

SHIP Down-Regulates FcεR1-Induced Degranulation at Supraoptimal IgE or Antigen Levels

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Cross-linking of the IgE-loaded high-affinity IgE receptor (FcεR1) by multivalent Ags results in mast cell activation and subsequent release of multiple proinflammatory mediators. The dose-response curve for FcεR1-mediated degranulation is bell-shaped, regardless of whether the IgE or the Ag concentration is varied. Although overall calcium influx follows this bell-shaped curve, intracellular calcium release continues to increase at supraoptimal IgE or Ag concentrations. As well, overall calcium mobilization adopts more transient kinetics when stimulations are conducted with supraoptimal instead of optimal Ag concentrations. Moreover, certain early signaling events continue to increase whereas degranulation drops under supraoptimal conditions. We identified SHIP, possibly in association with the FcεR1 β-chain, as a critical negative regulator acting within the inhibitory (supraoptimal) region of the dose-response curve that shifts the kinetics of calcium mobilization from a sustained to a transient response. Consistent with this, we found that degranulation of SHIP-deficient murine bone marrow-derived mast cells was not significantly reduced at supraoptimal Ag levels. A potential mediator of SHIP action, Bruton's tyrosine kinase, did not seem to play a role within the supraoptimal suppression of degranulation. Interestingly, SHIP was found to colocalize with the actin cytoskeleton (which has been shown previously to mediate the inhibition of degranulation at supraoptimal Ag doses). These results suggest that SHIP, together with other negative regulators, restrains bone marrow-derived mast cell activation at supraoptimal IgE or Ag concentrations in concert with the actin cytoskeleton. *The Journal of Immunology*, 2005, 174: 507–516.

The Ag-mediated cross-linking of high-affinity IgE receptors, FcεR1, on mast cells (MCs)² and the subsequent activation of these cells are important early events in the development of type I hypersensitivity reactions (1). The FcεR1 on MCs is composed of an α-subunit, a β-subunit, and two disulfide-bridged γ-subunits (2). Whereas the α-subunit binds to the constant Cε3 region of the IgE molecule, the β- and γ-subunits contain ITAM in their cytoplasmic domains and mediate the signal transduction of this receptor (2).

Critical steps involved in IgE-induced degranulation include, among others, intracellular calcium release, extracellular calcium entry, and the activation of the PI3K pathway (3, 4). With regard to the PI3K pathway, we demonstrated previously the positive role that this pathway plays in MC degranulation by analyzing bone marrow-derived MCs (BMMCs) from mice deficient for the Src homology 2-containing inositol polyphosphate 5'-phosphatase, SHIP, a critical negative regulator of PI3K signaling. SHIP-deficient BMMCs were found to be much more prone to Ag-induced degranulation than wild-type (WT) BMMCs (4) and even degranulated under conditions in which normal MCs did not, i.e., follow-

ing stimulation with Steel factor or IgE alone (4, 5). Relevant to this, it has recently been shown that basophils from a subset of hyperallergic individuals contain lower levels of SHIP than basophils from healthy donors. This implicates SHIP as a critical negative regulator of human basophil degranulation as well (6).

The degranulation of IgE-preloaded MCs after stimulation with increasing concentrations of multivalent Ag or anti-IgE Abs follows a bell-shaped dose-response curve. When studying anti-IgE-induced degranulation of IgE-loaded cells as well as the effect of increasing concentrations of monomeric Fab of anti-IgE Abs, Magro and Alexander (7) found that for a suboptimal to optimal concentration of anti-IgE, increasing concentrations of the monomer inhibited the release of histamine. Surprisingly, however, at optimal to supraoptimal concentration of anti-IgE Abs, increasing concentrations of the monomer-enhanced histamine release. Thus, they concluded that the descending portion of the dose-response curve might be the result of a turn-off mechanism caused by an excess of bridging (7). Related to this, it was demonstrated, using stable oligomers prepared by chemically cross-linking IgE, that no more than a few hundred trimers or an even smaller number of higher oligomers are required to induce a considerable amount of degranulation from RBL-2H3 MCs (8). Using mAbs directed against the α-chain of the FcεR1, a further study not only showed that FcεR1 aggregates as small as dimers are capable of providing an effective stimulus for degranulation but that only a small fraction of FcεR1 needs to be cross-linked to yield optimal degranulation (9).

How then can one envision the molecular mechanism by which degranulation is suppressed in the presence of supraoptimally cross-linked FcεR1? Baird and coworkers (10) reported that FcεR1 stimulation by polyclonal Abs against IgE induced a detergent-resistant association of these complexes with the cellular cytoskeleton. In dose-response studies, the extent of the cytoskeletal association followed the extent of FcεR1 bridging and, more

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² Abbreviations used in this paper: MC, mast cell; BMMC, bone marrow-derived MC; Btk, Bruton's tyrosine kinase; WT, wild type; HSA, human serum albumin; PLC, phospholipase C; PKB, protein kinase B; PKC, protein kinase C; IP₃, inositol 1,4,5-trisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; LatB, latrunculin B; I_{CRAC}, calcium release-activated calcium channel; SMC, submembrane cytoskeleton.

importantly, continued to increase beyond the stimulus concentration in which degranulation was maximal (10). Another study verified that detergent-insolubility of FcεR1-IgE-Ag complexes did not correlate with degranulation and, by using inhibitors of actin polymerization, they could increase Ag-stimulated degranulation at supraoptimal Ag concentrations, while detergent-insolubility was decreased (11). From these studies they concluded that Ag-triggered actin polymerization is more likely related to the termination/inhibition than to the stimulation of degranulation, particularly at supraoptimal Ag concentrations (11). Finally, Seagrave et al. (12) using scanning electron microscopy demonstrated that optimally cross-linked FcεR1 redistribute into chains and small clusters, whereas supraoptimally cross-linked receptors redistribute into clusters and large aggregates. Again, large aggregates at high stimulus concentrations were prevented from forming by adding either monovalent ligands or actin depolymerizing agents.

Because these results were not consistent with the notion that the inhibition of MC degranulation at high Ag concentrations is due to the formation of monovalent Ag-FcεR1 complexes, we set out to explore the signaling mechanism(s) responsible for this inhibition. A deeper understanding of this mechanism(s) should help to elucidate the molecular events that occur during hyposensitization of allergy patients. Our results suggest that SHIP plays a major role in this phenomenon.

Materials and Methods

Cell culture

Bone marrow cells (1×10^6 /ml) from 6- to 8-wk-old male mice (129/Sv) were cultured (37°C, 5% CO₂) as a single cell suspension in RPMI 1640 medium containing 20% FCS, 1% X63Ag8-653-conditioned medium as a source of IL-3 (13), 2 mM L-glutamine, 1×10^{-5} M 2-ME, 50 U/ml penicillin, and 50 mg/ml streptomycin. At weekly intervals, the nonadherent cells were reseeded at 5×10^5 cells/ml in fresh medium. By 4–6 wk in culture, >99% of the cells were *c-kit* and FcεR1 positive as assessed by PE-labeled anti-*c-kit* Abs (BD Pharmingen) and FITC-labeled IgE (anti-erythropoietin 26), respectively. SHIP^{+/+} and SHIP^{-/-} MCs were in vitro differentiated using the same protocol but starting from bone marrow cells of 6- to 8-wk-old SHIP^{+/+} and SHIP^{-/-} littermates (129/Sv × C57BL/6).

Reagents

Monoclonal anti-phosphotyrosine (4G10) and polyclonal anti-p85 Abs (no. 06-195) were purchased from Biozol. Polyclonal anti-phospholipase C (PLC)-γ1 (530), polyclonal anti-P PLC-γ1 (Y783) (sc-12943-R), monoclonal anti-SHIP (P1C1), and polyclonal anti-actin Abs (I-19) were obtained from Santa Cruz Biotechnology. Polyclonal anti-phospho protein kinase B (PKB, S473) and polyclonal anti-phospho-Bruton's tyrosine kinase (Btk, Y223) Abs were purchased from Cell Signaling Technology. Polyclonal anti-SHIP Abs (anti-N and anti-M) have been described (14). Monoclonal anti-β-chain Abs were a kind gift from Dr. R. Siraganian (Bethesda, MD). DNP-human serum albumin (HSA) containing 30–40 mol DNP per mole albumin, monoclonal IgE with specificity for DNP (SPE-7), and EGTA were purchased from Sigma-Aldrich. The protein kinase C (PKC) inhibitor bis-indolylmaleimide I, which preferentially inhibits classical and novel PKCs, was obtained from Calbiochem Merck Biosciences.

