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HPC-1/Syntaxin 1A Suppresses Exocytosis of PC12 Cells[1]

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The membrane protein syntaxin (originally named HPC-1) is involved in vesicle trafficking and required for neurotransmitter release at nerve terminals. The presence of syntaxin on target membranes is hypothesized to confer specificity to targeting and fusion *via* interactions with complementary vesicle-associated proteins. To elucidate the function of syntaxin 1A in exocytosis, HPC-1/syntaxin 1A-reduced PC12h cells (PC12h/Δsyx) that were stably transfected with a plasmid for antisense syntaxin 1A expression were constructed. Depolarizing stimulation of PC12h/Δsyx enhanced dopamine release, compared with PC12h. There was a strong inverse correlation between syntaxin 1A protein expression and enhancement of dopamine release. Reduction of syntaxin 1A had no effect on increase of the cytoplasmic free Ca^{2+} concentration by depolarized stimulation. Moreover, PC12h/Δsyx clones similarly enhanced of exocytosis by native secretagogues. These results indicate that syntaxin 1A has more than one function in exocytosis.

Key words: exocytosis, HPC-1, PC12h, SNARE, syntaxin.

The exocytotic process of hormones and neurotransmitters is central to intercellular communication and synaptic transmission. This process is accomplished by the calcium-triggered fusion of exocytotic vesicles with the plasma membrane (*1-3*). Syntaxin 1A (originally named HPC-1; *4*) is an integral plasma membrane protein and interacts with synaptotagmin, a synaptic vesicle membrane protein, and plasma membrane N-type calcium channels (*5, 6*). Syntaxin 1 is thought to be a receptor component for SNAPs [soluble *N*-ethyl-maleimide-sensitive factor (NSF) attachment proteins] and has a crucial role in the docking and fusion of synaptic vesicles with the plasma membrane.

Microinjection of soluble syntaxin 1A fragments suppresses calcium-regulated secretion in PC12 cells (*7*) and in neurons (*8*). Furthermore, clostridial botulinum neurotoxin C1 specifically cleaves syntaxin at a site located in the vicinity of the transmembrane domain and inhibits glutamate release from isolated nerve terminals (*9*). It is generally thought that the soluble and cleaved syntaxin fragments interfere with the formation of SNARE (SNAP receptor) complexes; therefore, syntaxin 1 may function as an accelerator of exocytosis. These results, however, are open to the opposite interpretation. If the fragments remain active or become functional following cleavage, the suppression of exocytosis would suggest that syntaxin 1A acts to suppress exocytosis. Thus, syntaxin 1A may have more than one function in the regulated secretary pathway. Indeed, it was reported recently that the overexpression of syntaxin 1A suppressed the release of coexpressed GH from COS-1 (*10*) and PC12 (*11*). Additionally, we have observed possible inhibitory roles of HPC-1/syntaxin 1A in the exocytosis process, as follows: (i) Anti-HPC-1/syntaxin 1A antibodies stimulate noradrenaline release from digitonin-permeabilized PC12h cells (*12*) and enhance the synaptic exocytosis in cultured hippocampal synapses (*13*); (ii) microinjection of capped RNAs for rat HPC-1 into newt embryonic cells inhibits the secretion of extracellular matrix (*14*); and (iii) overexpression of HPC-1/syntaxin 1A in the mouse β cell line βTC3 suppresses glucose-stimulated insulin release (*15*). Accordingly, dopamine release from HPC-1/syntaxin 1A-reduced PC12h cells (PC12h/Δsyn) was investigated to determine whether HPC-1/syntaxin 1A has another function as a suppressive regulator in exocytosis.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12h cells, a subline that expresses high levels of tyrosine hydroxylase activity (*16*), were maintained in Dulbecco's modified Eagle's medium with precolostrum newborn calf serum (5% v/v) and horse serum (5% v/v).

Plasmid Construction and Introduction into Cells—Antisense syntaxin 1A was constructed from the corresponding cDNA of rat HPC-1/syntaxin 1A (*4*). The antisense DNA expression vector was constructed as follows. An inverted 372 bp *Bam*HI/*Xba*I fragment of rat HPC-1/syntaxin 1A was ligated into a pRc/RSV expression vector (Invitrogen).

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; HBSS, Hanks' balanced salt solution; NSF, *N*-ethyl-maleimide-sensitive factor; PC12h/Δsyx, HPC-1/syntaxin 1A-reduced PC12h cells; SNAPs, soluble NSF attachment proteins; SNARE, SNAP receptor.

