Sustained Activation of the Tyrosine Kinase Syk by Antigen in Mast Cells Requires Local Ca$^{2+}$ Influx through Ca$^{2+}$ Release-activated Ca$^{2+}$ Channels*

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Siaw Wei Ng,$¹$ Joseph di Capite$²$, Karthika Singaravelu, and Anant B. Parekh$³$

From the Department of Physiology, Anatomy, and Genetics, University of Oxford, Sherrington Bldg., Parks Road, Oxford OX1 3PT, United Kingdom

Mast cell activation involves cross-linking of IgE receptors followed by phosphorylation of the non-receptor tyrosine kinase Syk. This results in activation of the plasma membrane-bound enzyme phospholipase Cγ1, which hydrolyzes the minor membrane phospholipid phosphatidylinositol 4,5-biphosphate to generate diacylglycerol and inositol trisphosphate. Inositol trisphosphate raises cytoplasmic Ca$^{2+}$ concentration by releasing Ca$^{2+}$ from intracellular stores. This Ca$^{2+}$ release phase is accompanied by sustained Ca$^{2+}$ influx through store-operated Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels. Here, we find that engagement of IgE receptors activates Syk, and this leads to Ca$^{2+}$ release from stores followed by Ca$^{2+}$ influx. The Ca$^{2+}$ influx phase then sustains Syk activity. The Ca$^{2+}$ influx pathway activated by these receptors was identified as the CRAC channel, because pharmacological block of the channels with either a low concentration of Gd$^{3+}$ or exposure to the novel CRAC channel blocker 3-fluoropyridine-4-carboxylic acid (2′,5′-dimethoxybiphenyl-4-yl)amide or RNA interference knockdown of Orai1, which encodes the CRAC channel pore, all prevented the increase in Syk activity triggered by Ca$^{2+}$ entry. CRAC channels and Syk are spatially close together, because increasing cytoplasmic Ca$^{2+}$ buffering with the fast Ca$^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N″-tetraacetic acid tetakis failed to prevent activation of Syk by Ca$^{2+}$ entry. Our results reveal a positive feedback step in mast cell activation where receptor-triggered Syk activation and subsequent Ca$^{2+}$ release opens CRAC channels, and the ensuing local Ca$^{2+}$ entry then maintains Syk activity. Ca$^{2+}$ entry through CRAC channels therefore provides a means whereby the Ca$^{2+}$ and tyrosine kinase signaling pathways can interact with one another.

The cell surface IgE receptor, FcεRI, recognizes the Fc region of antigen-specific IgE molecules. Cross-linking of FcεRI receptors with antigen is a critical early step in mast cell activation (1, 2). FcεRI receptors couple to a cascade of protein kinases, among which the non-receptor tyrosine kinase Syk plays a pivotal role in mast cell stimulation (3, 4). Syk binds to tyrosine-phosphorylated residues in the immunoreceptor tyrosine-based activation motif of the FcεRI β and γ chains via its two SH2 domains, increasing enzyme activity (5–7). Syk-null cells fail to degranulate following exposure to antigen and antisense oligonucleotides directed against Syk reduce antigen-driven responses in an in vivo rat model of asthma (8). In the RBL mast cell line, inhibition of Syk suppresses degranulation (9) as well as production of the pro-inflammatory cytokine leukotrienes (10).