Degranulation assays

For degranulation studies, cells were preloaded with 0.5 μg/ml IgE anti-DNP overnight at 37°C. The cells were then washed and resuspended in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA in 10 mM HEPES, pH 7.4). The cells were adapted to 37°C for 20 min and then treated for 30 min at 37°C as mentioned. The degree of degranulation was determined by measuring the release of β-hexosaminidase (5).

Calcium measurements

IgE-preloaded BMMCs were washed with RPMI 1640, resuspended at 5×10^6 cells/ml in RPMI 1640 containing 1% FCS, 30 μM Indo-1 AM (Molecular Probes), and 0.045% pluronic F-127 (Molecular Probes), and incubated for 45 min at 37°C. Cells were then pelleted, resuspended in RPMI

1640 containing 1% FCS, and analyzed in a FACStar^{Plus} (BD Biosciences) after the indicated stimulation procedures. Intracellular calcium release was recorded in the presence of 1 mM EGTA. The FACS profiles were converted to line graph data using the FCS Assistant version 1.3.1a application.

Ag stimulation, immunoprecipitation, and Western blotting

IgE-preloaded cells were washed twice in RPMI 1640/0.1% BSA and resuspended (2×10^7 /ml) in RPMI 1640/0.1% BSA. Cells were adapted to 37°C for 30 min and stimulated with the indicated concentrations of DNP-HSA. After stimulation, cells were pelleted and solubilized with 0.5% Nonidet P-40 and 0.5% deoxycholate in 4°C phosphorylation solubilization buffer. After normalizing for protein content, the postnuclear supernatants were either subjected directly to SDS-PAGE and Western blot analysis as previously described (15) or to immunoprecipitation with three subsequent washing steps using phosphorylation solubilization buffer containing 0.1% Nonidet P-40. The precipitate was separated by SDS-PAGE and analyzed by means of Western blotting.

Subcellular fractionation

Cytosol-membrane fractionation of BMMCs was performed as previously described (16). As a change to the published protocol, the membrane pellet obtained after the first round of ultracentrifugation was solubilized in hypotonic lysis buffer containing 0.5% of Nonidet P-40 and 0.5% of deoxycholate.

Coating of microscope cover glasses with poly-L-lysine

Glass coverslips were incubated in 65% nitric acid at 23°C overnight, rinsed twice in distilled H₂O and washed in distilled H₂O for 2 h at 23°C. The coverslips were then incubated in a freshly prepared poly-L-lysine solution (0.1 mg/ml in 1 M borate buffer, pH 8.5, P3626; Sigma-Aldrich) for 3 h. After washing three times in PBS, coated coverslips were briefly air dried and stored cool and dust-free.

Detection of actin and SHIP in BMMC by confocal microscopy

Poly-L-lysine-coated coverslips were rehydrated in PBS for 10 min. The coverslips were overlaid with BMMC growth medium containing 1×10^6 cells/ml for 3 h at 37°C. After washing twice with PBS, the cells were fixed with 3.7% formaldehyde solution in PBS for 20 min at 23°C and subsequently incubated with 50 mM NH₄Cl solution for 5 min at 23°C. The cells were permeabilized with 0.1% Triton X-100 for 5 min at 23°C and blocked with 5% goat serum for 30 min. For staining, cells were incubated with Alexa Fluor 546 phalloidin (1/50; Molecular Probes) and monoclonal anti-SHIP Ab (1/40) in PBS for 20 min at 23°C. After washing three times for 5 min with blocking buffer, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (1/200; Molecular Probes) in PBS for 20 min. Cells were washed twice with blocking buffer for 5 min and mounted using Confocal-Matrix (Micro-Tech Lab) according to the supplier's recommendation. Fluorochrome-labeled cells were visualized with a confocal laser scanner (Leica SP2; Leica Microsystems) mounted on an inverted microscope (Leica DM IRE2; Leica Microsystems) using a ×63 oil immersion lens (HCX PL APO ×63/1.32-0.6 Oil CS; Leica Microsystems). Image data were collected in sequential scanning mode at two different combinations of excitation and emission wavelengths: Alexa Fluor 488, excitation 488 nm/emission 503–530 nm; and Alexa Fluor 546, excitation 543 nm/emission 560–610 nm. Images were merged as RGB images using Photoshop 6.0 (Adobe Systems).

Results

The dose-response curve for Ag-induced degranulation does not correlate with calcium fluxes or early phosphorylation events

Although it is well established that IgE-induced degranulation declines at high levels of Ag, the notion that this is due to a reduction in cross-linked FcεR1 because of an increase in the number of monovalent IgE/FcεR1 complexes is likely incorrect (7). We therefore decided to investigate this further. A typical Ag dose-response experiment with BMMCs is shown in Fig. 1A. We then analyzed different FcεR1-mediated signaling events in response to increasing doses of Ag to learn more about the regulation of FcεR1 signaling, particularly at supraoptimal Ag doses. An examination of FcεR1-triggered calcium mobilization in BMMCs suggested a reasonably good correlation between degranulation and calcium

