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Cross-linking of FcεRI causes Ca²⁺ mobilization via a sphingosine kinase pathway in a clathrin-dependent manner

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Abstract

Clathrin-coated pits are now recognized to be involved in cell signaling in addition to receptor down-regulation. Here we tried to identify signaling pathways that might be dependent on clathrin. Our initial data with pharmacological inhibitors of formation of clathrin-coated pits or lipid-rafts indicated that Ca²⁺ response evoked by cross-linking of the high affinity receptors for IgE (FcεRI) was dependent on clathrin. To confirm this finding, we created clathrin-knockdown cells by transfecting the mast cell line RBL-2H3 with a shRNA-clathrin heavy chain construct. In these cells, the FcεRI-mediated Ca²⁺ response was almost completely abolished, which was accompanied by the inhibition of sphingosine 1-phosphate (S1P) production with no changes in inositol 1,4,5-trisphosphate (IP₃) production. This suggests that the Ca²⁺ signaling pathway via a sphingosine kinase (SK) is dependent on clathrin. Furthermore, antigen-induced tyrosine phosphorylation of p85 and p110 subunits of PI3K was almost completely inhibited in clathrin-knockdown cells. In contrast, antigen-induced tyrosine phosphorylation of phospholipase Cγ was not affected by clathrin knockdown and tyrosine phosphorylation of Syk and degranulation were partially inhibited in clathrin-knockdown cells. The present study identifies the SK/Ca²⁺ pathway to be dependent on clathrin.

Keywords

Ca²⁺ signaling; clathrin; sphingosine kinase; mast cell; FcεRI

1. Introduction

Allergic responses are mediated by the release of allergic inflammatory mediators from mast cells [1]. The release of these mediators is a Ca²⁺-dependent phenomenon. Crosslinking of high affinity receptors for IgE (FcεRI) leads to Ca²⁺ release from the ER, which is followed by Ca²⁺ influx from the outside of cell, causing an increase in the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i). The FcεRI-mediated Ca²⁺ mobilization is dependent on the production of inositol 1,4,5-trisphosphate (IP₃), a product of phospholipase Cγ (PLCγ) and sphingosine

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1-phosphate (S1P), a product of sphingosine kinase (SK) [2–4]. Activation of PLC γ by crosslinking of Fc ϵ RI is initiated by tyrosine phosphorylation of receptor subunits by a Src family tyrosine kinase, Lyn, which subsequently activates Syk (reviewed in [5]). This phosphorylation cascade is followed by the recruitment of adaptor proteins including LAT, SLP-76 and Grb-2 (reviewed in [6]). The activated PLC γ then produces IP $_3$, which binds its receptors in the ER. Although the IP $_3$ -mediated mechanism for Ca $^{2+}$ mobilization from the ER is well understood, not much is known about which isoform of SK is responsible for releasing Ca $^{2+}$ from the ER, how SK is activated, where S1P is produced and how it releases Ca $^{2+}$ from the ER.

S1P given extracellularly can induce various cellular responses, including regulation of cell migration, cell proliferation and survival and angiogenesis by acting through the G protein-coupled receptors of the endothelial differentiation gene (EDG) receptor family (reviewed in [7,8]). Five subtypes of S1P receptors have been identified to date and many tissues express one or more of these receptors [9,10]. Activation of cell surface S1P receptors by extracellular S1P can induce Ca $^{2+}$ mobilization by stimulating PLC to release IP $_3$ [11–13]. In addition to its extracellular actions, intracellularly generated S1P has been shown to mobilize Ca $^{2+}$ after the stimulation of various receptors such as Fc ϵ RI [2–4], Fc γ RI [14–16] and many G protein-coupled receptors [17,18]. S1P was also shown to be generated in the ER membrane and to release Ca $^{2+}$ from the ER [19]. Despite the indication of the involvement of intracellular S1P in Ca $^{2+}$ mobilization, the mechanism of S1P-induced Ca $^{2+}$ mobilization has not been elucidated. Although transactivation of S1P receptors by S1P that has been released from mast cells upon Fc ϵ RI cross-linking has been suggested [20], Fc ϵ RI-mediated Ca $^{2+}$ mobilization appears not to be due to the transactivation of S1P receptors since the inhibition of S1P secretion via the ATP binding cassette (ABC) C1 transporter did not affect antigen-induced Ca $^{2+}$ mobilization [21]. Thus, it is plausible to think that S1P has an intracellular target on the ER membrane. S1P, unlike IP $_3$, is not soluble in an aqueous cytoplasmic environment, which makes it impossible for S1P produced in the plasma membrane to diffuse to the ER. Therefore, in order to mobilize Ca $^{2+}$ from the ER, S1P has to be produced in the ER or to be transported to the ER. We hypothesized that endocytosis could be one mechanism for communication between signaling molecules in the plasma membrane and ones in the ER.