Activated Syk stimulates phospholipase Cγ1 in RBL cells (11), which hydrolyzes the phospholipid phosphatidylinositol 4,5-biphosphate to generate inositol trisphosphate (InsP$_3$) and diacylglycerol (12). InsP$_3$ binds to tetrameric InsP$_3$-gated Ca$^{2+}$ channels in the endoplasmic reticulum, releasing stored Ca$^{2+}$ into the cytosol. The ensuing store depletion then activates store-operated CRAC channels in the plasma membrane (13), which provide a substantial portion of Ca$^{2+}$ needed to activate mast cells. Genome-wide RNAi knockdown strategies have identified two key molecular components of the store-operated Ca$^{2+}$ entry pathway, STIM1 (14, 15) and Orai1 (16–18). STIM1, a protein that spans the endoplasmic reticulum, is the Ca$^{2+}$ sensor that detects the fall in Ca$^{2+}$ content within the store. It migrates from a relatively homogeneous distribution throughout the endoplasmic reticulum to discrete puncta within 25 nm of the plasma membrane, lining up opposite the plasma membrane protein Orai1 (19). Site-directed mutagenesis has established that Orai1 is all or part of the CRAC channel pore (20–22). The importance of STIM1 and Orai1 in mast cell function is underscored by the findings that degranulation, secretion of pro-inflammatory cytokine leukotrienes and chemokines, as well as the ability to mount an inflammatory response are all severely compromised in mice in which these genes have been ablated (23, 24).

Activation of immune cells often requires sustained Ca$^{2+}$ entry through CRAC channels (25). This in turn is dependent on a maintained elevation in InsP$_3$ levels (and thus phospholipase Cγ activity), which is needed to ensure the stores are depleted sufficiently for CRAC channels to remain open.

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$²$Held a Christopher Welch Scholarship.

$³$To whom correspondence should be addressed. Tel.: 44-1865-272439; Fax: 44-1865-272488; E-mail: anant.parekh@dpag.ox.ac.uk.

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4 The abbreviations used are: InsP$_3$, phosphatidylinositol 3-trisphosphate; CRAC, Ca$^{2+}$ release-activated Ca$^{2+}$; RNAi, RNA interference; PBS, phospho-buffered saline; TBS, Tris-buffered saline; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; eYFP, enhanced yellow fluorescent protein; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N″-tetraacetic acid.
Because phospholipase Cγ can be activated by Syk, we have examined whether the subsequent Ca²⁺ influx can feed back to maintain Syk activation. Our findings reveal a novel self-regenerative process whereby local Ca²⁺ influx through CRAC channels increases Syk activity, which in turn sustains CRAC channel activity by preventing store refilling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—RBL-1 cells were bought from ATCC. Cells were cultured (37 °C, 5% CO₂) in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM l-glutamine, and penicillin-streptomycin, as described previously (26). For Ca²⁺ imaging and patch clamp experiments, cells were passaged (using trypsin) onto glass coverslips and used 24–48 h after plating. Cells were sensitized to antigen (dinitrophenyl-bovine serum albumin, 80 μg/ml) by incubation in IgE (2.5 μg/ml) overnight in standard culture medium.

**Ca²⁺ Imaging**—Ca²⁺ imaging experiments were carried out using the IMAGO charge-coupled device camera-based system from TILL Photonics, as described previously (10). Cells were alternately excited at 356 and 380 nm (20-ms exposures, 0.5 Hz) using a Polychrome Monochromator. Images were analyzed offline using IGOR Pro. Cells were loaded with Fura-2-AM (2 μM) for 40 min at room temperature in the dark and then washed in standard external solution of composition (in mM) NaCl 145, KCl 2.8, CaCl₂ 2, MgCl₂ 2, d-glucose 10, HEPES 10, pH 7.4, with NaOH. Ca²⁺ signals are presented as the ratio 356/380.

**BAPTA-AM Loading**—Cells were incubated in BAPTA-AM (30 μM) for 45 min at room temperature as previously described (10).

**CRAC Channel Blocker**—The CRAC channel blocker used in this study, which we call Synta compound, was kindly provided by Dr. Valerie Morisset at GlaxoSmithKline, UK. The Synta compound is compound 66 from the WO2005/009954 A2 patent (3-fluoropyridine-4-carboxylic acid (2',5'-dimethoxybiphenyl-4-yl)amide (STRUCTURE 1)). The inhibitor was made up as a stock solution of 10 mM in DMSO.