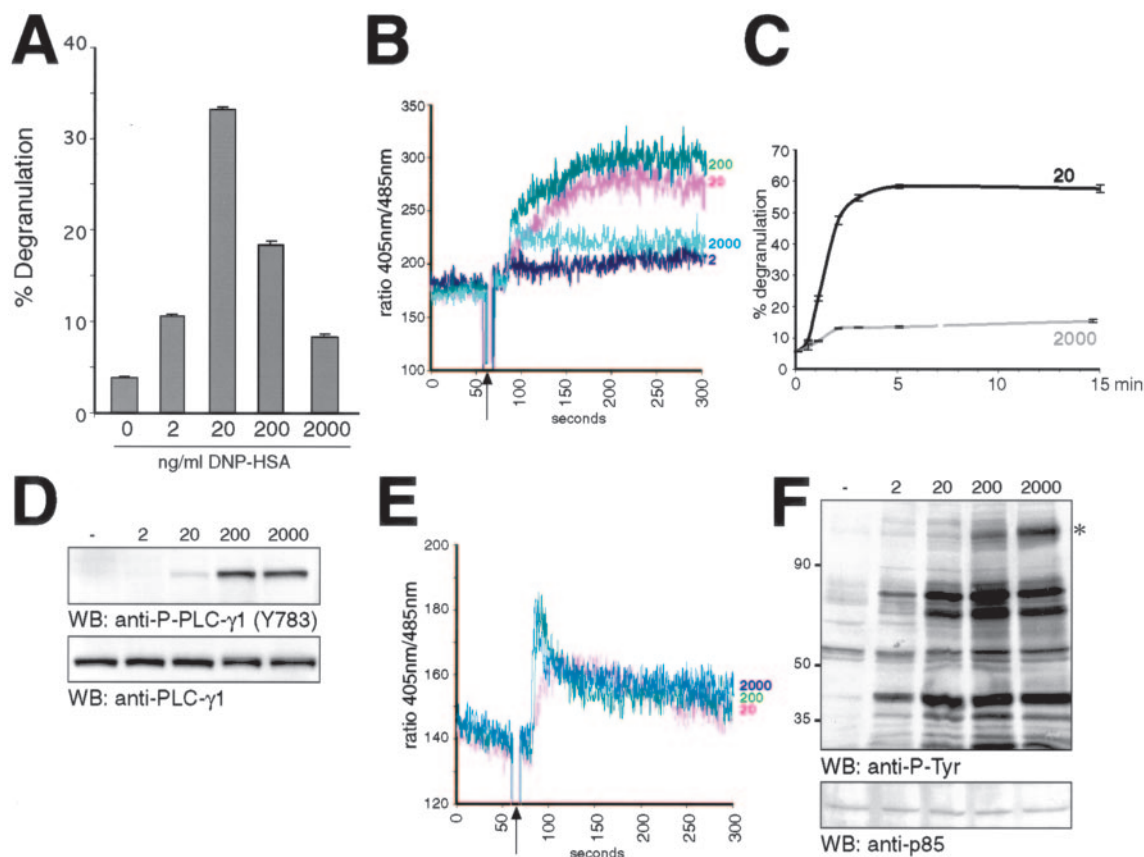


FIGURE 1. Ag-induced degranulation does not correlate with calcium fluxes or substrate tyrosine phosphorylation. **A**, BMMCs were preloaded with 0.5 μ g/ml IgE overnight. Cells were left unstimulated or stimulated with increasing concentrations of DNP-HSA (2, 20, 200, and 2000 ng/ml) for 30 min. Degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD. **B**, IgE-preloaded BMMCs were stimulated with different doses of Ag (2, 20, 200, and 2000 ng/ml) and calcium mobilization measured. The arrow marks the time point of Ag addition. Comparable results were obtained in three separate experiments. **C**, IgE-preloaded BMMCs were stimulated for increasing times (0.5, 1, 2, 3, 5, and 15 min) with 20 or 2000 ng/ml DNP-HSA. Degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD. **D**, BMMCs were loaded with 0.5 μ g/ml IgE overnight and stimulated for 1 min with increasing concentrations of Ag (2, 20, 200, and 2000 ng/ml DNP-HSA) or were left unstimulated (–). Postnuclear supernatants were analyzed for P-PLC- γ 1 (Y783) (top) as well as PLC- γ 1 (bottom) expression. **E**, IgE-preloaded BMMCs were stimulated with different doses of Ag (20, 200, and 2000 ng/ml) in the presence of 1 mM EGTA and intracellular calcium release measured. Arrows mark the time of Ag addition. Comparable results were obtained in three separate experiments. **F**, BMMCs were loaded with 0.5 μ g/ml IgE overnight and were left untreated (–) or stimulated for 1 min with increasing concentrations of Ag (2, 20, 200, and 2000 ng/ml DNP-HSA). Postnuclear supernatants were analyzed for substrate tyrosine phosphorylation (anti-P-Tyr) (top) as well as p85 (bottom) expression. A phosphoprotein (*) of $\sim$ 150 kDa is marked.

flux. Specifically, stimulation with suboptimal (2 ng/ml DNP-HSA) or supraoptimal (2000 ng/ml DNP-HSA) Ag concentrations resulted in weaker calcium influxes than at the optimal dose for degranulation (20 ng/ml) (Fig. 1B). However, stimulation with 200 ng/ml Ag, which is suboptimal for degranulation, produced the strongest calcium influx (Fig. 1B). As well, the initial rise in calcium was faster in supraoptimally than optimally stimulated cells, most likely because of the faster receptor occupancy under these conditions (see also later, Fig. 2C). However, the early signal at 2000 ng/ml was only of a transient nature (Fig. 1B).

Next, we performed degranulation time course studies at optimal and supraoptimal Ag concentrations to learn which time points are important to focus on in our further signaling analyses. Independent of the Ag dose, maximum degranulation was already reached within the first 3 min with a marked difference between optimally and supraoptimally stimulated degranulation being apparent already within the first minute (Fig. 1C).

Fc ϵ R1-induced calcium mobilization is a two-step process whereby intracellular calcium is first released in an inositol 1,4,5-trisphosphate (IP₃)-dependent manner from the endoplasmic reticulum. This release then triggers the influx of extracellular calcium

via store-operated calcium channels in the plasma membrane. PLC- γ is responsible for generating IP₃ (17) and thus we monitored the phosphorylation of PLC- γ 1 on Y783, a phosphorylation event known to be coupled to PLC- γ 1 activation (18). Interestingly, this phosphorylation of PLC- γ 1 on Y783 did not follow a bell-shaped dose-response curve (Fig. 1D, top panel). Equal loading was verified by anti-PLC- γ 1 reblotting (Fig. 1D, bottom panel). Intrigued by this finding we measured the release of intracellular calcium from the endoplasmic reticulum and found that in contrast to the overall calcium mobilization, intracellular calcium release was slightly stronger at supraoptimal than optimal levels of Ag (Fig. 1E). This indicated that the transient nature of the overall calcium signal at supraoptimal Ag concentrations was regulated by a step downstream of the initial calcium release. We also analyzed overall substrate tyrosine phosphorylation in response to increasing Ag concentrations. Signals at the optimal Ag concentration (20 ng/ml) were markedly stronger than at suboptimal Ag levels. At supraoptimal Ag concentrations signal strength did not decline, but even seemed to increase (in particular for the protein marked with an asterisk) (Fig. 1F, top panel). Comparable loading was verified by anti-p85 reblotting (Fig. 1F, bottom panel).

