Inhibitory Modulation of B Cell Receptor-mediated Ca\(^{2+}\) Mobilization by Src Homology 2 Domain-containing Inositol 5’-Phosphatase (SHIP)*

(Received for publication, October 8, 1998, and in revised form, January 20, 1999)

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 Src homology 2 domain-containing inositol 5’-phosphatase (SHIP) mediates inhibitory signals that attenuate intracellular Ca\(^{2+}\) mobilization in B cells upon B cell receptor (BCR) stimulation. To clarify the mechanisms affected by SHIP, we analyzed Ca\(^{2+}\) mobilization in the DT40 B cell line in which the SHIP gene was disrupted. In SHIP-deficient cells, Ca\(^{2+}\) transient elicited by BCR stimulation was more prolonged than that in control cells both in the presence and absence of extracellular Ca\(^{2+}\). Inositol 1,4,5-trisphosphate production following BCR stimulation was enhanced in SHIP-deficient cells. In SHIP-deficient cells in comparison with the control cells, BCR stimulation in the absence of extracellular Ca\(^{2+}\) induced a greater degree of Ca\(^{2+}\) store depletion and the Ca\(^{2+}\) influx upon re-addition of extracellular Ca\(^{2+}\) was also greater. However, store-operated Ca\(^{2+}\) influx (SOC) elicited by thapsigargin-induced store depletion was not affected by SHIP. These results indicate that the primary target pathway of SHIP is the Ca\(^{2+}\) release from the stores, and that Ca\(^{2+}\) influx by the SOC mechanism is secondarily controlled by the level of Ca\(^{2+}\) in the stores without direct inhibition of SOC. In this way, SHIP may play an important role in ensuring the robust tuning of Ca\(^{2+}\) signaling in B cells.

Intracellular calcium concentration ([Ca\(^{2+}\)]\text{i}) regulates cellular functions in various types of cells (1). In B lymphocytes, [Ca\(^{2+}\)]\text{i} controls cell proliferation, differentiation and apoptotic processes (2, 3). Cross-linking of B cell receptors (BCR) with specific antigens activates phospholipase C\(\gamma\) (PLC\(\gamma\)) through a series of tyrosine phosphorylations, resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). Inositol 1,4,5-trisphosphate (IP\(_3\)), a product of the hydrolysis of PIP\(_2\), then activates IP\(_3\) receptors to mobilize the intracellular Ca\(^{2+}\) stores. Ca\(^{2+}\) influx from the extracellular space is also activated in response to BCR cross-linking via the store-operated Ca\(^{2+}\) influx (SOC) pathway, which is activated by depletion of the intracellular Ca\(^{2+}\) stores (4, 5). BCR activation also results in the activation of phosphoinositide 3-kinase, which converts PIP\(_2\) to phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)).

In addition to BCR, B lymphocytes express another class of immunoreceptors, FcyRIIB. Co-cross-linking of BCR and FcyRIIB attenuates the BCR-induced Ca\(^{2+}\) response (6–10). The attenuation of the Ca\(^{2+}\) response requires the activation of the SH2 domain-containing inositol polyphosphate 5’-phosphatase (SHIP) (11–14), which catalyzes dephosphorylation of PIP\(_3\) and inositol 1,3,4,5-tetrakisphosphate at their 5-position of the inositol ring.

One of the possible target mechanisms of SHIP in the attenuation of the Ca\(^{2+}\) response is the SOC activity. Since the FcyRIIB-mediated inhibition of Ca\(^{2+}\) signaling was more prominent in the presence of extracellular Ca\(^{2+}\) than in its absence (8–10), SHIP was postulated to attenuate Ca\(^{2+}\) influx via the SOC channel (11–13, 17). Another potential target of SHIP is the PLC\(\gamma\) activity. Since PIP\(_3\) was reported to stimulate Bruton’s tyrosine kinase (Btk), which in turn tyrosine-phosphorylates PLC\(\gamma\) (18–20), degradation of PIP\(_3\) upon activation of SHIP by the co-cross-linking of FcyRIIB would decrease PLC\(\gamma\) activity and hence inhibit Ca\(^{2+}\) release from the stores. However, direct demonstration that SHIP affects SOC or Ca\(^{2+}\) release in FcyRIIB-mediated signaling remains to be reported.