Receptor-induced endocytosis is mediated by two different mechanisms, clathrin-dependent and clathrin-independent. Clathrin-dependent endocytosis is initiated by the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. This leads to the formation of clathrin-coated pits that bud and pinch off from the membrane in a dynamin-dependent manner and give rise to clathrin-coated vesicles, which are fused with the early endosome [22]. Receptors in the endosome can dissociate from the ligands and recycle back to cell surface or be degraded in the lysosome. Thus, the clathrin-dependent mechanism has long been thought to be a mechanism for the attenuation of receptor-activated responses. However, growing evidence suggests an important role of clathrin-dependent endocytosis in signal transduction [22–25]. Since the initial observation that activation of ERK and PI3K by EGF is dependent on clathrin-dependent endocytosis [26], endocytosis-dependent activation of ERK has been demonstrated in multiple receptor systems such as the insulin receptor [27], the 5-HT receptor [28], the m1 muscarinic receptor [29] and the μ and δ opioid receptors [30,31]. In contrast to clathrin-dependent endocytosis, clathrin-independent endocytosis is not well-understood. The major form of clathrin-independent endocytosis is dependent on lipid-rafts. A morphologically identifiable lipid-raft is caveolae, which contains sphingolipids, cholesterol and caveolin [22]. Immune cells, however, do not contain caveolin in their lipid-rafts [32].

Segregation of Fc ϵ RI and signaling molecules into distinct membrane domains has been shown in the rat mast cell line RBL-2H3 by biochemical studies [33] or studies performed by electron microscopy [32]. Although these studies have tried to identify the membrane domains where

receptor activation occurs, it has been controversial whether these sites involve clathrin [32, 34,35] or lipid rafts [36,37]. In the present study, we focused on the role of clathrin in FcεRI-mediated Ca²⁺ signaling and show the evidence that the FcεRI/SK/Ca²⁺ signaling pathway is dependent on clathrin.

2. Methods

2.1. Reagents

Materials were obtained from the following sources: radiolabelled compounds from NEN (Boston, MA); fura-2 AM from Molecular Probes Inc. (Eugene, OR); sphingosine from Biomol (Plymouth Meeting, PA); chlorpromazine and nystatin from Sigma (St. Louis, MO). Materials for cell culture were obtained from GIBCO-BRL (Gaithersburg, MD). Antibodies used included anti-clathrin heavy chain monoclonal antibody (mAb) (BD Biosciences, San Jose, CA), anti-α-tubulin mAb (Lab Vision Corporation, Fremont, CA), anti-phosphotyrosine (4G10) mAb (Upstate, Lake Placid, NY), anti-Lyn mAb, anti-p85 mAb, anti-p110 mAb, phospholipase Cγ1 and 2 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Syk polyclonal antibody (Top-Bio, Prague, Czech Republic).

2.2. Cell culture and transfection

RBL-2H3 cells were maintained as monolayer cultures in Earle's modified Eagle's medium supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). For transfection, cells were electroporated as previously described [38]. Cells (1.5×10^7 cells/150 µl) in Eagle's medium supplemented with 50 mM HEPES and 20% fetal bovine serum were electroporated (Gene Pulser, Bio-Rad) at 250V and a capacitance of 500 µF in the presence of 15–20 µg of the psiRNA-hH1zeo vector or the psiRNA-clathrin construct (InvivoGen, San Diego, CA). The culture medium was replaced with fresh medium containing 0.1 mg/ml of Zeocin 24 hr after transfection. Five to seven days after electroporation, the antibiotic resistant cells were cloned by plating less than one cell per well in 96-well plates.

2.3. Generation of an shRNA construct

A DNA construct containing specific 19 nucleotides of rat clathrin heavy chain cDNA was produced using the psiRNA-hH1-zeo cloning vector according to the manufacturer's instructions provided with the psiRNA system kit (InvivoGen, San Diego, CA). Briefly, two complementary oligonucleotides homologous to a 19-nucleotide segment of the clathrin heavy chain (5'-

TCCCAATTCGCTTTCAGGAGCATCCACCGATGCTCCTGAAAGCGAATTT-3' and 5'-CAAAAAATTCGCTTTCAGGAGCATCGGTGGGATGCTCCTGAAAGCGAATT-3') were made. Underlined sequences belong to the clathrin heavy chain and its complementary sequences. These two oligonucleotides were annealed and subcloned into the Bbs I restriction site of the psiRNA vector yielding psiRNA-clathrin. The DNA construct was transfected into RBL-2H3 cells by electroporation and cloned as described in the previous section.

2.4. Immunoprecipitation and immunoblotting

Cells (10^7 cells/sample) were washed twice with Tris-buffered saline and lysed by addition of ice-cold lysis buffer, containing 0.5% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, 10 µg/ml aprotinin and pepstatin A and 5 µg/ml leupeptin. Lysates were left on ice for 20 min and centrifuged for 30 min at $12,000 \times g$ in a microcentrifuge at 4 °C to remove nuclei. The supernatant was incubated with anti-phosphotyrosine antibody (4G10) overnight at 4 °C with rotation and further incubated for 2

hrs after addition of protein-G Sepharose (Roche Diagnostics, Indianapolis, IN). Immunoprecipitates were washed 5 times and subjected to SDS-PAGE.

After SDS-PAGE, proteins were transferred to PVDF membranes and blots were subjected to Western blot analysis as previously described [4]. To reprobe with another antibody, the blots were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL).