**Patch Clamp Recordings**—Whole cell patch clamp recordings were carried out as described (28). Sylgard-coated, fire-polished patch pipettes were filled with a solution that contained 145 mM cesium glutamate, 8 mM NaCl, 1 mM MgCl₂, 2 mM Mg-ATP, 10 mM HEPES, 10 mM EGTA, 30 μM InsP₃, pH 7.2, with CsOH. Pipette resistance was ~5 megohms when placed in an external solution containing 145 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 10 mM CaCl₂, 2 mM MgCl₂, 10 mM d-glucose, 10 mM HEPES, pH 7.4, with NaOH. The inwardly rectifying K⁺ current was measured with a pipette solution containing 145 mM potassium glutamate, 8 mM NaCl, 1 mM MgCl₂, 2 mM Mg-ATP, 10 mM HEPES, 0.1 mM EGTA, pH 7.2, with KOH. Bath solution for measuring the K⁺ current contained 108 mM NaCl, 50 mM KCl, 10 mM CaCl₂, 2 mM MgCl₂, 10 mM d-glucose, 10 mM HEPES, pH 7.4, with NaOH. A correction of +10 mV was applied for the subsequent liquid junction potential that arose from the glutamate-based pipette solutions. Ca²⁺ current through CRAC channels and K⁺ current through inwardly rectifying K⁺ channels were followed by applying voltage ramps (at 0.5 Hz) spanning −100 to +100 mV in 50 ms from a holding potential of 0 mV. Current amplitudes were measured from the ramps at −80 mV and normalized to cell size by dividing the amplitude by cell capacitance. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 μs.

**Preparation of Cell Lysates**—Attached cells from 6-cm plastic dishes were washed twice with PBS and lysed with PBS buffer containing 0.5% Triton X-100 and protease mixture inhibitor (Sigma), as described (10). Lysates were centrifuged at 8000 rpm for 5 min, and the supernatants were collected and stored at −80 °C until used. Protein concentrations were determined by Bio-Rad DC protein assay.

**Western Blotting**—Total cell lysates (40–50 μg) were separated by SDS-PAGE on a 10% gel and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin in TBS plus 0.1% Tween 20 (TBST) or 5% nonfat dry milk in PBS plus 0.1% Tween 20 (PBST) buffer for 2 h at room temperature. Membranes were washed with TBST/PBST three times and then incubated with primary antibody overnight at 4 °C or for 1 h at room temperature. Anti-phospho-Syk antibody was from New England Biolabs and used at a 1:2500 dilution. Total ERK2 antibody was from Santa Cruz Biotechnology and used at a dilution of 1:5000. The membranes were then washed with TBST/PBST again and incubated with a 1:2500–5000 dilution of goat anti-rabbit secondary antibody IgG from Santa Cruz Biotechnology and used at a 1:2500 dilution. Total ERK2 antibody was then washed with TBST/PBST and bands were developed for visualization using ECL-plus Western blotting detection system (GE Healthcare).

Gels were quantified using the UN-SCAN-IT software package (Silk Scientific). Total ERK2 is widely used as a control for gel loading (29–31). The antibody does not discriminate between phosphorylated (and hence active) and non-phosphorylated ERK2 and therefore detects the total amount of this protein, regardless of whether the kinase has been activated. The extent of Syk phosphorylation was therefore normalized to the total amount of ERK2 present in each lysate, to correct for differences in amount of cells used for each condition (10).

**Transfection and RNAi**—Cells were transfected with RNAi directed against ORAI1 using the Amaxa system, as previously described (27). All siRNA sequences were designed by using Invitrogen block-it software. The sequence for Orail (5’ to 3’) was GUCCACAAACCUCACUCCCTT and was from Invitrogen. Control cells were transfected under identical conditions.
with scrambled siRNA (Invitrogen) or enhance green fluorescent protein.

