Synaptotagmin II Negatively Regulates Ca\(^{2+}\)-triggered Exocytosis of Lysosomes in Mast Cells

By Dana Baram, Roberto Adachi, Ora Medalia, Michael Tuvim, Burton F. Dickey, Yoseph A. Mekori, and Ronit Sagi-Eisenberg

Summary

Synaptotagmins (Syts) I and II are believed to act as Ca\(^{2+}\) sensors in the control of neurotransmission. Here we demonstrate that mast cells express Syt II in their lysosomal fraction. We further show that activation of mast cells by either aggregation of Fc\(\varepsilon\)RI or by Ca\(^{2+}\) ionophores results in exocytosis of lysosomes, in addition to the well documented exocytosis of their secretory granules. Syt II directly regulates lysosomal exocytosis, whereby overexpression of Syt II inhibited Ca\(^{2+}\)-triggered release of the lysosomal processed form of cathepsin D, whereas suppression of Syt II expression markedly potentiated this release. These findings provide evidence for a novel function of Syt II in negatively regulating Ca\(^{2+}\)-triggered exocytosis of lysosomes, and suggest that Syt II–regulated secretion from lysosomes may play an important role in mast cell biology.

Key words: mast cells • lysosomes • calcium binding proteins • exocytosis • immunoglobulin E

Materials and Methods

Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; R BL, rat basophilic leukemia; RPMC, rat peritoneal mast cell; SG, secretory granule; SV, synaptic vesicle; Syt, synaptotagmin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Mast cells are specialized secretory cells that belong to the immune system. Through triggered exocytosis of their secretory granules (SGs), mast cells release biologically active substances, including vasoactive amines, proteases, and preformed cytokines. In addition, after their activation, mast cells produce and release arachidonic acid metabolites such as leukotrienes, prostaglandins, and multifunctional cytokines. Together, these mediators play central roles in both the immediate and late phase inflammatory reactions (1, 2). Although their physiological role in the body is less clear, mast cells importantly contribute to host defense against bacterial and parasite infections (3–8) as well as to cellular immune responses through their ability to present antigens and trigger antigen-specific T cell proliferation (9, 10).

Previous studies of exocytosis in mast cells indicate that the final trigger to exocytosis involves a late acting GTP-binding protein (11, 12) and Ca\(^{2+}\) (13, 14). The molecular identity of the mast cell exocytic Ca\(^{2+}\) sensor remains obscure. In the synapse, this role has been ascribed to synaptotagmins (Syts) I and II, abundant Ca\(^{2+}\) and phospholipid binding proteins localized on synaptic vesicles (SVs) (15–20). Binding of Ca\(^{2+}\) to Syt results in a conformational (21) or electrostatic (22) change that, by an as yet unresolved mechanism, allows exocytosis to occur. The finding that Syt I and II belong to a larger family of ubiquitously expressed proteins suggests that Syt isoforms may function as general Ca\(^{2+}\) sensors (23, 24). This hypothesis is supported by the recent demonstration of a role for a Syt isoform in controlling insulin secretion (25, 26).

We have recently reported that expression of Syt I in RBL-2H3 cells (a mucosal mast cell line) resulted in prominent potentiation and acceleration of Ca\(^{2+}\)-dependent exocytosis (27). Therefore, in this study we decided to identify the Syt isoform which is endogenously expressed in RBL cells, and explore its role in controlling exocytosis. We found that rat basophilic leukemia (RBL) cells endogenously express the Syt isoforms II, III, and V. The role of Syt II, the most abundant isoform in RBL cells, was investigated.
Isolation and Growth of Mast Cells. Bone marrow-derived mast cells (BM MCs) were obtained as previously described (28). In brief, femoral bone marrow cells from 6-wk-old BALB/c mice were cultured in 50% WEHI-3 cells conditioned medium. Culture medium was changed weekly, and nonadhering cells were used for further growth. After 3 wk, at least 99% of the cells were identified as mast cells by toluidine blue staining. Rat peritoneal mast cells (RPMCs) were obtained from Wistar rats by peritoneal lavage, and purified as previously described (29). In brief, a suspension of washed peritoneal cells was layered over a cushion of 30% Ficoll 400 (Pharmacia Biotech Inc.) in buffered saline and centrifuged at 150 g for 15 min. The purity of mast cells recovered from the bottom of the tube was >90%, as assessed by toluidine blue staining. RBL-2H3 cells (hereafter termed RBL cells) were maintained in adherent cultures in DMEM supplemented with 10% FCS in a humidified atmosphere of 6% CO₂ at 37°C.