2.5. Flow-cytometric analysis of FcεRI expression

Cells (1×10^5 cells/sample) were preincubated with 0.8 $\mu\text{g/ml}$ rat anti-mouse CD16/CD32 antibody (BD Pharmingen) in 0.2% BSA/PBS on ice to block IgG receptors and 5 min later, 1 $\mu\text{g/ml}$ mouse IgE (SPE-7, Sigma) was added and further incubated for 30 min. Cells were then washed with PBS and incubated with a 1:100 dilution of FITC-conjugated goat F(ab')₂ anti-mouse Ig's (Biosource) for 30 min on ice, washed and analyzed for cell surface expression of FcεRI on a FACS scan cytometer (Becton Dickinson FACSCalibur, Mountain View, CA).

2.6. In situ sphingosine kinase assay

Cells (2×10^6 cells/dish in 60 mm dishes) sensitized with DNP-IgE (50 ng/ml, Sigma, St. Louis, MO) were washed twice with a glucose-saline, PIPES buffer (pH7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 0.1% BSA and 1 mM CaCl₂. Cells were then stimulated or not in the presence of 0.5 mM 4-deoxypyridoxine and 0.1 mM L-canaline (Sigma) to prevent breakdown of S1P by S1P lyase. The reaction was quenched by placing the dishes on ice and the media was aspirated. Lipid extraction was performed by the addition of CHCl₃/MeOH/CHCl (100:200:1, v/v) and the subsequent addition of CHCl₃/0.1N HCl (1:1). The amount of radioactive S1P generated was determined by collection of the lower phase, followed by silica gel thin layer chromatography and autoradiography as described previously [2].

2.7. IP₃ assay

Cells were plated in culture dishes (60 mm diameter, 2×10^6 cells/dish) and sensitized with anti-DNP IgE (50 ng/ml) overnight. The next day, cells were washed twice with a glucose-saline, PIPES buffer (pH7.2) as described for the *in vivo* SK assay and stimulated in the same buffer at 37°C with or without DNP₃₅-HSA (10 ng/ml) for 5 min. The reaction was quenched by adding ice-cold 20% trichloroacetic acid. The amount of IP₃ was determined according to the manufacturer's instructions provided with IP₃ assay kit (Dupont/NEN, Boston, MA), with a modification that passed the aqueous phase of the extract through ultrafiltration units to exclude proteoglycans that interfere with the assay [39].

2.8. Ca²⁺ measurements

For the measurement of the cytosolic free Ca²⁺, cells were collected and incubated in complete growth medium containing DNP-IgE (50 ng/ml) at 37°C for 2 hrs with gentle rotation. The cells were then loaded with 2 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 45 min and measurement of cytosolic Ca²⁺ was performed in a buffer containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, 2.5 mM probenecid and 0.1% BSA, in a Deltascan bulk spectrofluorimeter (Photon Technology Inc., NJ) as previously described [39].

2.9. β-Hexosaminidase assay

Cells (2×10^5 cells/well in 24-well plates) sensitized with anti-DNP IgE (50 ng/ml) overnight were stimulated with the indicated concentrations of DNP₃₅-HSA in PIPES buffer at 37°C for 15 min. At the end of the incubation, aliquots (10 μl) of medium and cell lysates (in 500 μl 0.5% Triton X-100) were incubated with 10 μl of 1 mM p-nitrophenyl-N-acetyl β-D-

glucosaminide in 0.1 M sodium citrate buffer at 37°C for 1 hr. Finally, 250 μ l of a 0.1M Na_2CO_3 /0.1 M NaHCO_3 buffer was added. Absorbance was read at 410 nm as described previously [40].

3. Results

3.1. Fc ϵ RI-mediated Ca^{2+} mobilization is dependent on the formation of clathrin-coated pits at the cell surface

As mentioned in the previous section, we hypothesized that endocytosis could be a mechanism for communication between signaling molecules in the PM and ones in the ER that is responsible for antigen-induced Ca^{2+} mobilization from the ER. To test this hypothesis, we used two pharmacological inhibitors, chlorpromazine and nystatin, which inhibit one of two major endocytic mechanisms, clathrin-dependent and lipid-raft-dependent endocytosis, respectively. Pretreatment of cells with chlorpromazine, a compound that interferes with the formation of clathrin-coated pits [41–44], almost completely inhibited Fc ϵ RI-mediated Ca^{2+} responses. On the other hand, pretreatment with nystatin, a compound that disrupts lipid-rafts [41,45,46], only partially inhibited Fc ϵ RI-mediated Ca^{2+} responses (Fig. 1A). Ca^{2+} release from the ER as determined by measurements in the presence of EGTA (Fig. 1B) was also almost completely inhibited in the cells pretreated with chlorpromazine but only partially inhibited in those pretreated with nystatin. The partial inhibition of Fc ϵ R-mediated Ca^{2+} responses in nystatin-treated cells could be due to the fact that nystatin depletes cholesterol and cholesterol is a component of clathrin-coated pits. In fact, it was shown that the clathrin-enriched raised domains observed by atomic force microscopy was 50% reduced in height when cholesterol was extracted with methyl- β -cyclodextrin in RBL-2H3 cells [47]. To arbitrarily quantitate the Fc ϵ RI-mediated increase in $[\text{Ca}^{2+}]_i$, the net increase in the fluorescence ratio was determined in each experiment (Fig. 1C, 1D). The data suggested that Fc ϵ RI-mediated Ca^{2+} mobilization is dependent on the formation of clathrin-coated pits.

