to elicit Ca\(^{2+}\) release from its stores in DT40 B cells (7, 8). In Syk- or Btk-deficient cells, increase in IP\(_3\) production is impaired, and consequently Ca\(^{2+}\) release is inhibited. Lyn, a member of the src family of PTKs (9, 10), is also involved in the positive signal generation by BCR cross-linking. Deficiency in Lyn results in a considerably delayed increase in [Ca\(^{2+}\)]\(_i\) without impairment of IP\(_3\) production (8). However, Lyn seems to be also involved in producing the negative signal that inhibits increase in [Ca\(^{2+}\)]\(_i\). In Lyn-deficient B cells, BCR-mediated increase in [Ca\(^{2+}\)]\(_i\) was exaggerated (11). Thus, the regulation of Ca\(^{2+}\) release by these PTKs is well defined.

It has been suggested that PTKs may directly activate SOC. A member of the src family of PTKs, pp60\(^c-src\), was shown to be essential for the activation of SOC by using fibroblast cells from a gene-targeted mouse (12). Other reports are based on the results of experiments using a PTK inhibitor, genistein (13–16). However, there remain uncertainties with regard to the interpretation of results obtained in experiments using genistein. First, several PTKs decrease K\(^+\) channel activities (17, 18). A decrease in potassium channel activities may elicit membrane depolarization, which in turn attenuates SOC (4). Therefore, it is not clear whether PTKs have a direct effect on SOC or not. Second, it is probable that PTKs that affect IP\(_3\)-induced Ca\(^{2+}\) release may indirectly affect the SOC activation by changing the level of Ca\(^{2+}\) content in the Ca\(^{2+}\) stores. Indeed, Btk and Src homology 2 domain containing phosphatidylinositol 5'-phosphatase (SHIP), which have positive and negative effects on IP\(_3\) production, respectively, were found to regulate SOC as a secondary effect on the regulation of Ca\(^{2+}\) release (19–21). Third, Ser/Thr kinases such as protein kinases A, C, and G can be also inhibited by genistein due to the low specificity of this inhibitor (22). Inhibition of these kinases may also affect Ca\(^{2+}\) release as well as activation of K\(^+\) channels (18). Thus, the effects of PTKs downstream of the BCR cross-linking on SOC require further study.

In this study, we examined the direct effects of BCR cross-linking on SOC by bypassing the PLC\(_\gamma\)-IP\(_3\)-R pathway using thapsigargin (TG), and found a BCR-mediated inhibitory effect on SOC in DT40 B cells. The inhibition involves two pathways: depolarization-dependent inhibition and Lyn-mediated depolarization-independent inhibition of SOC. Thus, the present work revealed new negative signaling pathways for SOC regulation downstream of BCR cross-linking.
ical salt solution (PSS) (150 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, and 5.6 mM glucose; pH adjusted to 7.4 with NaOH) containing 0.1% BSA. Pairs of fluorescence images at emission wavelengths at 340 and 380 nm excitation was converted to $[\text{Ca}^{2+}]_i$ using the following equation: $[\text{Ca}^{2+}]_i = K'_i (R - R_{\text{min}})/(R_{\text{max}} - R)$, in which $K'_i$, $R_{\text{min}}$, and $R_{\text{max}}$ are the apparent dissociation constant and the maximal and minimal $R$ values, respectively (24). The values were determined in vitro under the equivalent optical conditions. The Ca$^{2+}$-free PSS had the same composition as PSS except for the omission of CaCl$_2$ and addition of EGTA (5 mM).

Measurement of membrane potentials

The cells were washed twice with PSS and suspended in cuvette at 2 × 10$^5$ cells/ml concentration. For measurement of the membrane potential, 200 mM DiSC$_3$ (5) (Molecular Probes) dissolved in DMPSO was added to 2 ml of cell suspension to obtain a final concentration of 200 nM. The fluorescence intensities at excitation wavelength of 580 nm and emission wavelength of 670 nm were monitored using a fluorometer (FP-750; Jasco, Tokyo, Japan) (25, 26). For calibration of membrane potential, all the measurements were followed by addition of 2 μM valinomycin and appropriate amounts of KCl. The membrane potential was calculated using the Nernst equation assuming the intracellular K$^+$ concentration ([K$^+$]) to be 150 mM. The Ca$^{2+}$-free PSS used in measuring the membrane potential contained 20 μM EGTA.

Statistical analysis

Statistical results are expressed as mean ± SE. Statistical comparisons were made using the paired $t$ test for the measurement of membrane potentials and the nonpaired $t$ test for all the other measurements. $[\text{Ca}^{2+}]_i$ were measured in single cells, and representative results are displayed in the following figures. The single cell data were pooled and the difference between pooled data was statistically analyzed to allow for cell to cell variations.