Reverse Transcription and PCR Amplification of Syt cDNA Fragments. RNA was isolated from trypsinized RBL cells collected by centrifugation at 400 g for 5 min, and from brains that were rapidly excised from 150–200-g Sprague-Dawley rats killed by asphyxiation with CO₂. Total RNA was isolated on a guanidine thiocyanate/CsCl gradient, extracted twice with phenol/chloroform, and then ethanol precipitated. The mRNA was isolated from total RNA by oligo-dT cellulose chromatography [Poly(A)Pure; Ambion], and frozen until used. The mRNA was isolated from total RNA by oligo-dT cellulose chromatography [Poly(A)Pure; Ambion], and 2 μg was reverse transcribed by 125 U of Moloney’s murine leukemia virus-reverse transcriptase (New England BioLabs) in a 50 μl reaction containing 2.5 μg each of (dT)₂₀ and random octamers, 1 mM of each dNTP, and 40 U of RNAsin (Promega) at 37°C for 30 min, 42°C for 30 min, and 50°C for 15 min. The first round of nested PCR was performed with 1 μl of AmpliTaq (Perkin-Elmer Cetus) in 100 μl reaction buffer supplemented with 1.5 mM MgCl₂, 10% (vol/vol) DMSO, 1 μM of each primer, 50 μM of each dNTP, and 1 μl of the reverse transcription reaction as template. The four primers correspond to RNA sequences encoding portions of Syt proteins schematized in Fig. 1A, and their sequences were: A, TCWGACCCYTAYGTACMGGAAGAC; B, AGACCACGTGCACMGGAAGAC; C, SYCUTTTSACRAATGGGRTCTGA; D, GGTTGGTSGSAGTGTCTTCTTCTT. For the first round of PCR, six cycles of 94°C for 1 min ramping to 49°C in 3 min, 49°C for 1 min, 72°C for 1 min were followed by 24 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 6 min. 1 μl of the PCR product obtained with RBL cell cDNA and the A and D primers was used for template in a second round of PCR identical to the first except that the initial six cycles with low annealing temperature were not included. The product of the second reaction using the B and C primers was purified by agarose gel electrophoresis, then ligated into the pCR-II vector (Invitrogen). DH5α cells were transformed with the ligation mixture and colonies were selected for sequencing.

Ribonuclease Protection Assay. Vectors containing the PCR-cloned Syt fragments were linearized with NotI for SP6-directed synthesis of riboprobes or with BamHI for T7-directed cRNA synthesis. cRNA hybridization controls were generated in a 50 μl reaction containing 3 μg of template DNA, 1.6 U/μl Rnase, 10 mM dithiothreitol (DTT), 0.1 μg/ml BSA, 1 mM of each NTP, 2.5 μM [32P]UTP (45 Ci/mmole; Amersham), and 100 U T7 RNA polymerase (New England Biolabs) in the manufacturer’s buffer. Reactions were incubated for 4 h at 37°C, 10 U DNase I was added, and the incubation was continued for another 20 min, and then cRNA was purified on a Nick-Scan column (Ambion). Riboprobes were transcribed in a 20 μl reaction using 1 μg of template DNA, 25 μM α-[32P]UTP (800 Ci/mmole; Amersham), 2 U/μl Rnase, 10 mM DTT, 0.1 mg/ml BSA, 0.5 mM each of other NTPs, and 10 U SP6 RNA polymerase (New England Biolabs) in the manufacturer’s buffer for 1 h at 40°C. After treatment with 5 U of DNase I, full-length transcripts were isolated by gel purification in 5% acrylamide/8 M urea gels. RNAase protection assays were performed using the RPA II kit (Ambion). Cognate cRNA (0.1 pmol) and yeast RNA were used as positive and negative controls. Each experiment contained 1 pmol riboprobe and varying amounts of RBL cell rRNA supplemented with yeast rRNA to complete a total of 40 μg rRNA. Hybridization was carried out overnight at 45°C. Protected probes were electrophoresed through 5% acrylamide/8 M urea gels and visualized by autoradiography.