3.2. Clathrin-knockdown almost completely inhibited Fc ϵ RI-mediated Ca^{2+} mobilization

To further test the involvement of the clathrin-coated pits in Fc ϵ RI-mediated Ca^{2+} signaling, clathrin was knocked down in RBL-2H3 cells by transfecting the cells with shRNA-clathrin heavy chain construct as described in the Methods. The expression level of clathrin heavy chain was markedly reduced in clathrin-knocked down clones as determined by Western blot analysis (Fig. 2A). The expression level of clathrin was quantified by densitometry. Density of the clathrin bands was normalized by that of α -tubulin bands and percent density was calculated against the density in RBL-2H3 cells. Expression of clathrin in the knockdown clones was slightly over 50% reduced compared to the vector-transfected control (Fig. 2B). The fact that only clones with incomplete knockdown of clathrin heavy chain have survived may indicate that clathrin is essential for cell survival [48]. To investigate the possibility of off-target effects of shRNA, surface expression of Fc ϵ RI was determined by FACS analysis. As shown in Figure 2C, the expression levels of Fc ϵ RI was almost identical in all the cells tested including RBL-2H3, vector control, cells transfected with shRNA scrambled sequence and 3 clathrin-knocked down clones.

As predicted from the chlorpromazine results (Fig. 1), antigen-induced Ca^{2+} mobilization was reduced in clathrin-knocked down cells (Fig. 3). Interestingly, the initial peak was almost completely abolished while the late phase increase was only partially inhibited compared to RBL-2H3, vector-transfected, or scrambled shRNA control cells, suggesting that clathrin is involved only in Ca^{2+} mobilization from the ER (Fig. 3A). These data revealed that the Fc ϵ RI-induced Ca^{2+} influx occurred without depletion of intracellular Ca^{2+} stores. Similarly, when Ca^{2+} was measured in the presence of EGTA, Ca^{2+} mobilization was almost completely inhibited in clathrin-knocked down cells (Fig. 3B). The changes in $[\text{Ca}^{2+}]_i$ were quantified as

described for Figure 1 and the data suggest that clathrin is necessary for FcεRI-mediated Ca^{2+} mobilization from the ER (Fig. 3C,D).

Since data shown in Figure 3 indicated that clathrin knockdown affected antigen-induced Ca^{2+} mobilization from the ER with only partially affecting Ca^{2+} influx, we performed experiments to confirm this. We measured Ca^{2+} influx after reproviding Ca^{2+} in the cells that had been stimulated with antigen in the presence of EGTA. Although Ca^{2+} mobilization from the ER occurred only in the control cell, the extent of Ca^{2+} influx was not significantly different except for the knockdown clone #3, in which Ca^{2+} influx was slightly reduced (Fig. 4 A,B). The data confirmed that Ca^{2+} mobilization from the ER, but not Ca^{2+} influx, was dependent on clathrin and Ca^{2+} influx could occur without depleting intracellular Ca^{2+} store.

To ensure that the inhibition of antigen-induced Ca^{2+} mobilization in clathrin-knocked down cells was not due to the direct effect of clathrin knockdown on Ca^{2+} transport mechanism, we measured Ca^{2+} mobilization induced by N^5 -ethylcarboxamidoadenosine (NECA), an agonist of the G protein-coupled adenosine receptor (Fig. 4C,D,E). We have previously shown that Ca^{2+} mobilization via the adenosine receptors in RBL-2H3 cells was mediated exclusively by the phospholipase C (PLC) pathway [4]. NECA-induced Ca^{2+} responses were almost identical in all the cells tested regardless of clathrin-knockdown. This suggests that clathrin knockdown does not directly regulate Ca^{2+} transport mechanisms but, instead, selectively affect the non-PLC pathway.

3.3. Activation of sphingosine kinase is dependent on clathrin

Since the FcεRI-mediated Ca^{2+} response could be mediated by both the SK and PLC pathways, we examined which of the Ca^{2+} signaling pathways is affected by clathrin-knockdown by measuring FcεRI-mediated production of S1P and IP_3 . FcεRI-mediated S1P production was measured at 1.5 min after antigen (100 ng/ml) stimulation, at which FcεRI-mediated S1P production was shown to be maximal [2]. On the other hand, antigen-induced production of IP_3 was measured 5 min after stimulation because IP_3 production was shown to gradually increase without a peak and reach the maximal level 5 min after antigen stimulation [39]. Antigen-induced S1P production was almost completely inhibited in clathrin-knocked down cells (Fig. 5A) while the IP_3 production was not affected (Fig. 5B). The data suggest that the activation of the FcεRI/SK/ Ca^{2+} signaling pathway is dependent on clathrin, perhaps the formation of clathrin-coated pits. It is interesting to note that FcεRI-mediated production of IP_3 is not affected at all by knockdown of clathrin. As suggested previously, the FcεRI/LAT/PLCγ pathway could have been activated in the lipid-rafts [32,49,50].