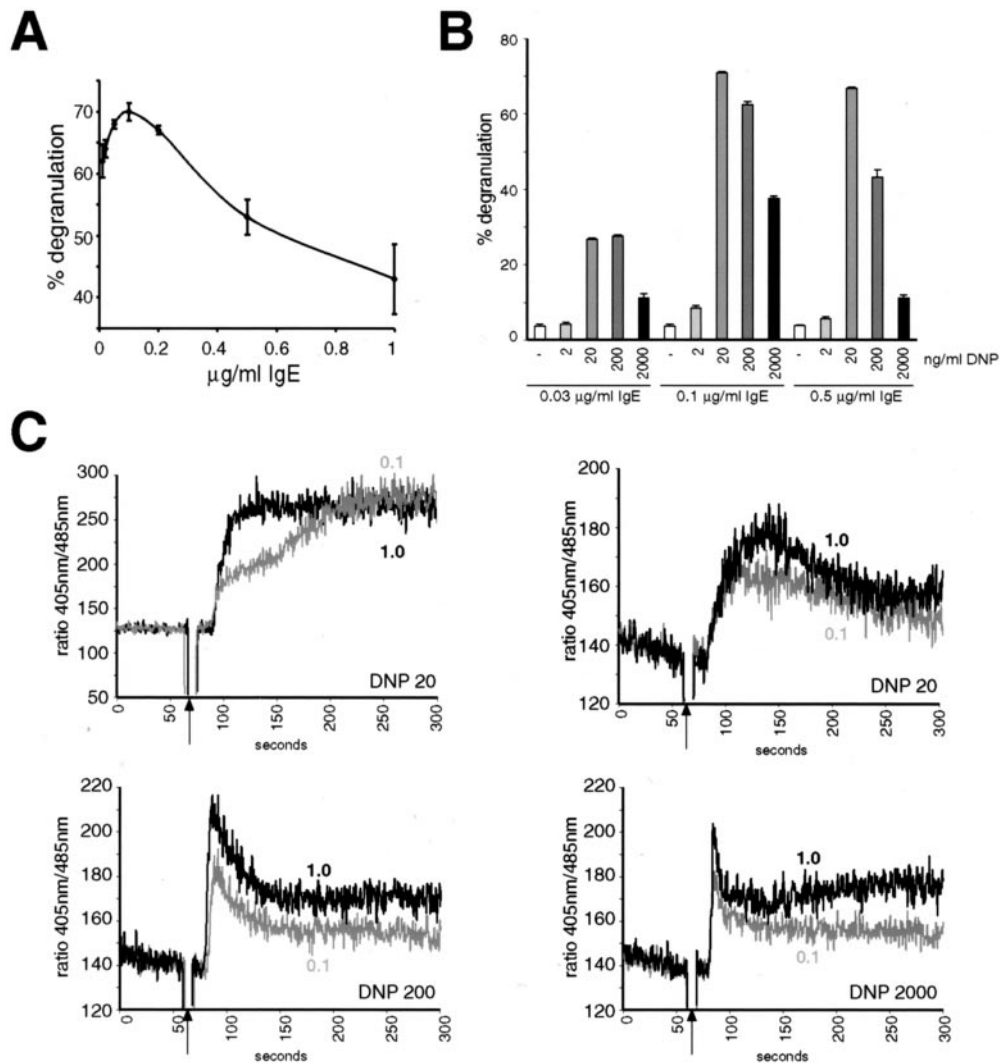


FIGURE 2. High concentrations of IgE reduce MC degranulation. **A**, BMMCs were preloaded with increasing levels of IgE (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, or 1.0 $\mu\text{g/ml}$) overnight and then stimulated with an optimal dose of Ag (20 ng/ml DNP-HSA) for 30 min and degranulation assessed by measuring the release of β -hexosaminidase. Each bar is the mean of quadruplicates $\pm$ SD. **B**, BMMCs were loaded with increasing levels of IgE (0.03, 0.1, or 0.5 $\mu\text{g/ml}$) overnight. Cells were then left untreated (—) or stimulated for 30 min with different doses of Ag (2, 20, 200, or 2000 ng/ml DNP-HSA). Subsequently, degranulation was assessed by measuring the release of β -hexosaminidase. Each bar is the mean of duplicates $\pm$ SD. **C**, BMMCs were preloaded with 0.1 (gray line) or 1.0 (black line) $\mu\text{g/ml}$ IgE overnight. Cells were stimulated either with 20 ng/ml Ag in the absence of EGTA (*upper left*) or with 20 (*upper right*), 200 (*lower left*), and 2000 ng/ml (*lower right*) Ag in the presence of 1 mM EGTA and calcium mobilization monitored. The arrows mark the time of Ag addition.

High concentrations of IgE also inhibit degranulation

To further explore the mechanism(s) underlying the reduced degranulation of BMMCs at supraoptimal Ag levels we considered whether we could duplicate this effect by keeping the Ag dose constant and increasing the IgE preloading concentration. Indeed, degranulation of MCs with an optimal Ag dose (20 ng/ml), after preloading overnight with increasing concentrations of IgE, resulted in a bell-shaped dose-response curve. As can be seen in Fig. 2A, 0.1 $\mu\text{g/ml}$ IgE was found to be the optimal concentration for degranulation. Moreover, this IgE-dependent bell-shaped dose-response curve was observed for all Ag concentrations tested (Fig. 2B). As well, degranulation induced by 0.5 $\mu\text{g/ml}$ IgE plus 2000 ng/ml Ag was lower than that induced by 0.1 $\mu\text{g/ml}$ IgE plus 2000 ng/ml Ag, indicating that supraoptimal doses of IgE and Ag can intensify each other's effect. Also of interest, when using a sub-optimal IgE concentration (0.03 $\mu\text{g/ml}$), the maximum degranulation shifted to higher Ag concentrations (from 20 to 200 ng/ml DNP-HSA) (Fig. 2B). To reach the optimal amount of FcεR1

cross-linking with respect to degranulation, most probably the higher Ag dose compensates for the lower IgE concentration.

Comparing the effects of optimal (0.1 $\mu\text{g/ml}$) vs supraoptimal (1 $\mu\text{g/ml}$) IgE concentrations on calcium mobilization, we found, as with Ag, that overall calcium mobilization in response to 20 ng/ml DNP-HSA was slightly stronger in cells preloaded with optimal than with supraoptimal IgE (Fig. 2C, *upper left panel*). The initial rise in calcium influx was again faster with the supraoptimal concentration and this again likely reflects faster receptor occupancy. As well, intracellular calcium release in response to 20 ng/ml DNP-HSA was more profound under supraoptimal IgE conditions (Fig. 2C, *upper right panel*). This was also the case for supraoptimal doses of Ag, i.e., 200 ng/ml (Fig. 2C, *lower left panel*) and 2000 ng/ml (Fig. 2C, *lower right panel*). The fact that high concentrations of IgE can mimic the reduction in degranulation seen with high Ag concentrations suggests that an increasing concentration of monovalent Ag/FcεR1 is not responsible for the bell-shaped IgE-induced dose-response curve and indicates, instead,

that negative signaling elements might be recruited in supraoptimally stimulated cells, which impact at a step subsequent to intracellular calcium release.

SHIP is strongly recruited under supraoptimal conditions and terminates supraoptimal FcεR1-induced calcium mobilization

We reported previously that SHIP restricts IgE-induced overall calcium mobilization in BMMCs while having no significant effect on intracellular release (5). Because stimulation with supraoptimal Ag concentrations led to a curtailed overall calcium flux with no effect on intracellular release (Fig. 1, *B* and *E*), we examined the role of SHIP in supraoptimally stimulated BMMCs. As shown in Fig. 3*A* (*top panel*), SHIP coprecipitated stronger with anti-phosphotyrosine Abs from lysates of supraoptimally stimulated BMMCs. Interestingly, the level of the FcεR1 β -chain in anti-phosphotyrosine precipitates was also much higher at supraoptimal Ag doses (Fig. 3*A*, *bottom panel*). Because both SHIP and the FcεR1 β -chain might be immunoprecipitating with anti-phosphotyrosine indirectly via association with an unrelated tyrosine-phosphorylated protein we assessed the tyrosine phosphorylation of SHIP and the FcεR1 β -chain directly by subjecting

postnuclear supernatants of stimulated BMMCs to anti-SHIP and anti-FcεR1 β -chain immunoprecipitation and subsequent anti-phosphotyrosine immunoblotting. As suggested by the anti-phosphotyrosine precipitations (Fig. 3*A*), both SHIP (Fig. 3*B*) and the β -chain (Fig. 3*C*) were more strongly tyrosine phosphorylated under supraoptimal conditions.

We next compared degranulation of WT and SHIP^{-/-} BMMCs in response to increasing levels of Ag to see whether the absence of SHIP had any effect on the typical bell-shaped dose-response curve. Not only did SHIP^{-/-} cells degranulate more strongly at suboptimal and optimal Ag concentrations (Fig. 3*D*) (4), but, unlike WT BMMCs, degranulation of SHIP^{-/-} BMMCs showed only a marginal reduction between 20 and 5000 ng/ml DNP-HSA (Fig. 3*D*). In a few experiments, we did observe more of a decline in the degranulation of SHIP^{-/-} BMMCs at supraoptimal Ag concentrations (2000 and 5000 ng/ml). However, even in these cases supraoptimal degranulation of SHIP^{-/-} BMMCs was still higher than optimal degranulation of WT BMMCs (data not shown). These results suggest that SHIP plays an important role in the down-regulation of MC degranulation at supraoptimal Ag levels. Time course studies demonstrated that degranulation of SHIP^{-/-}