Preparation of Mast Cell and Brain Lysates. Mast cells (10⁶) derived from different sources (RPMCs, BM MCs, and RBL-2H3) were washed in PBS and resuspended in 30 μl of lysi buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.2% SDS, 50 mM NaF, 10 mM Na3P, 2 mM NaVO₄, 1 mM PMF, and 10 μg/ml leupeptin) and centrifuged at 12,000 g for 15 min at 4°C. The cleared supernatants were mixed with 5× Laemmli sample buffer to a final concentration of 1×, boiled for 5 min, and subjected to SDS-PAGE and immunoblotting. For the preparation of brain homogenate, whole brain from a Wistar rat was homogenized in PBS at 4°C using a Polytron (Kinematica, GmbH, Switzerland; 20 s setting 7). Aliquots (5–10 μg protein) were mixed with 5× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and immunoblotting.

Subcellular Fractionation of RBL Cells. RBL cells (7×10⁵) were washed with PBS and suspended in homogenization buffer (0.25 M sucrose, 1 mM MgCl₂, 800 U/ml DNase I (Sigma Chemical Co.), 10 mM Hepes, pH 7.4, 1 mM PMF, and a cocktail of protease inhibitors [Boehringer Mannheim, Germany]). Cells were then disrupted by 3 cycles of freezing and thawing followed by 20 passages through a 21-gauge needle. Unbroken cells and nuclei were removed by sequential filtering through 5- and 2-μm filters (Poretics Co.). The final filtrate was then centrifuged for 10 min at 500 g and the supernatant loaded onto a continuous 0.45–2.0 M sucrose gradient (10 ml), which was layered over a 0.3 ml cushion of 70% (wt/wt) sucrose and centrifuged at 180,000 g at 4°C. Histamine was assayed fluorometrically after condensation in alkaline medium with o-phthalaldehyde (30). LDH activity was assayed using LDH reagent according to the manufacturer’s instructions (Merck Diagnostica, Germany). Secretion from RBL cells. RBL-2H3 cells were seeded in 24-well plates at 2×10⁵ cells per well and incubated overnight in a humidified incubator at 37°C. The cells were then washed three times in Tyrode’s buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA) and stimulated in the same buffer with the indicated concentrations of the calcium ionophore A23187 and the phorbol ester 12-O-tetradecanoyl-13-acetate (TPA; Calbiochem). Secretion was allowed to proceed for 30 min at 37°C. Aliquots from the supernatants were taken for measurements of reduced β-hexosaminidase activity. Cells in control wells were lysed by addition of 0.1% Triton X-100 to determine the total
enzyme content. For FcεR1 induced secretion, cells were passively sensitized by overnight incubation with DNP specific monoclonal IgE (SPE7, a gift of Dr. Z. Eshhar, the Weizmann Institute of Science, Rehovot, Israel), washed three times in Tyrode's buffer, and then stimulated with the indicated concentrations of the antigen, DNP-BSA. Activity of the released β-hexosaminidase was determined by incubating aliquots (20 µl) of supernatants and cell lysates for 90 min at 37°C with 50 µl of the substrate solution consisting of 1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) in 0.1 M citrate pH 4.5. The reaction was stopped by the addition of 150 µl of 0.2 M glycine, pH 10.7. OD was read at 405 nm, in an ELISA reader. Results were expressed as percentage of total β-hexosaminidase activity present in the cells. To assay the release of cathepsin D, supernatants of cells, stimulated as above, were concentrated in VivaSpin concentrators with a 10 kD cut-off (VivaScience, UK). The concentrates were mixed with 5× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and immunoblotting with anti–cathepsin D antibodies. For measurement of serotonin release, cells were incubated overnight with 2 µCi of [3H]5-hydroxytryptamine (NEN), washed, and stimulated as above. Aliquots from the supernatants were taken for measurement of radioactivity.

SDS-PAGE and Immunoblotting. Samples (normalized according to protein content or number of cells) were separated by SDS-PAGE using 10 or 12% polyacrylamide gels. Blots were blocked for 3 h in TBST (10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk followed by overnight incubation at 4°C with the indicated primary antibodies. Blots were washed three times and incubated for 1 h at room temperature with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit or anti–mouse IgG; Jackson Research Labs.). Immunoreactive bands were visualized by the enhanced chemiluminescence method according to standard procedures.