Results

BCR cross-linking elicits Ca$^{2+}$ release from Ca$^{2+}$ stores and subsequent activation of Ca$^{2+}$ influx via SOC, whose activation level is dependent on the state of the Ca$^{2+}$ stores. To determine whether BCR cross-linking has a direct effect on the regulation of SOC, independent of its effect on Ca$^{2+}$ release, we used TG, a potent inhibitor of sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase, to deplete the Ca$^{2+}$ stores. DT40 B cells were treated with 1 μM TG in Ca$^{2+}$-free solution for 600 s to deplete the Ca$^{2+}$ stores before extracellular Ca$^{2+}$ was reintroduced to elicit SOC (Fig. 1A). The increase in $[\text{Ca}^{2+}]_i$, via SOC reached a peak value of $\sim 1.11 ± 0.01$ μM in about 3–4 min ($n = 76$) and then $[\text{Ca}^{2+}]_i$ gradually decreased. Six hundred seconds after the reintroduction of Ca$^{2+}$, we introduced anti-chicken μ-chain IgM (M4) to induce BCR cross-linking (27). Following M4 application, the rate of decrease in $[\text{Ca}^{2+}]_i$ was significantly accelerated (Fig. 1, A and B). Five minutes after BCR cross-linking, the $[\text{Ca}^{2+}]_i$ was decreased to 54.6 ± 0.02% ($n = 76$) of the value of $[\text{Ca}^{2+}]_i$ just before the BCR cross-linking, while the corresponding $[\text{Ca}^{2+}]_i$ in the cells without BCR cross-linking was 81.1 ± 0.8% ($n = 76$).

Materials and Methods

Cells and culture

DT40 B cells were grown in RPMI 1640 medium containing 10% FCS, 1% chicken serum, 50 μM 2-ME, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO$_2$ at 0.5–1 × 10$^5$ cells/ml concentration. For measurement of the membrane potential, 200 nM TG was added to the culture medium (25, 26). For calibration of membrane potential, all the measurements were followed by addition of 2 μM valinomycin and appropriate amounts of KCl. The membrane potential was calculated using the Nernst equation assuming the intracellular K$^+$ concentration ([K$^+$]) to be 150 mM. The Ca$^{2+}$-free PSS used in measuring the membrane potential contained 20 μM EGTA.

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FIGURE 2. Depolarization-dependent inhibition of SOC by BCR cross-linking. A, BCR cross-linking by M4 (3 μg/ml) attenuated SOC (lower left) and evoked membrane depolarization (lower right) as compared with those without BCR cross-linking (upper left and right). B, Statistical analysis of the BCR cross-linking-mediated membrane depolarization. ○, Resting membrane potential; ●, membrane potential at the time point corresponding to 300 s after BCR cross-linking (right) or adding PSS as control (left), n = 5. C, SOC was elicited as in A, but in the presence of 2 μM valinomycin. An increase in the extracellular [K+] from 8 to 18 mM induced membrane depolarization (right) and attenuated SOC (left). Representative result of four experiments.

Cross-linking was 79.1 ± 0.01% (n = 115) (p < 0.0001). The decrease in [Ca2+] was not observed in cells treated with anti-mouse μ-chain IgG or anti-mouse IgG IgM (data not shown), indicating that the attenuation of SOC-mediated [Ca2+] increase is caused by BCR cross-linking. In another set of experiments, we cross-linked BCR before the application of extracellular Ca2+ to activate SOC and obtained consistent results. The initial rates of increase in [Ca2+] were significantly lower in the cells with BCR cross-linking (1.32 ± 0.15 nM/s, n = 33) than in the control cells (2.09 ± 0.19 nM/s, n = 36) (p = 0.002).

The observed attenuation by BCR cross-linking can be explained by its inhibitory effect on Ca2+ influx, or alternatively, by its potentiating effect on Ca2+ extrusion from the cytosol to the extracellular space. To examine the possible effect on the Ca2+ extrusion mechanism, we compared the rates of decrease in [Ca2+] after removal of the extracellular Ca2+ in the absence and presence of BCR cross-linking. Because the rate of Ca2+ extrusion is greatly affected by [Ca2+], (28), we plotted the extrusion rate vs [Ca2+] just before the removal of the extracellular Ca2+ (Fig. 1C). The plot shows there are no differences between the rates of decrease in [Ca2+] with and without BCR cross-linking. These results indicate that the BCR cross-linking does not affect Ca2+ extrusion mechanism and that the attenuation of SOC by BCR cross-linking is due to inhibition of Ca2+ influx.