3.4. Clathrin-knockdown selectively affects tyrosine phosphorylation of a subset of signaling molecules

Although our data indicate that clathrin knockdown interferes with Ca^{2+} signaling that is mediated by sphingosine kinase, the upstream signaling pathway is not known. In order to identify the possible upstream signaling pathway, FcεRI-mediated total tyrosine phosphorylation was determined by immunoprecipitating the tyrosine-phosphorylated molecules with anti-phosphotyrosine antibody (4G10) and detecting them with 4G10. As shown in Figure 6A, tyrosine phosphorylation of multiple bands was increased upon cross-linking of FcεRI in control cells as well as clathrin-knocked down cells and FcεRI-mediated tyrosine phosphorylation of some of the molecules were greatly reduced in clathrin-knocked down cells compared to RBL-2H3 or vector control cells. As indicated on the right hand side of the panel, we were able to identify that these individual bands contained PLCγ, subunits of PI3K, Syk and Lyn by reprobing blots with indicated antibodies (Fig. 6A). It is interesting to note tyrosine phosphorylation of p110 subunit of PI3K as previously observed in B cell antigen receptors-stimulated human, murine [51] and chicken B cells [52] and CD4-ligated T cells

[53]. Tyrosine phosphorylated p110 bands may also contain tyrosine phosphorylated Pyk₂ [54]. It is also possible that p110 subunit of PI3K might not be tyrosine phosphorylated but was coimmunoprecipitated with tyrosine phosphorylated p85 subunit of PI3K and tyrosine phosphorylation of p110 bands as detected with 4G10 might be attributed to Pyk₂ phosphorylation.

To confirm and quantitate tyrosine phosphorylation of Syk and p110 and p85 subunits of PI3K, which was greatly reduced in clathrin-knocked down cells, we reprobed each blot with anti-Syk and anti-p110 and anti-p85 PI3K antibodies. We assumed that the intensities of the bands detected with each antibody indirectly indicated tyrosine phosphorylation of the molecules because they have been immunoprecipitated with anti-phosphotyrosine antibody. As previously mentioned, even if p110 PI3K might not be tyrosine phosphorylated, the intensity of the band would still correlate with the association of the enzyme with p85 PI3K. Syk phosphorylation was markedly increased after antigen stimulation in RBL (411 ± 66 (mean $\pm$ SD) fold) and vector-transfected (289 ± 66 (mean $\pm$ SD) fold) cells. This phosphorylation was significantly inhibited up to 50 to 70 % in clathrin-knockdown cells (Fig. 6B). Unlike the partial dependence of Syk phosphorylation on clathrin, tyrosine phosphorylation of the p85 and p110 subunits of class I PI3K was almost completely abolished (Fig. 6C,D). Tyrosine phosphorylation of p85 after antigen stimulation of RBL and vector-transfected cells was 32 ± 13 (mean $\pm$ SD) fold and 20 ± 13 (mean $\pm$ SD) fold increased, respectively, while in clathrin knockdown cells, only 2 to 3 fold increased (Fig. 6C). Similarly, Fc ϵ RI-mediated increase in the intensities of p110 PI3K bands after Fc ϵ RI crosslinking in RBL and vector-transfected cells was 132 ± 32 (mean $\pm$ SD) fold and 137 ± 39 (mean $\pm$ SD) fold, respectively but that was markedly reduced to 12 to 27 fold in clathrin-knocked down cells (Fig. 6D). The data suggest that the activation of PI3K is absolutely dependent on clathrin.

3.5. Fc ϵ RI-mediated degranulation was partially impaired in clathrin knockdown cells

Since the rise in cytosolic Ca²⁺ concentration is important for degranulation and PI3K [55] has been suggested to play an important role in Fc ϵ RI-mediated degranulation, we compared Fc ϵ RI-mediated degranulation in control and clathrin-knocked down RBL-2H3 cells. As was the case with Ca²⁺ signaling, the extent of antigen-stimulated degranulation was lower in vector-transfected cells compared to RBL-2H3 cells. In clathrin knockdown cells, antigen-stimulated degranulation was partially inhibited, which could be attributed to the reduction in total Ca²⁺ flux (Fig. 7).

4. Discussion

The mechanisms of phosphorylation and activation of Fc ϵ RI and its downstream signaling cascade are well-studied. Cross-linking of Fc ϵ RI leads to the phosphorylation of β and γ subunits of the receptor on an ITAM motif by Lyn, which subsequently provides binding sites for the SH2 domain of Syk. However, the location on the plasma membrane where these processes occur remains obscure. Particularly, compartmentalization of Fc ϵ RI and early signaling molecules is controversial due to the lack of tools to clearly separate clathrin-coated pits from lipid-rafts. It could be also due to the fact that clathrin-coated pits and lipid-rafts are localized in close proximity [47]. Our current study focuses on the downstream signaling pathways that are dependent on clathrin in mast cells. Although vesicles such as early endosomes and caveolae have been shown to be involved in delivering signals from the plasma membrane to intracellular targets in some receptor systems [26–31], no study has investigated such roles of these vesicles in Fc ϵ RI-mediated downstream signaling in mast cells. Our data demonstrated that knockdown of clathrin led to an almost complete inhibition of S1P production (Fig. 5) and Ca²⁺ mobilization from the ER (Fig. 3). This suggests that clathrin-coated vesicles may be involved in transferring signals from the plasma membrane to the ER,

where the SK may produce S1P and release Ca^{2+} [19] Clathrin knockdown caused about 50% inhibition of Syk tyrosine phosphorylation (Fig. 6B) and nearly 90% inhibition of PI3K subunit phosphorylation (Fig. 6C,D), while Lyn kinase and PLC γ were not significantly affected (Fig. 6A). The data suggest that only a subset of Fc ϵ RI signaling molecules such as Syk and PI3K are clathrin-dependent. In agreement with previous findings showing that the Fyn/PI3K/PLD pathway indirectly activates SK1 [50], PI3K and SK were inhibited while the PLC/IP $_3$ pathway was not affected by clathrin knockdown. The data suggest that clathrin may selectively transduce the PI3K/SK pathway while not affecting the LAT/PLC pathway that is known to be mediated through lipid-rafts [49,56].