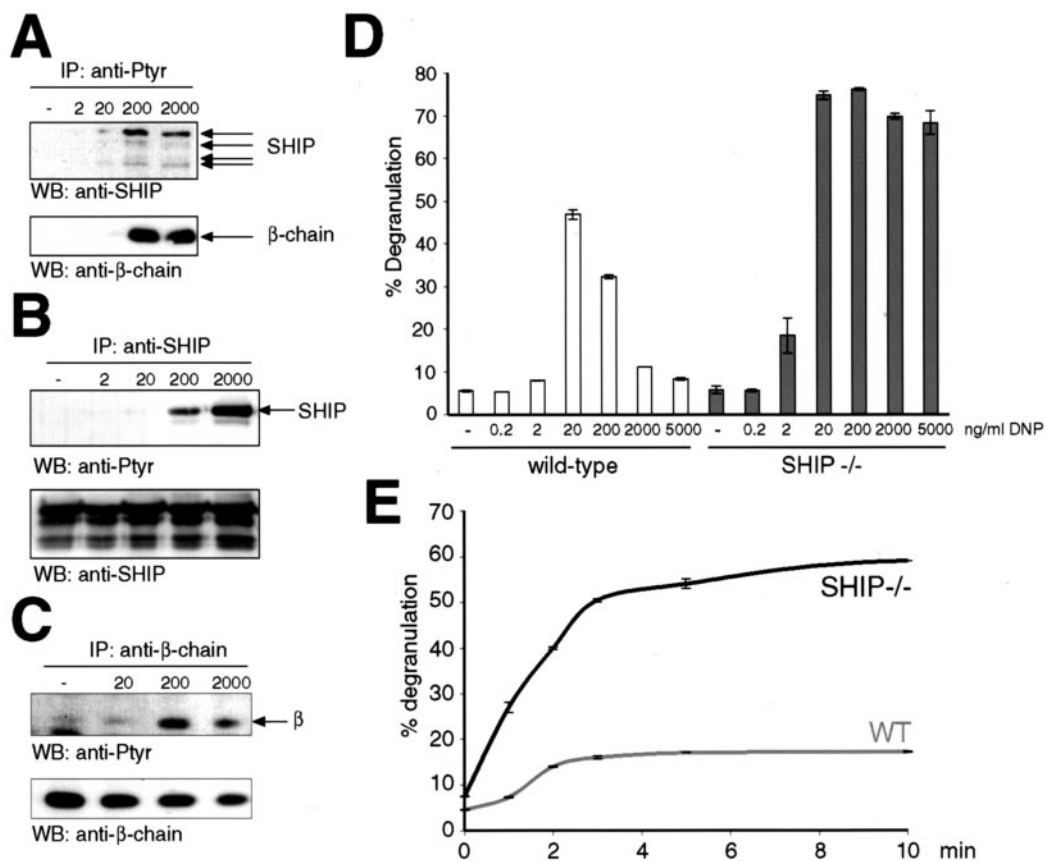


FIGURE 3. SHIP regulates the down-phase of the Ag-induced dose-response curve. (*A*) IgE-preloaded (0.5 μ g/ml) BMMCs were left untreated (–) or stimulated with 2, 20, 200, or 2000 ng/ml DNP-HSA for 1 min. Postnuclear supernatants were subjected to tyrosine phosphorylation (anti-Ptyr) immunoprecipitation. The precipitates were analyzed by anti-SHIP (*top*) and anti- β -chain (*bottom*) Western blotting. SHIP forms and the β -chain are indicated by arrows. *B*, Postnuclear supernatants obtained as in *A* were subjected to anti-SHIP immunoprecipitation with subsequent anti-phospho-tyrosine immunoblotting (*top*). Equal loading was verified by reprobating the membrane with anti-SHIP Abs (*bottom*). SHIP is marked by an arrow. *C*, Postnuclear supernatants obtained as in *A* were subjected to anti- β -chain immunoprecipitation with subsequent anti-phospho-tyrosine Western blotting (*top*). Equal loading was confirmed by reprobating the membrane with anti- β -chain Abs (*bottom*). The β -chain is marked by an arrow. *D*, IgE-preloaded (0.15 μ g/ml) WT and SHIP^{-/-} BMMCs were left unstimulated (–) or stimulated with 0.2, 2, 20, 200, 2000, or 5000 ng/ml DNP-HSA for 30 min and degranulation assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD. Comparable results were obtained in three separate experiments. Because SHIP^{-/-} BMMCs were already activated to a certain extent by preloading with 0.5 μ g/ml IgE (data not shown and Ref. 4), this and the following experiments were performed with 0.15 μ g/ml IgE only. *E*, IgE-preloaded WT and SHIP^{-/-} BMMCs were stimulated for increasing times (1, 2, 3, 5, and 10 min) with 2000 ng/ml DNP-HSA. Degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD.

and WT BMMCs in response to supraoptimal Ag concentrations reached plateau levels within the first 3 min (Fig. 3E; see also Fig. 1C), indicating the early nature of the SHIP-controlled signaling step.

Because the degree of degranulation correlated with the level of overall calcium mobilization in WT BMMCs, we compared overall calcium mobilization in WT and SHIP^{-/-} BMMCs, especially at high Ag concentrations. SHIP^{-/-} BMMCs showed a far higher overall calcium flux at optimal Ag concentrations (20 ng/ml) than WT BMMCs (Fig. 4, *upper panel*) (4). More importantly, even after stimulation with supraoptimal Ag levels (2000 ng/ml, Fig. 4, *middle panel*), and 5000 ng/ml (Fig. 4, *bottom panel*) SHIP^{-/-} BMMCs mobilized more calcium ions than optimally stimulated WT MCs. Particularly, when comparing the responses of WT and SHIP^{-/-} BMMCs at the highest supraoptimal Ag concentrations (2000 and 5000 ng/ml) the transient nature of overall calcium flux observed with WT cells was not seen with SHIP^{-/-} BMMCs (Fig. 4).

SHIP does not restrict early Btk activation at supraoptimal Ag concentrations

The tyrosine kinase Btk has been suggested to control sustained calcium mobilization in a phosphatidylinositol 3,4,5-trisphosphate (PIP₃)-dependent manner (19, 20). Thus, we sought to address the role of Btk in the observed dose-response behavior of FcεR1 signaling. For this purpose we used a phosphospecific Ab that recognizes Btk when autophosphorylated at Y223. We expected Ag-

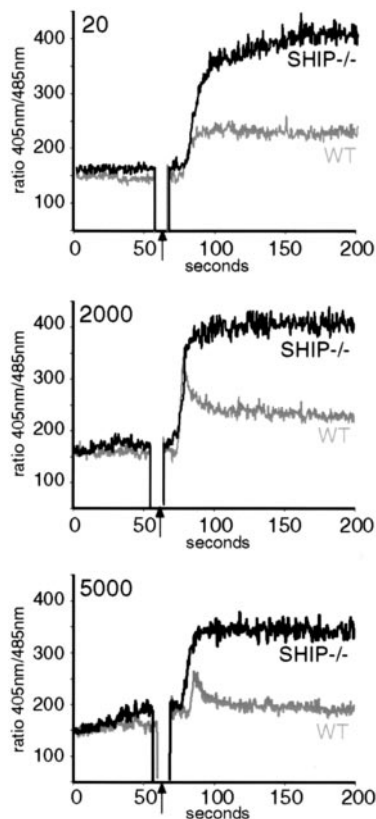


FIGURE 4. SHIP controls the transient nature of calcium mobilization at supraoptimal Ag concentrations. SHIP^{+/+} and SHIP^{-/-} BMMCs were preloaded with IgE (0.15 μ g/ml) and stimulated with 20 (*upper*), 2000 (*middle*), or 5000 ng/ml DNP-HSA (*lower*) at the time indicated by the arrows. Subsequently, cytosolic calcium levels were measured. Similar results were obtained in three separate experiments with different BMMC preparations.

triggered Btk autophosphorylation to be reduced in supraoptimally compared with optimally stimulated BMMCs. Furthermore, we expected a pronounced increase of Btk autophosphorylation in SHIP-deficient compared with WT BMMCs. Intriguingly, Btk autophosphorylation was enhanced under supraoptimal stimulation conditions, whereas PKB phosphorylation, an event strictly regulated by SHIP (21, 22), was not (Fig. 5A, *upper and middle panels*). Furthermore, SHIP deficiency did not result in a significant increase of Btk autophosphorylation over that seen in WT cells, whereas PKB phosphorylation was markedly augmented (Fig. 5A, *upper and middle panels*). Comparable loading was checked by anti-actin immunoblotting (Fig. 5A, *bottom panel*). To verify that autophosphorylation correlates with membrane translocation of Btk, cytosol-membrane fractionations were performed. Translocation of Btk to the membrane of Ag-stimulated BMMCs was stronger under supraoptimal compared with optimal stimulation conditions (Fig. 5B, *upper panel*), correlating with the observed autophosphorylation. Purity of membrane and cytosol fractions was confirmed by anti-FcεR1 β (Fig. 5B, *middle panel*) and anti-inhibitory protein I κ -B α immunoblotting (Fig. 5B, *bottom panel*), respectively.