**Reverse Transcriptase-PCR**—Total RNA was extracted from RBL cells by using an RNeasy Mini Kit (Qiagen). RNA was quantitated spectrophotometrically by absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer’s instructions. Following cDNA synthesis, PCR amplification was then performed using BIO-X-ACT™ Short DNA Polymerase (Bioline) with primers specific for rat oral-1 (sense, 5′-AGTCCCTACCTCCCACCTGG-3′, and antisense, 5′-GCCTTCTCCTCCACCTCG-3′, with an expected product size of 133 bp, 32 cycles) and Beta Actin (sense, 5′-TTGTACACACTGGGACGATAGT-3′, and antisense, 5′-GATCTGGATCTTCATGTTCTAGG-3′, with expected product size of 764 bp, 28 cycles). The PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining.

**Statistical Analysis**—Results are presented as means ± S.E. Statistical significance was assessed using Student’s t test and considered significant at *p* < 0.05 (*) or *p* < 0.01 (**) respectively.

**RESULTS**

**Antigen-evoked Responses Depend on Functional Syk**—Phosphorylation and thus activation of Syk is a central event following FcεRI receptor engagement (4). By regulating phospholipase Cγ, Syk drives the generation of cytoplasmic Ca2+ signals in response to antigen stimulation. Consistent with this, pre-treatment for 10 min with the Syk inhibitor 3-(1-methyl-1H-indoyl-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide prevented the generation of the antigen-evoked cytoplasmic Ca2+ rise in IgE-sensitized RBL cells (Fig. 1A). Acute application of the Syk inhibitor after the antigen-activated Ca2+ signal had developed also resulted in suppression of the Ca2+ signal (Fig. 1B). Hence Syk activity is critical for both the generation as well as subsequent maintenance of the Ca2+ signal following activation of FcεRI receptors.

**Ca2+ Influx Enhances Syk Activity**—The non-receptor tyrosine kinase Syk is activated following phosphorylation of tyrosine residues within the activation loop and on tyrosine 342 (32, 33). We monitored Syk activity by using a monoclonal antibody that specifically recognizes phosphorylated tyrosine residues in the active loop. Stimulation with antigen for 4 min in the presence of external Ca2+ resulted in robust Syk phosphorylation (upper panel of Fig. 1C; lower panel depicts total (combination of active and non-active) ERK2, which is taken as a control for gel loading; aggregate data from four independent experiments is summarized in Fig. 1D). However, when cells were stimulated with antigen for the same time but now in the absence of external Ca2+, significantly less (~2-fold) Syk phosphorylation occurred (Fig. 1, C and D). Because Western blotting involves a large population of cells, we were concerned that fewer cells in the population might respond to antigen in Ca2+-free solution than in the presence of Ca2+, thus accounting for the difference in Syk activation. However, the number of cells responding to either condition was similar (data not shown; see also Fig. 3).

We considered the possibility that Syk phosphorylation induced by antigen in the absence of external Ca2+ involved release of Ca2+ from internal stores. We exposed cells to thapsigargin (2 μM) in Ca2+-free solution to deplete the stores and then applied antigen, still in the absence of external Ca2+. Antibody now failed to trigger a Ca2+ response (data not shown). Ca2+ release to thapsigargin failed to activate Syk (data not shown and see also Fig. 6) but application of antigen after thapsigargin still resulted in Syk phosphorylation and to an extent similar to that seen when cells were stimulated with antigen in Ca2+-free solution but in the absence of thapsigargin (Fig. 1E). Hence the activation of Syk in the absence of external Ca2+ is independent of Ca2+ release from the stores.

In mast cells, Ca2+ influx can activate protein kinase C and β II thereby recruiting the MEK/ERK pathway (10). To see whether Syk activation was secondary to this signaling cascade, we directly stimulated protein kinase C with the phorbol ester phorbol 12-myristate 13-acetate. No Syk phosphorylation was observed, suggesting that activation of Syk in Ca2+-free solution is independent of Ca2+ release.
Store-operated Ca\(^{2+}\) Influx Sustains Syk Activity