Results

Expression Analysis of Endogenous Syt Isoforms. Primers were chosen from the conserved C2 domains (Fig. 1 A) that averaged 91% identity with known Syt isoforms. An initial round of PCR with four different primer pairs did not yield any visible product in reactions containing RBL cell cDNA, even though abundant product was obtained from brain cDNA using two different primer pairs (Fig. 1 B). When the PCR product of RBL cell cDNA with primers A and D was then used as template in a second round of PCR, the nested reaction with primers B and C yielded abundant product of the predicted size of 365 bp (Fig. 1 C). Sequencing the inserts of 21 colonies of subcloned PCR product yielded 10 colonies encoding a fragment of Syt II, 9 colonies encoding Syt III, and 2 colonies encoding Syt V. These findings were supported by the results of restriction digestions of the PCR product with multiple frequent cutting enzymes (data not shown) that were consistent with the presence of these three isoforms and did not indicate the presence of additional isoforms based on the known sequences of Syt isoforms.

For RNase protection assays were then performed to quantitatively assess the expression of Syt isoforms in RBL cells. Syt II was the most abundant (Fig. 2), and serial dilution of mRNA used to protect the Syt II probe indicated that this isoform was approximately fivefold more concentrated in RBL cells than in Syt III. The Syt V isoform was not protected even when mRNA was present at a level at least 10-fold higher than that which measurably protected the Syt II probe (Fig. 2), suggesting that Syt V mRNA is present in RBL cells at a concentration less than one-tenth the level of Syt II.

Because Syt II, which shares the highest homology with the predominant neural isoform Syt I (31), was the most abundant isoform, we chose to focus this study on Syt II. We next examined the expression of the Syt II protein using specific antibodies (mAb 8G2B, directed against the NH2 terminus of Syt II). A single immunoreactive protein was detected in RBL cells (Fig. 3, lane 2). Immunoreactivity in RBL cells (Mr ~80 kD) had less mobility on SDS-PAGE than immunoreactivity in the brain (Fig. 3, lane 1).
Nevertheless, an 80-kD Syt II–immunoreactive protein was also detected in lysates from fully differentiated, connective tissue-type, RPMCs (Fig. 3, lane 3) and primary murine BMMCs (Fig. 3, lane 4). These size differences in Syt II may thus arise from tissue-specific posttranslational modifications.

Effect of Syt II Overexpression on Ca\(^{2+}\)-induced Exocytosis.

To study the functional role of Syt II, we stably transfected RBL cells with neural Syt II cDNA and selected clones with increased levels (approximately twofold) of Syt II expression (Syt II\(^{-}\); Fig. 4, lanes 1–3) for further studies. Notably, transfection with neural Syt II cDNA resulted in overexpression of the same 80-kD Syt II–immunoreactive protein, strengthening the concept that the increased apparent Mr of RBL-Syt II was due to tissue-specific posttranslational modifications.

Overexpression of Syt II had no effect on the spontaneous release of the SG-associated enzyme, \(\beta\)-hexosaminidase (32). In the absence of any stimulus, both control cells (empty vector-transfected) and cells overexpressing Syt II released up to 5% of their total \(\beta\)-hexosaminidase (Fig. 5 A). However, in contrast to transfection with Syt I (27), overexpression of Syt II failed to potentiate \(Ca^{2+}\)-dependent exocytosis evoked by a \(Ca^{2+}\) ionophore alone (Fig. 5 A), or in the presence of phorbol ester (Fig. 5 B). Instead, a mild inhibition could be observed when the cells were triggered with low (<10 \(\mu\)M) concentrations of the \(Ca^{2+}\) ionophore.

Figure 2. RNase protection assay of RBL cell transcripts. Autoradiogram of the products of an RNase protection assay after PAGE. The riboprobes used for hybridization are listed in the top row, the amount of mRNA loaded in each lane is listed in the second row, and a size marker is shown on the left side. This figure is a representative example of an experiment that was repeated four times.

Figure 3. Expression of Syt II protein in mast cells. Whole lysates (10\(^6\) cell equivalents) derived from RBL-2H3 cells (lane 2), RPMCs (lane 3), BMMCs (lane 4), and a crude brain homogenate (lane 1, 10 \(\mu\)g protein), were resolved by SDS-PAGE and immunoblotted using the mAb 8G2B directed against the N\(_2\) terminus of Syt II.