It has been shown that cross-linking of BCR alone leads to tyrosine phosphorylation of SHIP (21, 22). Furthermore, SHIP seems to inhibit the BCR-mediated Ca\(^{2+}\) response even without co-cross-linking of FcyRIIB, because SHIP-deficient cells exhibit prolongation of the [Ca\(^{2+}\)]\text{i} transient upon BCR activation (23). While SHIP is recruited to the immunoreceptor tyrosine-based inhibitory motif on the intracellular region of FcyRIIB upon activation of this receptor (11), there is no immunoreceptor tyrosine-based inhibitory motif on the BCR complex. Therefore, SHIP is not a unique molecule for FcyRIIB-mediated inhibitory signaling, but plays an important role in BCR-mediated signaling; the mechanisms of the recruitment of SHIP seem different in the absence or presence of FcyRIIB-mediated signaling. It remains to be clarified how Ca\(^{2+}\) mobilization is attenuated by SHIP in BCR-mediated signaling.

In an effort to understand the role of SHIP in BCR-mediated signaling, we addressed the following questions in this work: 1) how are the patterns of Ca\(^{2+}\) mobilization elicited by BCR stimulation modulated by SHIP, and 2) which Ca\(^{2+}\) mobilization pathway is affected by SHIP: Ca\(^{2+}\) release or SOC. To clarify these points, we compared the patterns of Ca\(^{2+}\) mobilization in control and SHIP-deficient DT40 cells at the single
cell level. We found that the prolongation of [Ca\(^{2+}\)] transient in SHIP-deficient cells is due to enhanced Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores with enhancement of IP\(_3\) production. We also found that SHIP regulates SOC not by a direct interaction but through its effect on Ca\(^{2+}\) release. SHIP thus plays an important role in shaping the Ca\(^{2+}\) mobilization patterns after BCR stimulation.

**Experimental Procedures**

**Cell Lines and Culture**—Chicken B cells (DT40) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO\(_2\) at 0.5–1 × 10\(^6\) cells/ml. The generation of SHIP-deficient cells and transfection of either mouse wild-type SHIP or mutant SHIP with disrupted phosphatase activity (P671A, D675A, R676G) were described elsewhere (13).

**Measurement of Intracellular Ca\(^{2+}\) Concentration**—About 30 min before the experiments, cells were attached to collagen-coated coverslips. Cells on the coverslips were incubated with 5 μM Fura-2 AM for 30 min at room temperature in physiological salt solution (PSS) (150 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, 5.6 mM glucose; pH adjusted to 7.4 with NaOH) containing 0.1% bovine serum albumin followed by rinsing with PSS. The coverslips with Fura-2-loaded cells were mounted on the stage of an inverted epifluorescence microscope (TMD 300, Nikon). Cells were examined under a 40× immersion objective (numerical aperture: 0.7, Olympus). Pairs of fluorescence images with 340 and 380 nm excitations were collected using a cooled CCD camera (PXL-37, Photometrics), at either 0.5 or 0.33 Hz. The ratio (R) between the fluorescence intensities at 340 and 380 nm excitations was converted to [Ca\(^{2+}\)] using the following equation (24):

\[
\text{[Ca}^{2+}\text{]} = K_i(R - R_{\text{min}})/(R_{\text{max}} - R),
\]

where \(K_i\), \(R_{\text{max}}\), and \(R_{\text{min}}\) are the dissociation constant and maximal and minimal R values, respectively.

The values were determined in vitro under equivalent optical conditions. \(R_{\text{max}}\) and \(R_{\text{min}}\) were multiplied by a factor of 0.85 for viscosity correction (25).

**Immunoblotting**—Cells were harvested and washed with phosphate buffered saline. The cells were precipitated in 10% trichloroacetic acid (2 × 10\(^6\) cells/ml). The precipitates were dissolved in solution containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, and 1% SDS. The samples were separated by SDS-polyacrylamide gel electrophoresis and were electrotransferred to polyvinyldene difluoride membranes (Trans-Blot, BioRad). The membranes were incubated with the primary polyclonal antibody (anti-mouse SHIP, Upstate Biotechnology, Inc.) and then with anti-rabbit secondary antibody. The blots were detected with enhanced chemiluminescence (Renaissance, NEN Life Science Products).