Our data to this point suggested that Btk autophosphorylation showed an inverse correlation with PI3K pathway activation. PKC- β has been described to phosphorylate Btk at S180 in the TH domain, thereby suppressing its activation (23). Moreover, we have recently demonstrated enhanced PKC- β membrane recruitment in Ag-triggered SHIP^{-/-} compared with WT BMMCs (21). In accordance, translocation of PKC- β to the membrane was stronger under optimal rather than supraoptimal stimulation conditions (Fig. 5C). Finally, we sought to determine the effect of the PKC inhibitor bis-indolylmaleimide on Ag-triggered Btk autophosphorylation in WT and SHIP^{-/-} BMMCs. In each cell type and under every stimulation condition applied, Btk autophosphorylation was dramatically enhanced in the presence of bis-indolylmaleimide (Fig. 5D). This view was intensified by using less postnuclear supernatant than for the analysis shown in Fig. 5A. Preincubation of BMMCs with phorbol ester (PMA) had the opposite effect in that it reduced Ag-stimulated Btk autophosphorylation (data not shown). Moreover, in the situation of PKC inhibition, Btk autophosphorylation seemed to be slightly stronger in SHIP^{-/-} BMMCs (Fig. 5D). In conclusion, SHIP does not seem to negatively regulate Btk activation in a comparable straightforward manner as it does with PKB. Thus, the link between SHIP and Btk with respect to the regulation of early supraoptimal Ag stimulation appears questionable.

SHIP colocalizes with the actin cytoskeleton

The actin cytoskeleton has been implicated as a negative regulator of MC degranulation after stimulation with supraoptimal Ag concentrations (10). Because both SHIP and the actin cytoskeleton appeared to play an important role in the down-phase of the bell-shaped dose-response curve we finally investigated whether there is a physical interaction between SHIP and the microfilament system in BMMCs. Because previous degranulation studies involving actin depolymerizing drugs (like latrunculin B (LatB)) have been conducted primarily with the RBL-2H3 cell line, we first verified that preincubation of primary BMMCs with LatB (10 μ M) increased degranulation after stimulation with 20–2000 ng/ml Ag. This was indeed the case and although LatB increased Ag-induced degranulation at every Ag concentration used, the increase (with respect to percentage) was most pronounced at the highest Ag dose tested (2000 ng/ml) (Fig. 6A). Conversely, LatB treatment decreased Ag-triggered tyrosine phosphorylation of SHIP (Fig. 6B),

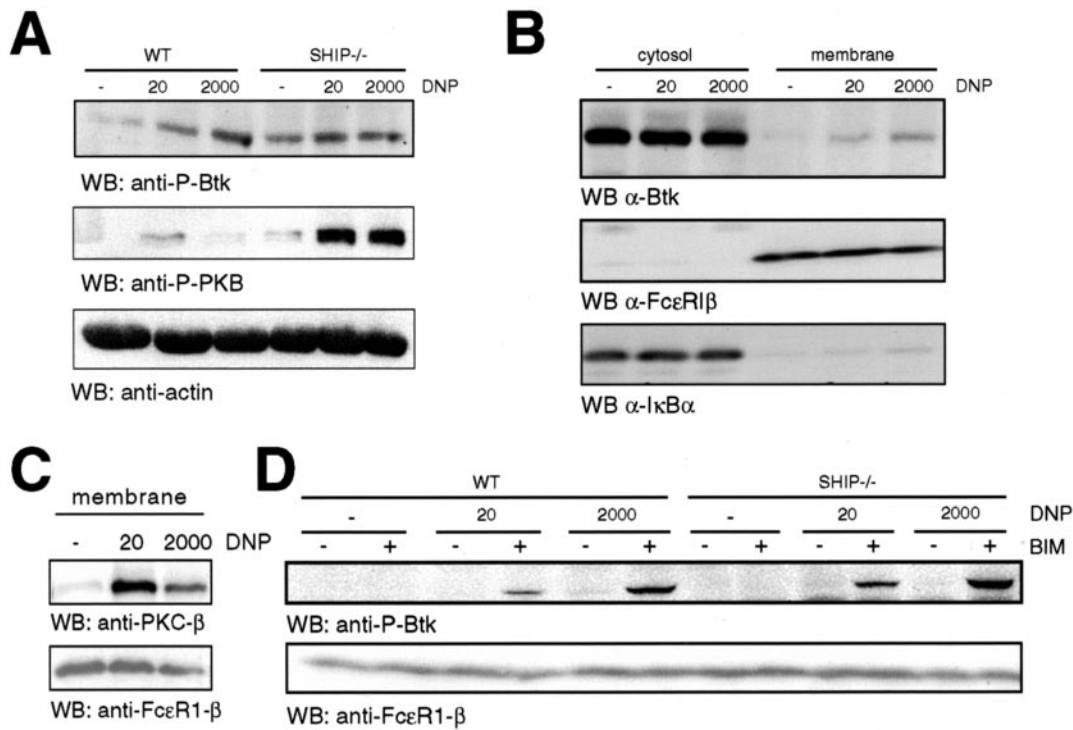


FIGURE 5. SHIP is not restricting Btk activation in BMMCs. *A*, IgE-preloaded (0.15 μ g/ml) WT and SHIP^{-/-} BMMCs were left unstimulated (–) or stimulated with 20 or 2000 ng/ml DNP-HSA for 1 min. Postnuclear supernatants were analyzed for P-Btk (Y223) (upper), P-PKB (middle), and actin (lower) expression. *B*, WT BMMCs were preloaded with IgE (0.5 μ g/ml) and stimulated as described for *A*. Subcellular fractionations into cytosol and membrane fractions were performed and the obtained fractions were analyzed by anti-Btk (upper), anti-FcεR1β (middle), and anti-IκBα (lower) immunoblotting. *C*, WT BMMCs were treated as described in *B* and membrane fractions were analyzed by anti-PKC-β (top) and anti-FcεR1β immunoblotting (bottom). *D*, IgE-preloaded WT and SHIP^{-/-} BMMCs were either treated with vehicle (DMSO) or 5 μ M bis-indolylmaleimide (BIM) for 20 min. Cells were then left untreated or stimulated with 20 or 2000 ng/ml DNP-HSA for 1 min. Postnuclear supernatants were analyzed by anti-P-Btk (top) and anti-FcεR1β Western blotting (bottom).

suggesting functional interaction between SHIP and the actin cytoskeleton. This was corroborated by our finding that actin coprecipitated with anti-SHIP Abs (Fig. 6*B*, bottom panel). Furthermore, by performing subcellular fractionations of BMMCs, we observed a significant portion of SHIP within the submembrane cytoskeleton (SMC) independent of the activation state of the cells (data not shown). However, because we were not able to find an actin-specific Ab capable of immunoprecipitating substantial amounts of actin itself, we used the technique of confocal microscopy, to learn whether SHIP colocalizes with F-actin in BMMCs. Resting as well as activated BMMCs, stained for F-actin with Alexa Fluor 546 phalloidin, exhibited prominent rings of cortical actin (Fig. 6*C*, upper right panel). Staining with a monoclonal anti-SHIP Ab (P1C1) gave a different pattern. In addition to peripheral submembrane areas, cytoplasmic staining was observed in unstimulated cells as well as stimulated cells with no detection of SHIP in the nucleus (Fig. 6*C*, upper left panel). No staining was observed in the absence of the primary Ab as well as in SHIP^{-/-} BMMCs (data not shown). Merging the F-actin and SHIP images clearly demonstrated colocalization of SHIP with F-actin in peripheral submembrane areas (Fig. 6*C*, lower left panel in yellow). In comparison to unstimulated and supraoptimally stimulated cells, ~5–10% of the optimally stimulated cells displayed a dramatic shape change with the formation of various protrusions and lamellipodia within 10 min (Fig. 6*D*). The percentage increased to nearly 50% after 30 min of stimulation. Strong colocalization of SHIP and F-actin was observed in these cells (Fig. 6*D*).