Figure 4. Overexpression of Syt II in RBL cells. Whole lysates derived from G418-resistant RBL clones (1.5 \(\times\) 10\(^6\) cell equivalents), transfected with either the pcDNA3-Syt II recombinant vector (Syt II\(^{-}\), lanes 1-3) or with the empty pcDNA3 vector (control, lanes 4-6) were resolved by SDS-PAGE and immunoblotted using monoclonal 8G2B anti-Syt II antibodies.
Effects of Syt II on the Release of Cathepsin D. Lysosomes were recently shown to behave as Ca\textsuperscript{2+}-regulated exocytic vesicles (34). Since β-hexosaminidase is distributed between histamine-containing SGs and procathepsin D–containing lysosomes in RBL cells, it was important to determine whether secretion of the content of the latter compartment is negatively regulated by Syt II. To address this question, we examined whether Syt II could modulate release of the lysosomally processed form of cathepsin D (mature cathepsin D, Mr 43 kD) (35). Concentrating the cell supernatants by 20-fold allowed the detection of cathepsin D in supernatants from Ca\textsuperscript{2+} ionophore- or antigen-triggered cells (Fig. 9 A, lanes 1–3). The amount of secreted mature cathepsin D was significantly inhibited or increased in the Syt II\textsuperscript{+} or Syt II\textsuperscript{−} cells, respectively (Fig. 9). Ca\textsuperscript{2+} ionophore was more effective than the immunological stimulus in the Syt II\textsuperscript{+} cells, but did not differ significantly in Syt II\textsuperscript{−} cells. The precursor form of cathepsin D (53 kD) was detected in supernatants of both triggered and nontriggered cells (data not shown), reflecting the constitutive release of unprocessed cathepsin D (34). These results demonstrate that mast cell activation triggers exocytosis of a lysosomal fraction distinct from histamine-containing SGs, and that mobilization of this compartment depends substantially on the expression level of Syt II.

Effects of Syt II on the Release of Serotonin. We have also evaluated the effects of Syt II on the triggered release of serotonin, to exclusively monitor exocytosis of SGs (36). Overexpression of Syt II had no significant effect on serotonin release triggered by either secretagogue (Fig. 10, A–C). However, reducing its level of expression in the Syt II\textsuperscript{−} cells had a small but significant stimulatory effect (Fig. 10, A–C).

Discussion

Previous studies have already alluded to the possibility that Syt isoforms may serve the role of general Ca\textsuperscript{2+} sensors, controlling regulated exocytosis also in nonneuronal cells.
secretory cells (23, 25, 26). We and others have previously shown that mast cells express Syt and SNAREs that probably function to control mediators released from these cells (27, 37). Here, we demonstrate that RBL cells endogenously express at least three distinct isoforms of Syt, including Syt II, III, and V. Syt II was identified both by RNAase protection assays (Fig. 2) and at the protein level, on the basis of its immunoreactivity with specific antibodies (Fig. 3). However, in contrast to its location on SVs or SGs in neurons or endocrine cells, in the RBL cells, Syt II cofractionates with the lysosomal fraction rather than with the histamine-containing SGs (Fig. 8). Furthermore, transfection of the RBL cells with neural Syt II cDNA resulted in overexpression of Syt II (Fig. 4) and its targeting to the same fraction (Fig. 8).