Unlike early findings demonstrating the role of intracellular S1P in Fc ϵ RI-mediated Ca^{2+} mobilization from the ER of RBL-2H3 and human mast cells [2–4], studies performed in mouse fetal liver-derived mast cell suggested no role of S1P in Ca^{2+} mobilization from the ER based on the differential knockout of SK1 and/or SK2 [57]. This discrepancy could be attributed to the species differences as previously noted [58,59]. In fact, SK2 but not SK1 was shown to be required for cytokine production and degranulation in these cells [57], while SK1 but not SK2 played a critical role in antigen-induced degranulation and cytokine release in cord blood-derived and LAD2 human mast cells [60]. In addition, the relative SK2 activity versus SK1 has been shown to be higher in mouse than in human or rats [61], suggesting that different roles could be played by different SK isoforms in different species. In line with this, the fact that intracellular S1P is not involved in antigen-induced Ca^{2+} mobilization from the ER in mouse mast cells [57] may suggest that mouse mast cells do not express the isoform of SK that is responsible for releasing Ca^{2+} from the ER. Recently, we have cloned a long form of SK1 that has a 75 amino acid extension at the N-terminal end of SK1 from rat kidney and RBL-2H3 cells [62] and this isoform was highly homologous with human long-SK1 (71% identity) that was cloned by the NIH Mammalian Gene Collection (MGC) project. The mouse homolog has not been identified or cloned yet. Human long-SK1 appeared to be distributed through the cytosol and perinuclear region and further translocated to the ER upon antigen stimulation and caused an increase in Ca^{2+} mobilization from the ER when transfected in RBL-2H3 cells (ID Jung and OH Choi, unpublished observation). As in the case of mouse mast cells [57,63], it is possible that the activation of an SK isoform and the production of S1P on the plasma membrane may lead to Ca^{2+} influx possibly by removing sphingosine that is shown to inhibit SOC entry [64] and the activation of an SK isoform and the production of S1P in the ER may lead to the release of Ca^{2+} from the ER. Our data showing that clathrin knockdown does not affect Ca^{2+} influx but inhibit Ca^{2+} release from the ER (Fig. 3 and 4A,B) suggest that SK activation on the PM is not affected by clathrin knockdown but the translocation and/or activation of an SK in the ER is inhibited. Although understanding of the precise mechanism of action of clathrin needs further study, it is possible that clathrin may play an important role in the process of translocation of SK to the ER by delivering early signaling molecules and possibly SK in early endosome to the close proximity of the ER.

The results shown in the present study indicate that the Fc ϵ RI-mediated Ca^{2+} signaling is dependent on clathrin as measured using pharmacological inhibitors of clathrin-coated pits (Fig. 1) or using clathrin-knocked down cells (Fig. 3). In clathrin-knocked down RBL-2H3 cells, Fc ϵ RI-induced production of S1P was almost completely inhibited while Fc ϵ RI-induced production of IP $_3$ was not affected (Fig. 5). In these cells, the initial peak of the Fc ϵ RI-mediated Ca^{2+} response was completely inhibited while the second phase of the Ca^{2+} response was not (Fig. 3 and 4A,B). The data suggest that production of S1P is necessary for Ca^{2+} mobilization from the ER. Ca^{2+} influx has been shown to occur through the opening of store-operated Ca^{2+} (SOC) channels upon depletion of intracellular Ca^{2+} stores. However, our data suggesting that Ca^{2+} influx can occur without depletion of intracellular stores are consistent with our previous findings in PI3KC2 β -overexpressed RBL-2H3 cells [4]. In these cells, phosphatidylinositol 4,5-bisphosphate (PIP $_2$) was depleted and Fc ϵ RI-mediated Ca^{2+}

mobilization from the ER was completely abolished while Ca^{2+} influx was not. Agonist-induced Ca^{2+} influx, independent of store depletion, has been shown to require interaction between IP_3 and IP_3 receptors in DT40 chicken B lymphocytes made to express IP_3 receptor mutants that are incapable of releasing Ca^{2+} from the ER [65]. It has also been shown that $\text{PLC}\gamma 1$ and $\text{PLC}\gamma 2$, independent of their phospholipase activities, are required for agonist-induced Ca^{2+} entry [66,67]. These findings suggest a role of the $\text{PLC}\gamma$ pathway in Ca^{2+} influx. In line with these findings, Ca^{2+} influx seen in our clathrin-knocked down cells could have occurred through the interaction between IP_3 and IP_3 receptors or by the action of $\text{PLC}\gamma$. Another mechanism by which Ca^{2+} influx could occur without depletion of the intracellular Ca^{2+} stores was suggested by the study by Olivera et al. [57], which demonstrated that, in fetal liver-derived mast cells of SK2 knockout mice, $\text{Fc}\epsilon\text{RI}$ -mediated Ca^{2+} influx was partially reduced. Authors speculated that the conversion of sphingosine to S1P by the SK2 activation might have lowered the amounts of sphingosine that had been shown to inhibit SOC channels [64]. Then, it could be speculated that $\text{Fc}\epsilon\text{RI}$ -mediated Ca^{2+} influx in clathrin-knocked down RBL-2H3 cells could have been mediated by the activation of SK2, which might be activated independent of clathrin.