**FIGURE 3. Pharmacological block of CRAC channels reduced antigen-evoked Syk phosphorylation.** A, exposure to antigen in the presence of 2 \(\mu\)M Gd\(^{3+}\) prevented Ca\(^{2+}\) influx-dependent Syk phosphorylation. The upper gel compares the extent of Syk phosphorylation in 2 mM Ca\(^{2+}\) and then 2 mM Ca\(^{2+}\) and Gd\(^{3+}\); lower gel, total ERK2 levels. B, the CRAC channel blocker Synta compound (10 \(\mu\)M) inhibited store-operated Ca\(^{2+}\) entry. In these experiments, cells were loaded with Fura-2 and pre-treated for 10 min with thapsigargin (2 \(\mu\)M) in Ca\(^{2+}\)-free solution. Ca\(^{2+}\) was readdmitted as indicated. C, the rate of rise of the Ca\(^{2+}\) signal from experiments as in panel B are summarized. Each bar represents >80 cells. D, pre-treatment with 10 \(\mu\)M Synta compound suppresses development of \(I_{\text{CRAC}}\). E, current-voltage relationships from the recordings in panel D (100 s) are shown. F, the relation between \(I_{\text{CRAC}}\) and Synta compound concentration is shown. G, 10 \(\mu\)M Synta compound inhibited antigen-evoked Ca\(^{2+}\) influx in Fura-2-loaded cells (>60 cells per point). H, antigen-triggered phosphorylation of Syk was reduced by 10 \(\mu\)M Synta compound. I, aggregate data from four experiments, as in H, is summarized. In \(H\) and \(I\), antigen (10 \(\mu\)g/ml) was applied in 2 mM Ca\(^{2+}\).
Store-operated Ca\(^{2+}\) Influx Sustains Syk Activity

![Graph](image)

FIGURE 4. The Synta compound does not impair other plasma membrane ion transporters. A, current-voltage relationships for inwardly rectifying K\(^{+}\) current are shown for a control cell and for one exposed to 10 \(\mu\)M Synta compound for 10 min. B, aggregate data for control cells (n = 5) and for those exposed to the Synta compound (n = 6) are compared. Current amplitudes were measured from voltage ramps at −80 mV. C, plasma membrane Ca\(^{2+}\) ATPase activity was monitored in control cells and in those exposed to the Synta compound by measuring the rate of decline of the cytoplasmic Ca\(^{2+}\) following stimulation with thapsigargin in Ca\(^{2+}\)-free solution (with 0.1 mM EGTA). D, aggregate data from experiments as in panel C are compared. For both conditions, number of cells was >60.

ence of 2 mM Ca\(^{2+}\)) in the absence and then presence of 2 \(\mu\)M Gd\(^{3+}\). Antigen-triggered Syk phosphorylation was significantly reduced in the presence of Gd\(^{3+}\) (Fig. 3A), and the extent of the remaining Syk phosphorylation was similar to that seen when cells were stimulated with antigen in Ca\(^{2+}\)-free solution (Fig. 1).

We extended the pharmacological strategy by using a novel CRAC channel blocker, called the Synta compound (see “Experimental Procedures”). Store-operated CRAC channels were activated by exposing Fura-2-loaded cells to the sarcoplasmic-endoplasmic reticulum calcium ATPase pump blocker thapsigargin in Ca\(^{2+}\)-free solution. By blocking Ca\(^{2+}\) reuptake, thapsigargin gradually depletes the stores of Ca\(^{2+}\), thereby opening CRAC channels. Readmission of external Ca\(^{2+}\) results in Ca\(^{2+}\) entry through the CRAC channels, generating a cytoplasmic Ca\(^{2+}\) rise. We measured the rate of rise of cytoplasmic Ca\(^{2+}\) following Ca\(^{2+}\) readmission, because this is a more reliable indicator of CRAC channel activity than the steady-state amplitude. As shown in Fig. 3B, 10 \(\mu\)M Synta compound substantially slowed the rate of rise of the cytoplasmic Ca\(^{2+}\) signal after readmission of external Ca\(^{2+}\), and this amounted to almost 90% block of the CRAC channels (Fig. 3C).