**Mast cells** belong to immune cells of the hemopoietic lineage, where an intimate connection exists between lysosomes and SGs (38). The SGs of mast cells include, in addition to their secretory cargo of vasoactive amines (e.g., histamine and serotonin), lysosomal enzymes such as β-hexosaminidase, β-glucuronidase, arylsulfatase, and carboxypeptidases (32), as well as lysosomal integral membrane proteins (LIMPs) (39). Therefore, mast cell SGs can be defined as secretory lysosomes. Nevertheless, in consistence with previous data (40, 41), our data indicate that in addition to the lysosomal, amine-containing SGs, mast cells also contain lysosomes which lack biogenic amines and with which Syt II is associated (Fig. 8). Such amine-free lysosomes were previously reported to resist cell triggering by immunologic or Ca$$^{2+}$$ ionophore stimulation (40, 41). Whether the two popula-
tions of granules are sequentially formed and by what mechanism selective retention of the nonsecretory lysosomes is achieved, remained unknown. We now demonstrate that mast cells can, to some extent, release also their lysosomal pool of hydrolases, upon both an immunologic and a Ca$^{2+}$ ionophore trigger. In this process both lysosomal enzymes, which are distributed between both SGs and lysosomes, such as $\beta$-hexosaminidase, as well as hydrolases localized exclusively to the amine-free lysosomal fraction, such as cathepsin D (Fig. 9), are released. However, this release is inhibited by overexpression of Syt II and markedly potentiated by reducing the level of Syt II expression (Fig. 9). Recently, three types of granules were ultrastructurally distinguished in IFN-$\gamma$-treated mast cells (Table I). Type I and type II granules were both labeled by a fluid phase endocytic marker and both contained MHC class II as well as lysosomal markers (42). These results have therefore suggested their position in the endocytic pathway, similarly to lysosomal compartments (42). Serotonin was localized to type II and type III granules, of which the latter type did not internalize the fluid phase endocytic marker, nor did it contain MHC class II (42). Based on these results, it was suggested that a fusion event between type I (amine-free lysosomes) and III (e.g., SGs) granules may account for the formation of type II granules (42). Our results are compatible with this model and define Syt II as the molecular entity, which may control this fusion event and effect selective retention of the nonsecretory lysosomes during cell activation (see model shown in Fig. 11). Furthermore, this model predicts that downregulation of Syt II should also indirectly affect SG exocytosis by facilitating the fusion event between the amine-free lysosomes and SGs. Indeed, we found that suppression of Syt II level of expression also moderately potentiates serotonin release (Fig. 10).

The molecular mechanism by which Syt II inhibits lysosome exocytosis is currently unknown. Syts fall into three distinct classes that for syntaxin binding require either high Ca$^{2+}$ concentrations (200 $\mu$M) (class A) or low Ca$^{2+}$ concentrations ($\leq$1 $\mu$M) (class B), or do not bind syntaxin in a

| Granule type | MHC class II | $\beta$-hexosaminidase | Serotonin |
|--------------|--------------|------------------------|-----------|
| I            | +            | +                      | –         |
| II           | +            | +                      | +         |
| III          | –            | +                      | +         |

Based on Raposo et al. (42).

**Figure 9.** Release of Cathepsin D. (A) Control RBL cells (lanes 1–3), Syt II$^+$ cells (lanes 4–6), and Syt II$^-$ cells (lanes 7–9) were incubated for 30 min at 37°C with buffer (lanes 1, 4, and 7), 50 ng/ml of the DNP-BSA antigen (lanes 2, 5, and 8), or 10 $\mu$M of the Ca$^{2+}$ ionophore A23187 (lanes 3, 6, and 9). The concentrated cell supernatants were resolved by SDS-PAGE and immunoblotted using anti–cathepsin D (Cat D) antibodies. (B) The intensity of the band corresponding to mature cathepsin D was quantitated by densitometry (using a B.I.S. 202D densitometer, Dinko & Rheimni, Israel) and is presented as fold stimulation of the level in control, nonstimulated cells.