In summary, our present work demonstrates that clathrin is necessary for $\text{Fc}\epsilon\text{RI}$ -mediated Ca^{2+} mobilization from the ER in RBL-2H3 mast cells. We also show that a subset of $\text{Fc}\epsilon\text{RI}$ -mediated signaling pathways that leads to both the production of S1P and release Ca^{2+} from the ER is dependent on clathrin.

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FIG. 1. Fc ϵ RI-mediated Ca²⁺ responses were almost completely inhibited by the treatment with chlorpromazine, an inhibitor of clathrin-coated pit formation

The antigen-induced Ca²⁺ response was measured in the presence of 1 mM extracellular Ca²⁺ (A) or immediately after the addition of EGTA to determine the increase in [Ca²⁺]_i due to the release from the ER (B) in RBL-2H3 cells that have been preincubated with 2 μ l DMSO (Control), 25 μ M chlorpromazine or 50 μ M nystatin for 3 min at 37 °C. Responses are expressed as an increase in the fluorescence ratio measured at 510 nm when excited alternating between 340 nm and 380 nm. Traces are representatives of at least 3 similar experiments. (C,D) Fluorescence ratio was arbitrarily quantitated by calculating the net increase in the ratio at the peak or 1 min after antigen stimulation (Total), at 3 min after antigen stimulation (Sustained) or at the peak or 30 sec after antigen stimulation in the presence of EGTA (ER Release). Concentrations of DNP₃₅-HSA (Ag) and EGTA were 10 ng/ml and 1.2 mM, respectively. Values are means $\pm$ SD and * indicates significant differences at $p < 0.001$ versus DMSO or #, versus chlorpromazine as determined by one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test.

FIG. 2. Clathrin heavy chain is knocked down

The clathrin heavy chain was knocked down in RBL-2H3 cells by transfecting a specific shRNA plasmid. (A) Western analysis was performed using total cell lysates of RBL-2H3 cells (RBL), and the cells transfected with shRNA vector alone (Cont) and a shRNA construct (shRNA Clones). Clathrin was detected using mouse anti-clathrin heavy chain antibody.

Expression of α -tubulin was detected as a loading control. (B) Clathrin expression was quantified by densitometry and normalized with α -tubulin levels. The percent expression was calculated in comparison to the expression levels in RBL-2H3 cells. Values are means $\pm$ SD of 4 separate experiments performed. The asterisks indicate a significant decrease compared to control cells at $p < 0.001$ (***) as analyzed by ANOVA and Tukey-Kramer multiple comparisons test. (C) RBL-2H cells, vector control, cells transfected with shRNA of scrambled sequences and 3 clathrin-knocked down clones were analyzed for the surface expression of Fc ϵ RI by flow cytometry. Closed histogram is the isotype control and open histograms are Fc ϵ RI staining of all the cells tested.

FIG. 3. $\text{Fc}\epsilon\text{RI}$ -mediated Ca^{2+} responses were inhibited in clathrin-knocked down cells
 The antigen-induced Ca^{2+} response was measured in the presence of 1 mM extracellular Ca^{2+} (A) or immediately after the addition of EGTA to determine the increase in $[\text{Ca}^{2+}]_i$ due to the release from the ER (B) in RBL-2H3 cells (RBL), vector control (Vec), cells transfected with shRNA of scrambled sequences (Scr) and clathrin-knocked down cells (Clones #1, #2 and #3). Responses are expressed as an increase in the fluorescence ratio measured at 510 nm when excited alternating between 340 nm and 380 nm. Traces are representatives of at least 3 similar experiments. (C,D) Fluorescence ratio was arbitrarily quantitated by calculating the net increase in the ratio at the peak or 1 min after antigen stimulation (Total), at 3 min after antigen stimulation (Sustained) or at the peak or 30 sec after antigen stimulation in the presence of EGTA (ER Release). Concentrations of DNP₃₅-HSA (Ag) and EGTA were 10 ng/ml and 1.2 mM, respectively. Values are means $\pm$ SD and asterisks indicate significant differences compared to the control cells at $p < 0.05$ (*) and 0.001 (***) as determined by ANOVA and Tukey-Kramer multiple comparisons test.