Discussion

The engagement of the IgE-bound FcεR1 by multivalent Ag results in a multitude of signaling events and biological responses. Dose-response analyses reveal bell-shaped dose-response curves for Ag-triggered degranulation (controlled by the amount of Ag as well as the amount of IgE) (Figs. 1 and 2). The reduced response at high, supraoptimal Ag concentrations seems to be due to an excess of FcεR1 cross-linking and an increased involvement of the actin microfilament system (7, 9, 10). Reduced responses after preloading the cells with supraoptimal IgE concentrations might be due to the fact that cytokinergic IgEs (e.g., IgE α -DNP; clone SPE-7) are able to precluster FcεR1 and thus lower the threshold for Ag-mediated FcεR1 aggregation (24, 25). One could envision that an excess of cross-linking not only reduces the biological response, like degranulation, but also the activation of so-called positive signaling pathways leading to the respective response. This appears to be true for overall calcium mobilization (Figs. 1 and 2), which follows the bell-shaped dose-response curve. Another possibility is that negative regulatory molecules might only be weakly activated under optimal stimulation conditions but are activated/recruited in a more pronounced fashion when excessive FcεR1 cross-linking is caused by supraoptimal Ag or IgE levels, thereby down-regulating the biological responses. In this respect, tyrosine phosphorylation of SHIP, a major gatekeeper of MC degranulation (4), was found to be far greater at supraoptimal than optimal Ag concentrations (Fig. 3). This was corroborated by our finding that SHIP^{-/-} BMMCs exerted stronger degranulation and calcium mobilization at supraoptimal Ag concentrations than WT BMMCs at

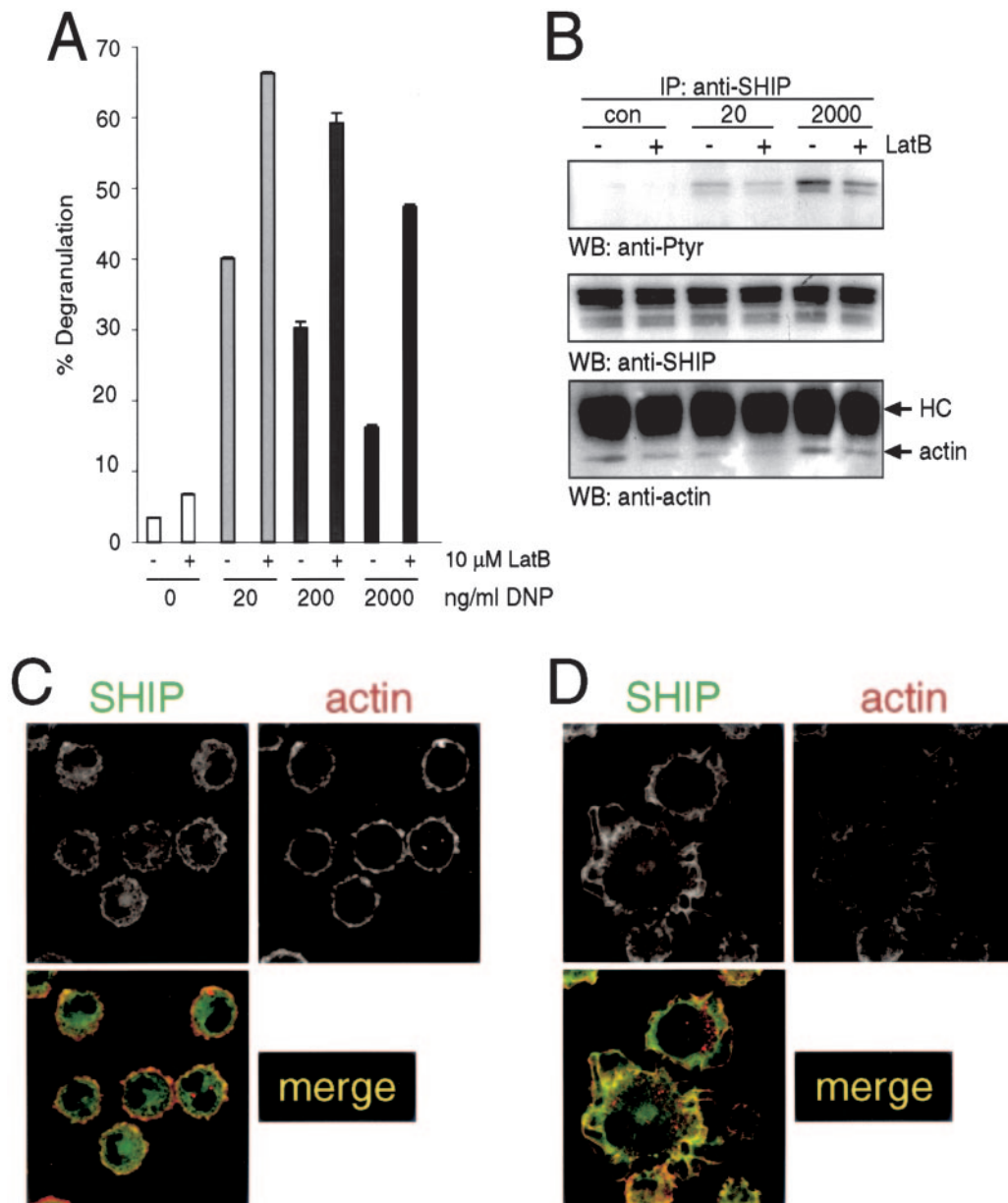


FIGURE 6. SHIP colocalizes with the actin cytoskeleton. *A*, IgE-preloaded BMMCs were treated for 15 min with vehicle (–) or 10 μ M LatB (+) and were subsequently left unstimulated or stimulated with increasing concentrations of Ag (20, 200, and 2000 ng/ml DNP-HSA (DNP)) for 30 min. Degranulation was assessed by β -hexosaminidase assays. Each bar is the mean of duplicates $\pm$ SD. *B*, IgE-preloaded BMMCs were incubated for 15 min with vehicle (–) or 10 μ M LatB (+) and subsequently left unstimulated or stimulated with Ag (20 and 2000 ng/ml DNP-HSA) for 1 min. Postnuclear supernatants were subjected to immunoprecipitation with anti-SHIP Abs and precipitates were analyzed by anti-phospho-tyrosine (*upper*), anti-SHIP (*middle*), and anti-actin Western blotting (*bottom*). IgE-preloaded BMMCs were left unstimulated (*C*) or were stimulated (*D*) for 10 min with 20 ng/ml Ag. The cells were fixed and stained with monoclonal anti-SHIP Ab followed by Alexa Fluor 488 goat anti-mouse IgG (*top left*; green) and Alexa Fluor 546 phalloidin (*top right*; red). The respective overlay (merge) are shown in the *bottom* panels in yellow.

optimal Ag levels (Figs. 3 and 4). Related to this, Oliver and coworkers (26) found a nonparallel relationship between the overall level of tyrosine phosphorylation and degranulation, using anti-phospho-tyrosine flow cytometry.

Because we saw a slight decrease in degranulation and calcium mobilization at supraoptimal Ag concentrations in the SHIP^{–/–} BMMCs in some experiments (data not shown and Fig. 4), another PIP₃ phosphatase might be weakly activated in BMMCs under these conditions. Consistent with this notion we have detected low levels of SHIP-2 and PTEN (phosphatase and tensin homologue deleted on chromosome 10) in both WT and SHIP^{–/–} BMMCs (22, 27). Recently, BMMCs deficient for the Src family kinase Lyn

have been demonstrated to be hyperresponsive to stimulation with Ag, resulting in a complete loss of the bell-shaped dose-response curve for degranulation at supraoptimal Ag concentrations (28). Interestingly, Lyn is capable of phosphorylating SHIP (28) as well as PKC- δ (29), for which we have previously shown negative regulatory potential at supraoptimal Ag concentrations (29). This suggests that SHIP-independent, Lyn-controlled negative signaling elements might contribute to the observed decrease in degranulation and calcium mobilization at supraoptimal Ag concentrations in SHIP^{–/–} BMMCs. Furthermore, the inhibitory region of the Ag dose-response curve might also include the activation of one or more protein tyrosine phosphatases. However, these phosphatases

do not appear to act on SHIP or the Fc ϵ R1 β -chain because these two proteins are more strongly phosphorylated under supraoptimal conditions (Fig. 3).