**Figure 10.** Release of serotonin. Control (○), Syt II$^+$ (●) and Syt II$^-$ (■) cells, loaded with $[^{3}H]$-5-hydroxytryptamine (serotonin), were stimulated for 30 min at 37°C with the indicated concentrations of the Ca$^{2+}$ ionophore A23187 alone (A), together with 50 nM TPA (B), or with the antigen DNP-BSA (C). The extent of serotonin release is presented as percentage of the total radioactivity in the cells. The data points presented are means ± SEM of 8–12 determinations and include three independent clones stably transfected with the empty pcDNA3 vector, three independent clones stably transfected with the pcDNA3-Syt II recombinant vector, and three independent clones stably transfected with pcDNA3-Syt II in the antisense orientation. Statistical analysis was performed using two-tailed Student's t test. *P < 0.05; **P < 0.01. Inset: serotonin release of individual clones (1–3 stably transfected with the empty pcDNA3 vector and 4–6 stably transfected with pcDNA3-Syt II in the antisense orientation) at a representative concentration of agonist: A, 10 $\mu$M A23187; B, 1 $\mu$M A23187; and C, 10 ng/ml DN P-BSA.
Ca\textsuperscript{2+}-dependent manner (class C) (20). Syt II, the major R B L isoform, and Syt V, the least abundant isoform, are class A proteins. However, although Ca\textsuperscript{2+} concentrations measured in neurons during an action potential are high enough to support Ca\textsuperscript{2+}-dependent interaction of class A Syts with syntaxins, the rise of intracellular Ca\textsuperscript{2+} concentrations (44), calcium-dependent Syts negatively regulate neuronal exocytosis at basal Ca\textsuperscript{2+} concentrations (44), whereas positive effects on exocytosis are observed only at elevated Ca\textsuperscript{2+} concentrations and are thought to depend on interaction with syntaxin (20). In the mast cells Syt II seems to increase the Ca\textsuperscript{2+} requirements for lysosomal exocytosis, since Ca\textsuperscript{2+} ionophore is far more effective than immunologic stimulation in triggering cathepsin D release from control cells, but both are equally potent in Syt II–cells (Fig. 9). It is of great interest that Syt II appears to be used in mast cells as a negative regulator of Ca\textsuperscript{2+}-dependent exocytosis and of a subclass of secretory vesicles, and is the first example to our knowledge. Syt II inhibitory function appears to be linked to its lysosomal association since Syt I, although highly homologous to Syt II, potentiated Ca\textsuperscript{2+}-dependent exocytosis of SG when transfected into the R B L cells, alongside its SG targeting (27). The reasons for this differential targeting of Syt I and Syt II remain unknown.

Although not proven here, the remaining Syt isoform expressed in R B L cells, Syt III, which is a class B protein, would be an adequate candidate to serve as the positive regulator of SG exocytosis, whose action is mimicked by transfected Syt I.

In conclusion, our findings provide unequivocal evidence for an active role of Syt II in negatively controlling Ca\textsuperscript{2+}-regulated lysosomal exocytosis. This observation extends the function of Syt II to regulation of exocytosis of secretory organelles exclusive to SVs or SGs. Specifically, mast cells regulation by Syt II may have important implications on their function as APCs in host defense mechanisms, as this process requires uptake and lysosomal processing of antigens, followed by presentation of M H C class II–peptide complexes on the mast cell surface (10, 45). Our model predicts that the cellular level of Syt II could be up- or downregulated to determine the balance of mast cell effector function between the secretion of inflammatory mediators from SG exocytosis and the presentation of antigen by externalization of M H C class II–containing lysosomes. Syt II may thus play a central role in controlling the physiological functions of mast cells.

We thank Drs. Y. Zick and D. Neumann for helpful discussions and a critical reading of this manuscript; and Drs. T. C. Sudhof, M. Takahashi, and Z. Eshhar for their generous gifts of cDNA and antibodies.

This work was supported by grants from the Israel Science Foundation, founded by the Israel Academy for Sciences and Humanities, and by the Thyssen Stiftung (to R. Sagi-Eisenberg).

Address correspondence to Ronit Sagi-Eisenberg, Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, 69978, Israel. Phone: 972-3-640-9500; Fax: 972-3-640-7432; E-mail: histol3.ccsyg.tau.ac.il

Received for publication 12 January 1999 and in revised form 22 March 1999.

References

1. Stevens, R. L., and K. F. Austen. 1989. Recent advance in the cellular and molecular biology of mast cells. Immunol. Today. 10:381–385.

2. Galli, S. O., J. R. Gordon, and B. K. Wershil. 1991. Cytokine production by mast cells and basophils. Curr. Opin. Immunol. 3:865–872.

3. M etcafe, D. D., M. K aliner, and M. A. Donlon. 1981. The mast cell. Crit. Rev. Immunol. 3:23–74.

4. M alaviya, R., T. Ikeda, E. R oss, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through T N F-alpha. Nature. 381:77–80.

5. Prodeus, A. P., X. Zhou, M. Maurer, S. J. Galli, and M. C. Carroll. 1997. Impaired mast cell-dependent natural immunity in complement C3-deficient mice. Nature. 390:172–175.

6. Echtenacher, B., D. N. Mannel, and L. Hultner. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. Nature. 381:75–77.

7. Abraham, S. N., and R. Malaviya. 1997. Mast cells in infection and immunity. Infect. Immun. 65:3501–3508.

8. Galli, S. J. 1997. The Paul Kallos memorial lecture. The mast