The rate of Ca2+ influx may depend on the membrane potential, which alters the electromotive force of Ca2+ influx. Therefore, we examined whether the membrane potential changed upon BCR cross-linking using DiSC3 (5), a fluorescent indicator of membrane potential (25, 26). The membrane potential was depolarized by about 20 mV within 5 min after BCR cross-linking in wild-type (WT) cells (Fig. 2, A and B). We then examined whether the SOC activity was inhibited solely by depolarization without BCR cross-linking. To mimic the depolarization by BCR cross-linking, the membrane potential was controlled by alteration of the extracellular [K+] in the presence of a K+ ionophore, valinomycin. SOC was induced in PSS containing 8 mM K+ maintaining the membrane potential about −75 mV. Six hundred seconds after the induction of SOC, [K+] was increased to 18 mM, resulting in a subsequent increase in the membrane potential to −55 mV. We observed that SOC was inhibited by the K+-induced depolarization (Fig. 2C). The rate of decrease in [Ca2+], changed from −0.045 ± 0.03 nM/s to −1.22 ± 0.11 nM/s (p < 0.0001, n = 37).

Next, we examined whether the BCR cross-linking has depolarization-independent effect on SOC. Cells were treated with valinomycin and 8 mM K+ to keep membrane potential about −75 mV. Treatment with valinomycin abolished the change in the membrane potential by BCR cross-linking (Fig. 3B). Under this condition, the BCR cross-linking still inhibited SOC (Fig. 3A). The rate of change in [Ca2+], was significantly accelerated from −0.071 ± 0.02 nM/s to −0.60 ± 0.07 nM/s (p < 0.0001, n = 38) after BCR cross-linking.

Molecules in the downstream of BCR, such as Lyn, Syk, Btk, and SHIP, have been shown to be important in the regulation of Ca2+ release from its stores (3, 8, 23). To study whether these molecules were also involved in the inhibition of SOC, we tested the effects of BCR cross-linking on SOC in DT40 B cells deficient in one of these molecules (Fig. 4). In Syk−, Btk−, or SHIP-deficient cells, BCR cross-linking enhanced the rate of decrease in [Ca2+], as was seen in the WT cells. In contrast, the rate of [Ca2+] decrease in Lyn-deficient cells was little changed upon BCR cross-linking (Fig. 4). These results suggest that Lyn is essential for the BCR-mediated inhibitory effect on SOC.

To study the functional role of Lyn in the BCR-mediated inhibition of SOC, membrane potential was measured in Lyn-deficient cells. Although BCR cross-linking induced membrane depolarization in these cells (Fig. 5A, lower right panel), the magnitude of depolarization (9.2 ± 1.1 mV, n = 4) was significantly smaller than that in WT cells (21.2 ± 2.2 mV, n = 5, p = 0.003) (Figs. 2B and 5B). Furthermore, the time course of depolarization was significantly delayed in Lyn-deficient cells. The rate of increase in membrane potential was 0.04 ± 0.01 mV/s (n = 5) for Lyn-deficient cells and 0.18 ± 0.03 mV/s (n = 6) for WT cells (p =...
These results indicate that Lyn is partly involved in BCR-mediated depolarization. Whereas modest depolarization was observed, inhibition of SOC by depolarization was scarcely observed under this condition (Fig. 5A, lower left panel). However, the mechanism of SOC inhibition by membrane depolarization was not disrupted in Lyn-deficient cells. The SOC in Lyn-deficient cells were inhibited by high [K\(^+\)] treatment (Fig. 5C). The rate of [Ca\(^{2+}\)]\(_i\) decrease was increased from 0.11 ± 0.03 nM/s to 0.62 ± 0.07 nM/s (p < 0.001, n = 20). These results indicate that the BCR-mediated depolarization in Lyn-deficient cells was too small and slow to induce significant SOC inhibition. We further examined the involvement of Lyn in the depolarization-independent mechanism of SOC inhibition. When Lyn-deficient cells were treated with valinomycin and 8 mM [K\(^+\)] to keep membrane potential about −75 mV, no inhibition of SOC by BCR cross-linking was observed (Fig. 5D). The rate of [Ca\(^{2+}\)]\(_i\) decrease was little changed (−0.064 ± 0.03 nM/s to −0.091 ± 0.03 nM/s, p = 0.53, n = 20). Thus, Lyn was required for the depolarization-independent inhibition of SOC after BCR cross-linking.

**Discussion**

In this study, we showed a couple of novel pathways in the BCR-mediated negative signaling, i.e., inhibition of SOC. One of the pathways involves membrane depolarization, which in turn may reduce the driving force of Ca\(^{2+}\) influx and SOC channel activity. The other is membrane potential-independent Lyn-mediated inhibition of SOC. In general accordance with this notion, the increase in the decay rate of [Ca\(^{2+}\)]\(_i\), induced by BCR cross-linking (∼1.4 nM/s, Fig. 1B) was comparable with the addition of the corresponding values of the depolarization-dependent effect (∼1.1 nM/s, Fig. 2C) and the depolarization-independent effect (∼0.5 nM/s, Fig. 3A), although the quantitative comparison should be taken as a rough estimate because the time course of BCR-mediated depolarization may not be the same as that of high-K\(^+\)-induced depolarization.