To measure CRAC channel activity directly, we carried out whole cell patch clamp recordings in which we activated the CRAC current (I\(_{\text{CRAC}}\)) by dialyzing cells with InsP\(_{3}\) in 10 mM EGTA. The time course of I\(_{\text{CRAC}}\) development is shown in Fig. 3D (filled circles) and the current-voltage relationship, taken when I\(_{\text{CRAC}}\) had reached steady-state, is depicted in Fig. 3E. Pre-treatment for 5 min with 10 \(\mu\)M Synta compound abolished I\(_{\text{CRAC}}\) (Fig. 3, D and E). Similarly, after activation of I\(_{\text{CRAC}}\) perfusion with the Synta compound resulted in a loss of the current (data not shown). We constructed a dose-inhibition curve for the Synta compound, which is summarized in Fig. 3F. These data could be fitted with a Hill-type equation, yielding a Hill coefficient of 1.1 and an IC\(_{50}\) of 3 \(\mu\)M.

Having established that the Synta compound blocks CRAC channels, we went on to examine the effects of the drug on antigen-evoked Ca\(^{2+}\) influx and Syk activation. The Synta compound suppressed Ca\(^{2+}\) influx evoked by antigen (Fig. 3G) and reduced the extent of Syk phosphorylation (Fig. 3, H and I). Strikingly, the fall in Syk activity (∼50%, Fig. 3I) in the presence of Synta compound was similar to that seen when external Ca\(^{2+}\) was removed (Fig. 1D), consistent with suppression of Ca\(^{2+}\) influx by Synta compound.

We tested the selectivity of the Synta compound for CRAC channels by examining whether the drug interfered with two other major ion transport mechanisms in the mast cell membrane: inwardly rectifying K\(^{+}\) channels and the plasma membrane Ca\(^{2+}\) ATPase pump. The current-voltage relationship and the amplitude of the inwardly rectifying K\(^{+}\) current was unaffected by 10 \(\mu\)M Synta compound (Fig. 4, A and B). Similarly, the rate of Ca\(^{2+}\) removal from the cytoplasm after stimulation with thapsigargin in Ca\(^{2+}\)-free solution, which reflects activity of the plasma membrane Ca\(^{2+}\) ATPase pump (36), was similar in the absence and presence of the Synta compound (Fig. 4, C and D).

RNAi Knockdown of Orai1 Reduces Antigen-dependent Syk Activation—To strengthen the pharmacological approach, we used an RNAi knockdown strategy to suppress expression of the protein Orai1, which is central to the CRAC channel pathway. Orai1 is a plasma membrane protein that comprises the pore of the CRAC channel (16, 17, 20–22). Transfection with siRNA to Orai1 resulted in a reduction in Orai1 mRNA levels, measured using reverse transcription-PCR (Fig. 5A). Knockdown was only partial, because our transfection efficiency was ∼60%, judged by expression of eYFP. Co-transfection of Orai1 siRNA with an eYFP construct enabled us to identify trans-
Store-operated Ca\(^{2+}\) Influx Sustains Syk Activity

**FIGURE 5.** Knocking down expression of Orai1 suppressed antigen-evoked Ca\(^{2+}\) influx and subsequent Syk phosphorylation. A, reverse transcriptase-PCR shows that transfection with siRNA to Orai1 results in reduced Orai1 expression. Upper panel, Orai1; lower panel, β-actin controls for the samples used in the upper panel. B, store-operated Ca\(^{2+}\) influx is significantly reduced by siRNA to Orai1. Cells were transfected with eYFP alone, eYFP and nonsense siRNA (control), or eYFP and Orai1 siRNA. 48–60 h post-transfection, cells were exposed to thapsigargin in Ca\(^{2+}\)-free solution and then 2 mM Ca\(^{2+}\) was readmitted. The extent of Ca\(^{2+}\) release by thapsigargin was similar for the two conditions. C, aggregate data from several experiments as in panel B are shown. The peak amplitude of the Ca\(^{2+}\) signal was measured. Control denotes 77 cells and Orai1 siRNA 61 cells. A similar reduction in the rate of Ca\(^{2+}\) influx was also observed. D, transfection of cells with RNAi to Orai1 significantly reduced Ca\(^{2+}\) influx-dependent activation of Syk in response to antigen stimulation. The upper gel compares Syk activation to antigen for control transfected cells and for those in which Orai1 had been knocked down. The lower gel is the total ERK2 loading control. The histogram depicts averaged data from four independent experiments.