Intriguingly, tyrosine phosphorylation of the Fc ϵ R1 β -chain, which has been shown to be the amplifier subunit of the Fc ϵ R1 (30), was found to follow the more linear dose-response curve observed for SHIP and was strongest at supraoptimal Ag doses (Fig. 3), which is in line with a recent report by Draberova et al. (31). The β -chain may also directly recruit SHIP after Fc ϵ R1 engagement, thus playing a part in the negative regulation of MC responses (32, 33). Unfortunately, the β -chain-SHIP interaction has only been demonstrated by using a yeast-tribrid system (32) or by pull-down assays using phosphorylated β -ITAM peptides (33) and we have not been able to demonstrate a direct *in vivo* association in coimmunoprecipitation studies so far (data not shown). It is conceivable that the low level of tyrosine phosphorylation of the Fc ϵ R1 β -chain that occurs at optimal Ag concentrations recruits mostly positive regulators of MC activation, whereas pronounced β -chain phosphorylation under supraoptimal conditions preferentially attracts SHIP (either directly or indirectly), which itself is most strongly tyrosine phosphorylated under these conditions, most likely by Lyn (28).

Because PLC- γ activation is known to be up-stream of IP $_3$ production, intracellular calcium release, and thus extracellular calcium influx (17), we were also intrigued by the finding that Ag-triggered activation/phosphorylation of PLC- γ 1 was strongest under supraoptimal conditions in which overall calcium mobilization was reduced and transient (Fig. 1). This enhanced activation of PLC- γ 1 correlated with augmented Btk phosphorylation (Fig. 5) and translated into a stronger release of intracellular calcium ions (Figs. 1 and 2). It has been previously demonstrated in RBL-2H3 cells that the phorbol ester PMA accelerated the inactivation of the calcium release-activated calcium (I $_{CRAC}$) channel, whereas PKC inhibitors reduced inactivation of the I $_{CRAC}$ channel (34). We verified these effects of PMA and PKC inhibitors on Ag-mediated calcium mobilization in BMMCs (data not shown). Because activated PLC- γ not only provides IP $_3$ but also the classical and novel PKC activator diacylglycerol (17), PLC- γ at supraoptimal Ag concentrations might be involved in the reduction of extracellular calcium influx via activated PKC isotypes. Coincidentally, the novel PKC isotype PKC- δ has been demonstrated to be a negative regulator of Ag-mediated calcium mobilization and degranulation under supraoptimal conditions (29).

Btk activation has been suggested to be important for the induction of sustained calcium signals. It has been shown that Btk contributes to the activation of PLC- γ , thereby enabling sustained IP $_3$ production, which balances store refilling and thus promotes sustained influx via I $_{CRAC}$ channels (19, 20, 35). In this line, Btk-deficient BMMCs have a reduction in maximal calcium mobilization and degranulation (36, 37). These data suggested to us that under supraoptimal Ag conditions, Btk is negatively controlled by SHIP and thus might be responsible for the shift to transient calcium mobilization and suppressed degranulation. Our data from Ag-stimulated WT and SHIP $^{-/-}$ BMMCs (Fig. 5), however, demonstrate that at least at early time points relevant for the control of the degranulation reaction, Btk is not regulated in a direct SHIP-dependent fashion (in strong contrast to PKB). Interestingly, Setoguchi et al. (38) reported, using electron microscopy, that Btk $^{-/-}$ BMMCs display abnormal secretory granules with translucent contents suggesting a role for Btk in the development of BMMCs or maturation of secretory granules. This of course raises the question of whether impaired degranulation in Btk $^{-/-}$ BMMCs represents a primary or secondary effect. In addition to the control of Btk activity and membrane translocation by the SHIP

substrate PIP $_3$ via the Btk PH domain (39), PKC- β has been shown to negatively regulate Btk via phosphorylation of Ser ^{180} within the Tec homology domain of Btk (23). Because we demonstrated previously that SHIP restricts PKC- β membrane translocation after Fc ϵ R1 triggering (21) we suggest a model in which SHIP controls Btk in a dual mode: on the one hand negatively by hydrolyzing PIP $_3$ and interfering with Btk membrane translocation, conversely, positively (via double-negative feedback regulation) by hydrolyzing PIP $_3$ and thus negatively regulating PKC- β . From this scenario, it is evident that the final regulation of Btk will depend on the ratio of expression of the different players (Btk, SHIP, PI3K, and PKC- β) and cannot be mimicked by viral reconstitution systems omitting one of the players. The PKC- β path also suggests that there might be a negative role for PI3K in the activation of Btk. Indeed, this has been demonstrated recently by Suzuki et al. (40). BCR stimulation of B cells from WT and p85 $^{PI3K-/-}$ mice revealed that Btk phosphorylation was stronger in the mutant cells. Furthermore, Btk phosphorylation was not blocked by the PI3K inhibitor wortmannin in WT cells (40). In conclusion, regulation of Btk by SHIP does not seem to play a role in the suppression observed in BMMCs at supraoptimal Ag concentrations.

MC activation is tightly coupled to rearrangements of cytoskeletal components. Microtubule-disrupting drugs, for instance, have been demonstrated to inhibit degranulation of MCs (41). In contrast, inhibitors that disrupt actin microfilaments do not cause significant degranulation on their own, but they do enhance Fc ϵ R1-mediated degranulation of RBL-2H3 cells and BMMCs (Fig. 6A) (42). MCs and other hemopoietic cells contain a cage-like SMC and after subcellular fractionation of resting and stimulated BMMCs, we found that SHIP was present in the Triton X-100 soluble cytosolic fraction as well as in the SMC fraction (data not shown). Moreover, we could demonstrate that SHIP and actin can be copurified and that SHIP and F-actin colocalize particularly in the submembranal area of unstimulated as well as stimulated BMMCs (Fig. 6).

An interaction of tyrosine-phosphorylated SHIP with the actin cytoskeleton has been suggested in thrombin-stimulated human platelets (43). As well, the SHIP-related protein, SHIP-2, has been found to bind to actin-binding proteins via its proline-rich C terminus and this interaction may be responsible for SHIP-2 localizing to the actin cytoskeleton and regulating PIP $_3$ levels (44). Coincidentally, the proline-rich tail of SHIP has been shown to be important for SHIP's attenuating action on MC degranulation (45) and thus might be involved in SHIP interacting with actin or actin-binding proteins in MCs. Currently, this issue is under investigation.

Finally, one can envision a model in which SHIP is localized to the SMC via its proline-rich C terminus in unstimulated as well as optimally stimulated BMMCs. However, it is not interacting with the Fc ϵ R1 in a pronounced fashion due to the latter not being recruited to F-actin-rich regions and its β -chain not being heavily tyrosine-phosphorylated. In the case of supraoptimal Fc ϵ R1 engagement, the Fc ϵ R1 β -chain is strongly phosphorylated on ITAM tyrosines enabling the SHIP Src homology 2-domain to bind and form a ternary complex with F-actin, and the Fc ϵ R1 β -chain. Within this ternary complex, SHIP might change its conformation, thus exposing its 5-phosphatase domain to the inner leaflet of the plasma membrane and hydrolyzing its substrate, PIP $_3$. Related to this, it is worthy of note that during the process of heterologous desensitization of the Fc ϵ R1, receptor aggregates that cannot be uncoupled by monovalent Ag and most likely are located within the Triton-insoluble fraction seem to constitute the "memory" for the desensitizing signal (46). Our data suggest that SHIP could be part of this memory system. Also worthy of note is a recent paper by Rivera and coworkers (47) addressing the IgE- and Ag-concentration dependence of lymphokine secretion by BMMCs. In

this interesting report they provide evidence that secretion of lymphokines that promote allergic inflammation is potentially induced at low Ag concentrations or at low receptor occupancy with IgE, whereas some lymphokines that down-regulate this response require high receptor occupancy (47). This paper also permits the conclusion that perturbations in the regulation of intracellular signaling pathways, in particular the PI3K-SHIP pathway, might have a dramatic impact on the course of an allergic reaction even when Ag and/or IgE levels are within an otherwise inhibitory concentration range.

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