**IP\(_3\) Measurement**—Cells were harvested and washed with PSS. The BCR-stimulated cell suspension (2 × 10\(^6\) cells) was mixed with 15% trichloroacetic acid for termination of the reaction. After centrifugation, the supernatant was extracted with water-saturated diethylether and the water phase was neutralized with NaHCO\(_3\) to pH 7.5. 3-Hilinositol-1,4,5-trisphosphate assay system (TRK1000, Amersham Pharmacia Biotech) was used for measurement of IP\(_3\) levels (27). Radioactivity was measured for 3 min in a β-scintillation counter, and converted to IP\(_3\) concentration using the calibration curve.

**Statistical Analysis**—Statistical results are expressed as mean ± S.E. Statistical comparisons were made using the paired t test for the IP\(_3\) assay and the non-paired t test for all the other measurements.

**Materials**—RPMI 1640 medium, glutamine, penicillin, and streptomycin were purchased from Life Technologies, Inc. Chicken serum, immonycin, and thapsigargin were obtained from Sigma. Fura-2 AM was purchased from Molecular Probes. All other chemicals were of the highest reagent grade.

**Results**

Prolonged Ca\(^{2+}\) Mobilization in SHIP-deficient DT40 Cells—We compared the time course of [Ca\(^{2+}\)] mobilization in control and SHIP-deficient cells at the single cell level. BCR was activated by the application of anti-μ mouse IgM monoclonal antibody, M4. Ca\(^{2+}\) mobilization in control cells showed either a single peak or attenuating oscillations with a few peaks, and [Ca\(^{2+}\)] returned to the resting value within 200 s (Fig. 1A). In contrast, SHIP-deficient cells showed a plateau-like response or oscillations persisting for over 200 s (Fig. 1B). Quantitative analysis of [Ca\(^{2+}\)] showed that the peak level of [Ca\(^{2+}\)] increase was slightly greater in SHIP-deficient cells (675 ± 29 nm, n = 140) than in control cells (556 ± 23 nm, n = 140; p < 0.002). The difference was more conspicuous in the late phase, and at 150 s after the BCR stimulation, [Ca\(^{2+}\)] remained at a higher level in SHIP-deficient cells (350 ± 31 nm, n = 70) than in control cells (110 ± 14 nm, n = 70; p < 0.0001) (Fig. 1, A and B; Fig. 2A).

To confirm that the observed prolongation of Ca\(^{2+}\) mobilization in SHIP-deficient cells was due to the absence of SHIP, we transfected the SHIP-deficient cells with mouse SHIP or mutant SHIP lacking phosphatase activity. The expression of wild-type SHIP activity shortened the duration of [Ca\(^{2+}\)] increase (Fig. 1C), whereas transfection of SHIP lacking phosphatase activity was without effect (Fig. 1D). The levels of expression of the wild-type and mutant SHIP were almost the same (Fig. 1E). These results indicate that the shortening of the Ca\(^{2+}\) mobilization pattern by SHIP required its phosphatase activity.

**Effect of SHIP on Ca\(^{2+}\) Release from Ca\(^{2+}\) Stores**—There exist two main pathways for Ca\(^{2+}\) mobilization: Ca\(^{2+}\) release from the Ca\(^{2+}\) stores and Ca\(^{2+}\) entry from the extracellular space. Both pathways are potential targets of SHIP. To determine the role of SHIP in the Ca\(^{2+}\) release pathway, we analyzed the time course of Ca\(^{2+}\) mobilization by BCR stimulation in the presence and absence of extracellular Ca\(^{2+}\). The prolongation of Ca\(^{2+}\) mobilization in SHIP-deficient cells in the pres-
The time course of changes in [Ca\(^{2+}\)], with (A) or without (B) extracellular Ca\(^{2+}\) in control cells (thick trace) or in SHIP-deficient cells (thin trace). The extracellular Ca\(^{2+}\) was removed 20 s before BCR stimulation. Arrows indicate the time point of M4 antibody application.

**Fig. 2.** Prolongation of [Ca\(^{2+}\)] transient elicited by BCR stimulation in SHIP-deficient cells in the presence and absence of extracellular Ca\(^{2+}\).

The time course of changes in [Ca\(^{2+}\)], with (A) or without (B) extracellular Ca\(^{2+}\) in control cells (thick trace) or in SHIP-deficient cells (thin trace). The extracellular Ca\(^{2+}\) was removed 20 s before BCR stimulation. Arrows indicate the time point of M4 antibody application.

**Fig. 3.** Enhancement of IP\(_3\) production in SHIP-deficient cells.

IP\(_3\) production was enhanced in SHIP-deficient cells compared to control cells. This enhancement was measured using an IP\(_3\) binding protein assay system.