**FIGURE 6.** Antigen and CRAC channels interact synergistically to sustain Syk phosphorylation. A, upper panel; Western blot showing Syk phosphorylation induced by antigen was increased by thapsigargin. Aggregate data from three experiments is summarized in the lower panel. B, upper panel shows that Syk phosphorylation following stimulation with antigen in Ca\(^{2+}\)-free solution was not enhanced by thapsigargin. Aggregate data from two experiments is summarized in the lower panel. C, activation of CRAC channels by thapsigargin alone did not lead to any detectable Syk phosphorylation (upper panel), compared with control (non-stimulated) cells. Averaged data from three experiments are summarized in the lower panel, which compares Syk phosphorylation to antigen in 2 mM Ca\(^{2+}\) versus thapsigargin in 2 mM Ca\(^{2+}\).

fected cells and thus measure the impact of Orai1 knockdown on store-operated Ca\(^{2+}\) entry. Readmission of external Ca\(^{2+}\) to cells pre-treated with thapsigargin resulted in robust store-operated Ca\(^{2+}\) influx in cells transfected with either eYFP alone or eYFP and scrambled siRNA (Fig. 5B). However, the rate and extent of Ca\(^{2+}\) influx was significantly reduced when cells were transfected with Orai1 siRNA (Fig. 5B, aggregate data are summarized in Fig. 5C). Hence knockdown of Orai1 results in significantly less store-operated Ca\(^{2+}\) entry. Following Orai1 knockdown, antigen activation of Syk was significantly reduced (Fig. 5D, aggregate data are summarized in the lower panel), demonstrating a role for CRAC channels in Syk activation. Because our transfection efficiency was ~60%, the fall in Syk phosphorylation following Orai1 knock down was not as extensive as that seen in Ca\(^{2+}\)-free solution (where no Ca\(^{2+}\) entry occurs). Collectively, the pharmacological and RNAi experiments reveal that it is antigen-evoked Ca\(^{2+}\) entry through CRAC channels that maintains the activity of Syk.

Synergy between CRAC Channels and FcγR Receptors in Phosphorylating Syk—Although antigen activates I<sub>CRAC</sub>, it does so to a submaximal extent even when applied at a supra-maximal concentration (37). The size of the current can be increased by further depletion of stores (37). To see whether antigen activation of Syk could be increased by recruiting more CRAC channels, we applied antigen together with thapsigargin. Thapsigargin depletes stores sufficiently to activate I<sub>CRAC</sub> maximally (38). The combination of antigen and thapsigargin resulted in substantially more Syk phosphorylation than was observed with antigen alone (Fig. 6A, aggregate data are depicted in the lower panel). By blocking sarcoplasmic-endoplasmic reticulum calcium ATPase pumps, thapsigargin not only depletes stores but also reduces cytoplasmic Ca\(^{2+}\) buffering. This would increase the extent and time course of the Ca\(^{2+}\) signal evoked by antigen. To test whether the dramatic potentiating effects of thapsigargin on antigen-evoked Syk phosphorylation were indeed due to the reduction in cytoplasmic Ca\(^{2+}\) buffering, we applied antigen with thapsigargin but now in Ca\(^{2+}\)-free external solution. Under these conditions, the combination of antigen and thapsigargin was only as effective as antigen alone (Fig. 6B); no potentiation occurred. Reduced cytoplasmic Ca\(^{2+}\) clearance therefore cannot explain the significant increase in Syk phosphorylation seen in response to the combination of antigen and thapsigargin. Stimulation with thapsigargin in the presence of external Ca\(^{2+}\) failed to evoke any detectable Syk phosphorylation (Fig. 6C). Hence receptor-independent activation of CRAC channels and subsequent Ca\(^{2+}\) entry cannot mimic the effects of antigen stimulation on Syk phosphorylation. Instead, Ca\(^{2+}\) entry acts synergistically with an early
consequence of FcεRI receptor cross-linking and this interaction sustains Syk activity.