FIG. 4. Clathrin knockdown does not affect Fc ϵ RI-mediated Ca^{2+} influx or Ca^{2+} mobilization mediated by a G protein coupled adenosine receptors

(A) The antigen-induced Ca^{2+} influx was measured by reprovding extracellular Ca^{2+} in the cells that had been stimulated with antigen in the presence of 1.2 mM EFTA. The cells tested were RBL-2H3 cells (RBL), vector control (Vec), transfected with shRNA of scrambled sequences (Scr) and clathrin-knocked down cells (Clones #1, #2 and #3). Responses are expressed as an increase in the fluorescence ratio measured at 510 nm when excited alternating between 340 nm and 380 nm. Traces are representatives of at least 3 similar experiments. (B) Fluorescence ratio was arbitrarily quantitated by calculating the net increase in the ratio at the peak or 1 min after the reprovding of extracellular Ca^{2+} . Concentrations of DNP₃₅-HSA (Ag), EGTA and extracellular Ca^{2+} were 10 ng/ml 1.2 mM and 1.2 mM, respectively. Values are means $\pm$ SD and asterisks indicate significant differences compared to the vector control cells at $p < 0.01$ (**) as determined by ANOVA and Tukey-Kramer multiple comparisons test. The NECA-induced Ca^{2+} response was measured in the presence of 1 mM extracellular Ca^{2+} (C) or immediately after the addition of EGTA to determine the increase in $[\text{Ca}^{2+}]_i$ due to the release from the ER (D) in RBL-2H3 cells (RBL), vector control (Vec), cells transfected with shRNA of scrambled sequences (Scr) and clathrin-knocked down cells (Clones #1, #2 and #3). Responses are expressed as an increase in the fluorescence ratio measured at 510 nm when excited alternating between 340 nm and 380 nm. Traces are representatives of at least 3 similar experiments. (E) Fluorescence ratio was arbitrarily quantitated by calculating the net increase in the ratio at the peak after NECA stimulation (Total) or at the peak after antigen stimulation in the presence of EGTA (ER Release). Concentrations of NECA and EGTA were 10 μ M and 1.2 mM, respectively. Values are means $\pm$ SD and significant differences compared to the control cells were determined by ANOVA and Tukey-Kramer multiple comparisons test.

FIG. 5. FcεRI-mediated SK activity was almost completely abolished in clathrin-knocked down cells while IP₃ production was not affected

(A) Production of S1P was measured in RBL-2H3 (RBL), vector-transfected (Cont) and clathrin-knocked down cells stimulated (■) or not (□) for 1.5 min with 100 ng/ml DNP₃₅-HSA. The fold increase in S1P production was calculated by dividing by the amounts of S1P produced in unstimulated RBL-2H3 cells. Values are means ± SD of 4 separate experiments. The pound signs indicate significant differences compared to unstimulated control cells ($p < 0.01$ (##) and 0.001 (###) and asterisks, compared to stimulated control cells ($p < 0.001$ (***) and 0.01 (**)) as determined by ANOVA and Tukey-Kramer multiple comparisons tests. (B) FcεRI-mediated production of IP₃ was measured 5 min after antigen stimulation (DNP₃₅-HSA, 10 ng/ml) in RBL-2H3 cells (RBL), vector-transfected (Cont) and clathrin-knocked down cells (shRNA clones). Values are means ± SEM of 3 separate experiments performed in duplicate. The pound signs indicate significant differences between unstimulated and stimulated cells at $p < 0.05$ (#) and 0.01 (##) as determined by paired t-tests.

FIG. 6. Fc ϵ RI-mediated increases in tyrosine phosphorylation were variably affected in clathrin-knocked down cells

(A) RBL-2H3 (RBL), vector-transfected (Cont) and clathrin-knocked down (shRNA Clones) cells were stimulated (S) or not (U) for 5 min with DNP₃₅-HSA (10 ng/ml) and cell lysates were immunoprecipitated with anti-phosphotyrosine (4G10) antibody. Immunoprecipitates were subjected to SDS-Page and Western blot analysis with 4G10. The prominent bands were identified by stripping and reprobing with antibodies detecting proteins indicated on the right side of the figure. (B,C,D) For confirmation and quantitation, blots from Figure 5A were reprobed with anti-Syk (B), anti-p85 PI3K (C) and anti-p110 PI3K (D) antibodies. For quantification, fold increase in the intensities of corresponding bands was calculated in comparison to those in unstimulated RBL-2H3 cells. Values are means $\pm$ SD of 5 separate experiments. The pound signs indicate significant differences between unstimulated and stimulated cells at $p < 0.05$ (#), 0.01 (##) and 0.001 (###) as determined by paired t-tests. The asterisks indicate significant differences compared to stimulated control cells at $p < 0.05$ (*), 0.01 (**) and 0.001 (***) as analyzed by ANOVA and Tukey-Kramer multiple comparisons test.

FIG.7. FcεRI-mediated degranulation was partially inhibited in clathrin-knocked down cells RBL-2H3 (RBL), vector-transfected (Cont) and clathrin-knocked down (Clones #1, #2, #3) cells sensitized overnight with 50 ng/ml DNP-IgE were stimulated with DNP₃₅-HSA at 37 °C for 15 min. Values are mean ± SEM obtained from 3 separate experiments performed in triplicate. The asterisks indicate significant differences compared to stimulated control cells at each concentration of DNP₃₅-HSA at p<0.01(**) and 0.001 (***) as analyzed by ANOVA and Tukey-Kramer multiple comparisons test.