How does Lyn regulate SOC channel activity? It is possible that Lyn directly regulates SOC channel activity, because Lyn and related src family of kinases have been shown to regulate various ion channel activities. In T lymphocytes, Lck phosphorylates and inactivates Kv1.3 (17), while Fyn phosphorylates and activates IP\(_3\) R (29). In neurons, activation of Src increases the mean open time of N-methyl-d-aspartate (NMDA) receptors (30), and Fyn phosphorylates and activates Kv1.5 and Kv2.1 (31). Functional coupling between Lyn and an \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor in the cerebellum has been reported (32).

**FIGURE 3.** BCR-mediated inhibition of SOC without depolarization. A, SOC was elicited in the presence of 2 μM valinomycin and 8 mM [K\(^+\)]. BCR cross-linking by M4 (3 μg/ml) attenuated SOC without membrane depolarization. B, The membrane potential was kept near −75 mV under this condition. Representative result of five experiments.

**FIGURE 4.** BCR-mediated effects on SOC in Lyn-, Syk-, Btk-, and SHIP-deficient DT40 B cells. A, SOC was elicited in Lyn-, Syk-, Btk-, and SHIP-deficient cells, as shown in Fig. 1. BCR cross-linking by M4 (3 μg/ml) attenuated SOC except for the case of Lyn-deficient cells. B, Statistical analysis of the BCR-mediated inhibition of SOC. Means and SEM of the decay rate of [Ca\(^{2+}\)]\(_i\) at 60 s before (□) and after (■) the start of BCR cross-linking in WT cells (n = 115) and cells deficient in Lyn (n = 53), Syk (n = 48), Btk (n = 47), or SHIP (n = 46).
The SOC channel activity may be regulated by protein kinase C (PKC). In rat basophilic leukemia-2H3 cells, Parekh et al. (33) showed that the Ca\(^{2+}\) release-activated Ca\(^{2+}\) current induced by Ca\(^{2+}\) store depletion was inhibited by the application of a PKC activator (PMA) and enhanced by a PKC inhibitor (bisindolylmaleimide) under the condition that the membrane potential was held constant. Thus, we also examined the effect of PKC activator, PMA on SOC, and observed an inhibition of SOC in DT40 B cells (unpublished observation). Because PKC is activated following BCR cross-linking (34), PKC is a candidate molecule that inhibits SOC channel in the Lyn-mediated inhibitory pathway. However, the following lines of evidence argue against the hypothesis that PKC plays an essential role in the BCR-mediated SOC inhibition in DT40 B cells. First, we observed that a PKC inhibitor (bisindolylmaleimide) had no effect on the BCR-mediated SOC inhibition in DT40 B cells (unpublished observation). Because PKC is activated following BCR cross-linking (34), PKC is a candidate molecule that inhibits SOC channel in the Lyn-mediated inhibitory pathway. However, the following lines of evidence argue against the hypothesis that PKC plays an essential role in the BCR-mediated SOC inhibition in DT40 B cells. First, we observed that a PKC inhibitor (bisindolylmaleimide) had no effect on the BCR-mediated SOC inhibition in DT40 B cells (unpublished observation). Second, the SOC inhibition was still observed in Syk- or Btk-deficient cells (Fig. 4). In these cells, PKC activation by BCR cross-linking should be minimal because the activation of PLC\(\gamma\) downstream of Syk or Btk is impaired (3, 8). Third, the presence of Lyn decreased PKC activity in response to BCR cross-linking (35).

Accumulated lines of evidence suggest that the BCR-mediated negative signals are critical in the regulation of B cell functions. An enhancement of B cell proliferation and an increase in the number of self Ag-reactive plasma cells were observed concomitantly with enhanced Ca\(^{2+}\) signaling in response to BCR cross-linking in murine B cells deficient in one of the molecules involved in the negative signaling, i.e., CD22, Src homology domain containing phosphatase-1, or Lyn (36–40). Furthermore, the B cell tolerance is likely to require the negative Ca\(^{2+}\) signals. BCR cross-linking by self Ag elicited lower and oscillatory increases in [Ca\(^{2+}\)]\(_i\), in self-tolerant B cells than in naive cells, and this altered pattern of Ca\(^{2+}\) signaling presumably causes distinct activation patterns of transcription factors (41). Thus, the BCR-mediated inhibition of SOC may be one of the important mechanisms that regulate B cell functions.

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References