**DISCUSSION**

Our findings reveal a novel positive feedback cascade between Syk activity and Ca²⁺ influx through CRAC channels that sustains mast cell activation. Stimulation of Syk is an early event after antigen stimulation where it contributes to activation of phospholipase Cγ and subsequent Ca²⁺ release from intracellular stores (4). This fall in store Ca²⁺ content results in activation of CRAC channels (39). When Syk activity is blocked after the development of a Ca²⁺ plateau (reflecting store-operated Ca²⁺ entry) to antigen, the Ca²⁺ signal falls rapidly. Hence Syk activity is required both to initiate and then sustain Ca²⁺ entry, presumably by maintaining InsP₃ at levels sufficient to ensure partial store depletion (25). Syk activity is not independent of CRAC channels, however. Local Ca²⁺ entry through these channels feeds back to sustain Syk activity, providing a mechanism for the prolonged Ca²⁺ influx seen with FcεRI receptor activation and which is needed for appropriate stimulation of mast cells. The finding that Ca²⁺ influx sustains Syk activity provides a molecular mechanism that helps explain how certain G protein-coupled receptors, which themselves fail to trigger degranulation, can potentiate the antigen-driven responses in mast cells (40).

In some other cell types, cytoplasmic Ca²⁺ has been reported to activate Syk. In platelets, Syk was activated by the rise in cytoplasmic Ca²⁺ that accompanied Ca²⁺ ionophore application (41). In a human B cell line, platelet-activating factor stimulated Syk in a Ca²⁺-dependent manner (42). Finally, in PC12 cells, Ca²⁺ influx led to the rapid activation of the non-receptor tyrosine kinase PYK2 (43). Although the mechanism whereby cytoplasmic Ca²⁺ activated these tyrosine kinases was not resolved, addition of Ca²⁺ to either cell lysates (42) or the isolated kinase (43) did not increase enzyme activity. Hence Ca²⁺ is unlikely to stimulate Syk directly. Consistent with this is our finding that Ca²⁺ influx through CRAC channels following stimulation with thapsigargin failed to evoke any detectable Syk activation. Instead, an additional signal associated with FcεRI receptors is required. Syk is activated by binding, via SH2 domains, to phosphotyrosine residues in the immunoreceptor tyrosine-based activation motif signaling units on the β and γ chains of the receptor, which are phosphorylated by receptor-associated Src kinases like Lyn (4). It is conceivable that Syk binding to the immunoreceptor tyrosine-based activation motif region is increased by Ca²⁺²⁴, that a phosphorylated immunoreceptor tyrosine-based activation motif region and a Ca²⁺ rise are both needed to increase Syk activity, or that Lyn activity, once triggered by receptor cross-linking, is potentiated by Ca²⁺.

Finally, the fact that Ca²⁺ influx through CRAC channels but not Ca²⁺²⁴ release from intracellular stores was able to activate Syk adds to the growing list of examples where the spatial location of the Ca²⁺²⁴ rise is important in selectively activating a target (44, 45). It is striking that Ca²⁺²⁴ entry is able to support Syk activity even in the presence of the fast Ca²⁺²⁴ chelator BAPTA in the cytoplasm. BAPTA restricts Ca²⁺²⁴ entry to within a few nanometers/tens of nanometers of its point of entry (46), and therefore it is local Ca²⁺²⁴ entry through CRAC channels that maintains Syk. Hence FcεRI receptors, Syk, and CRAC channels might be closely apposed, possibly co-localized in a signaling complex. Our functional studies lend support to recent observations that FcεRI-dependent signaling occurs in lipid raft domains (47), where store-operated channels can also be found (48).

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