**Fig. 4.** Prolongation of Ca\(^{2+}\) transient due to SOC elicited by BCR stimulation in SHIP-deficient cells.

The Ca\(^{2+}\) transient was prolonged due to SOC elicited by BCR stimulation in SHIP-deficient cells. This result was measured using an IP\(_3\) binding protein assay system.

**Fig. 5.** Prolongation of Ca\(^{2+}\) transient due to SOC elicited by BCR stimulation in SHIP-deficient cells.

The Ca\(^{2+}\) transient was prolonged due to SOC elicited by BCR stimulation in SHIP-deficient cells. This result was measured using an IP\(_3\) binding protein assay system.

**Fig. 6.** Prolongation of Ca\(^{2+}\) transient due to SOC elicited by BCR stimulation in SHIP-deficient cells.

The Ca\(^{2+}\) transient was prolonged due to SOC elicited by BCR stimulation in SHIP-deficient cells. This result was measured using an IP\(_3\) binding protein assay system.

**Fig. 7.** Prolongation of Ca\(^{2+}\) transient due to SOC elicited by BCR stimulation in SHIP-deficient cells.

The Ca\(^{2+}\) transient was prolonged due to SOC elicited by BCR stimulation in SHIP-deficient cells. This result was measured using an IP\(_3\) binding protein assay system.
from control and SHIP-deficient cells were superimposable, indicating no difference in the level of activity of the Ca\textsuperscript{2+} extrusion mechanism between control and SHIP-deficient cells.

**DISCUSSION**

We examined the Ca\textsuperscript{2+} mobilization patterns in SHIP-deficient B cells at the single cell level and showed that the main role of SHIP in BCR-mediated Ca\textsuperscript{2+} signaling was to abbreviate the duration of Ca\textsuperscript{2+} mobilization. SHIP prevented continuous Ca\textsuperscript{2+} release from the intracellular Ca\textsuperscript{2+} stores via inhibition of IP\textsubscript{3} production. Furthermore, we showed that SHIP regulates SOC not by a direct effect on the SOC mechanism but by controlling the depletion level of the Ca\textsuperscript{2+} stores. This inhibitory cascade of SHIP is shared by the Fc\textgamma RIIB pathway despite the difference in the way for recruitment of SHIP.

In B lymphocytes, accumulation of PIP\textsubscript{3} via the activation of phosphoinositide 3-kinase was reported to stimulate Btk, which then phosphorylates PLC\textgamma (18–20). In other studies, accumulation of PIP\textsubscript{3} was reported to lead to PH domain-mediated membrane targeting of PLC\textgamma (29, 30). In either case, PIP\textsubscript{3} functions as a potent activator of PLC\textgamma. Therefore, degradation of PIP\textsubscript{3} by SHIP is expected to inhibit PLC\textgamma and hence Ca\textsuperscript{2+} release from the Ca\textsuperscript{2+} stores. We now show direct evidence that IP\textsubscript{3} production in BCR-mediated signaling is enhanced in SHIP-deficient cells (Fig. 3).

In Fc\textgamma RIIB-mediated inhibitory signaling, SHIP was implicated not only in IP\textsubscript{3} production but also in the inhibition of SOC, or store depletion-induced Ca\textsuperscript{2+} influx (11, 13). We, therefore, examined the effects of SHIP on Ca\textsuperscript{2+} influx elicited after BCR activation, and found that Ca\textsuperscript{2+} influx in SHIP-deficient cells was indeed greater than that in control cells (Fig. 4). SOC has been postulated to be a mechanism of Ca\textsuperscript{2+} influx in B lymphocytes, as is the case in other nonexcitable cells (4, 31). Thus, we also examined the effect of SHIP on SOC after store depletion by thapsigargin, which is a potent inhibitor of SERCA and depletes the Ca\textsuperscript{2+} stores without IP\textsubscript{3} production (28). However, thapsigargin-induced SOC was not affected by the presence of SHIP either with or without BCR cross-linking (Fig. 5). These results clearly indicate that SHIP does not have a direct inhibitory effect on either SOC channel activity or the SOC activation mechanism itself in BCR-mediated signaling. Then, how did SHIP inhibit the Ca\textsuperscript{2+} influx after BCR stimulation? The activation of SOC was shown to be regulated by the level of depletion the Ca\textsuperscript{2+} stores (32–34). We therefore postulated that the effect of SHIP on Ca\textsuperscript{2+} influx resulted from the difference in the extent of Ca\textsuperscript{2+} store depletion in control and SHIP-deficient cells after BCR cross-linking. To test this hy-
Ca$^{2+}$ Mobilization Pathway Affected by SHIP

Fig. 6. Ca$^{2+}$ remaining in the Ca$^{2+}$ stores evaluated by ionomycin treatment after BCR stimulation. BCR stimulation with M4 antibody (arrow) for 600 s was followed by 600-s application of 1 μM ionomycin (closed bar) in control (A) and SHIP-deficient cells (B). Extracellular Ca$^{2+}$ was removed 20 s before the BCR stimulation (open bar). Control experiments were performed under the same protocol without BCR stimulation in control (D) and SHIP-deficient cells (E). C and F show the average and S.E. of [Ca$^{2+}$]$_i$ increase during the ionomycin application with and without BCR stimulation, respectively.

Fig. 7. The Ca$^{2+}$ extrusion rates in control and SHIP-deficient cells. The Ca$^{2+}$ extrusion rate, which was represented by the initial rate of decrease in [Ca$^{2+}$], upon removal of extracellular Ca$^{2+}$, was plotted against the [Ca$^{2+}$]$_i$ just before the Ca$^{2+}$ removal. The closed and open circles are data from control and SHIP-deficient cells, respectively. The initial rate of Ca$^{2+}$ extrusion was estimated from the results of experiments shown in Fig. 5 (D and E) as the slope of the dotted line that was fitted to the data points for 120-s period after the removal of extracellular Ca$^{2+}$ (inset).

The Ca$^{2+}$ mobilization pathway affected by SHIP. In SHIP-deficient cells, the amount of Ca$^{2+}$ remaining in the stores was much smaller than that in control cells (Fig. 6). The results indicate that SHIP causes early termination of Ca$^{2+}$ release making SOC activation minimal in normal BCR signaling. On the other hand, the Ca$^{2+}$ stores were likely to be depleted enough to activate SOC in thapsigargin-treated cells and in BCR-stimulated SHIP-deficient cells. A similar inhibitory cascade after FcRI co-cross-linking was shown recently (20).

The differential effects of SHIP on Ca$^{2+}$ influx elicited by BCR cross-linking and SOC elicited by thrombin-induced platelet activation (36, 37). In FcεRI-stimulated RBL-2H3 cells, SHIP was reported to be phosphorylated and recruited to the immunoreceptor tyrosine-based activation motif of FcεRI (38, 39). However, the role of SHIP in these cells is unclear. Therefore, it will be important to examine whether SHIP plays the same inhibitory role in Ca$^{2+}$ signaling in different cell types as shown here in B cells.

Accumulating evidence suggests that the cellular responses are more often controlled by the temporal pattern of Ca$^{2+}$ mobilization than by the peak or average levels of [Ca$^{2+}$]$_i$ (1, 34, 40–42). Although the mechanism underlying the Ca$^{2+}$ mobilization pattern-mediated control of cell function is not fully understood, the temporal pattern of [Ca$^{2+}$]$_i$ increase has been implicated in the differential activation of subsets of proteins and/or genes due to different Ca$^{2+}$-mediated activation kinetics. Our present finding indicates that the Ca$^{2+}$ mobilization is curtailed by the inhibition of IP$_3$ production by SHIP. This conclusion was supported by the results obtained from the DT40 cells in which all three IP3 receptor genes were disrupted. In these IP3 receptor-deficient cells, there was no Ca$^{2+}$ response to BCR stimulation, although thapsigargin-elicited Ca$^{2+}$ increase was clearly observed (31). These results suggest the absence of a Ca$^{2+}$ influx pathway other than the SOC in BCR signaling in DT40 cells.

SHIP has been found in a variety of hematopoietic cells other than B cells and may play a role in cell signaling in these cells (21, 35). For example, SHIP may be involved in thrombin-induced platelet activation (36, 37). In FcεRI-stimulated RBL-2H3 cells, SHIP was reported to be phosphorylated and recruited to the immunoreceptor tyrosine-based activation motif of FcεRI (38, 39). However, the role of SHIP in these cells is unclear. Therefore, it will be important to examine whether SHIP plays the same inhibitory role in Ca$^{2+}$ signaling in different cell types as shown here in B cells.

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Acknowledgments—We thank Mari Kurosaki, Toshiko Yamazawa, and Tomoya Miyakawa for helpful discussion.

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