This construct was used to transfect PC12h cells by DOSPER™ (Boehringer Mannheim, Mannheim, Germany) and transfected cells were selected by use of Genetecin (400 mg/liter)(GIBCO).

Preparation of Cell Lysate—Cells were washed two times with phosphate-buffered saline (PBS) and treated with 20% trichloroacetic acid for 30 min on ice to precipitate proteins and inactivate proteinase. The precipitated proteins were scraped off the dishes using a rubber policeman and centrifuged. The pellets were dissolved and sonicated in ice-cold solubilizing solution containing 9 M urea, 2% Triton X-100, 1% dithiothreitol, and 5% lithium sulfate. The protein concentration was determined by the Bradford method (*17*) with bovine immunoglobulins as a standard. Solubilized samples were stored at −80°C.

Immunoblotting and Analysis—Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% gels, then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated in blocking buffer containing 5% (w/v) skim milk, then exposed to primary antibody, 14D8, a HPC-1 specific monoclonal antibody (*18*). After washing, the membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG, then, washed and soaked in enhanced chemiluminescence reagent (Amersham). The amount of HPC-1/syntaxin 1A protein was estimated by densitometric analysis and compared with an authentic HPC-1/syntaxin 1A protein.

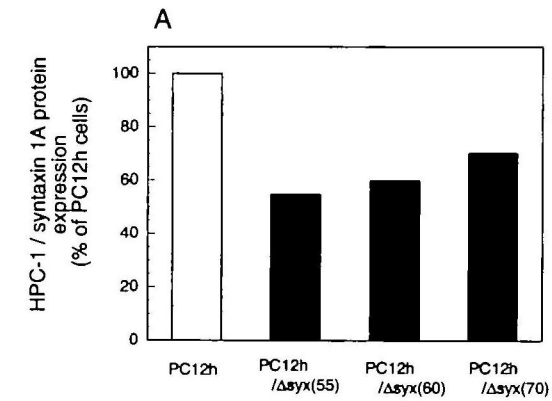
Electron Microscopy—The cells were fixed in pre-fixative solution (2.5% glutaraldehyde, 3% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4) for a day. After washing with the same buffer solution, they were post-fixed in 1% OsO_4 and 0.1 M cacodylate buffer at pH 7.4 for 2 h. They were then washed briefly in the buffer solution, dehydrated in alcohol, and embedded in epoxy resin. Ultrathin sections were cut and double-stained with uranyl acetate and lead citrate. The sections were examined with a JEM-100C transmission electron microscope (JEOL, Tokyo).

Quantification of Dopamine—Two days before the experiments, PC12h/Δsyx and PC12h were plated on collagen-coated 24-well plates. Dopamine release was measured as described previously (*19, 20*) with minor modifications. After replacing the medium in the plate with 500 μl of Hanks' balanced salt solution containing 0.05% bovine serum albumin (HBSS-BSA), the cells were preincubated for 10 min, then HBSS-BSA was replaced with a high K^+ solution (85 mM NaCl, 60 mM KCl, 2.5 mM $CaCl_2$, 5 mM $MgSO_4$, 11 mM glucose, 0.05% BSA, and 15 mM HEPES-KOH, pH 7.3). For native secretagogue stimulation, PACAP or carbachol was added to HBSS-BSA. After incubation for 15 min at 37°C, the amount of dopamine released in the medium was measured by high performance liquid chromatography with an electrochemical detector.

Measurement of Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_i$)—Cells plated on collagen-coated cover slips were used for single-cell $[Ca^{2+}]_i$ measurements. The medium was replaced with HBSS-BSA containing 4 μM fura-2/AM, and the cells were incubated at 25°C for 30 min. The cells were washed twice with normal solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 5 mM $MgSO_4$, 11 mM glucose, 15 mM HEPES-NaOH, pH 7.3) and fluorescence was measured in the perfusion bath with an image processor (ARGUS-50, Hamamatsu Photonics). The $[Ca^{2+}]_i$ was measured in normal solution, high-K^+ solution (60 mM), and high-K^+ solution containing 10 μM nifedipine. Data were obtained every 5 s and expressed as the means ± SE for 30 s from approximately 100 cells.

RESULTS

Antisense HPC-1/syntaxin 1A was constructed from the corresponding cDNA of rat HPC-1 (*4*) and introduced into PC12h cells. Quantitative analysis of this protein was carried out by immunoblotting analysis of total cell lysate prepared from antisense transformants. Three selected



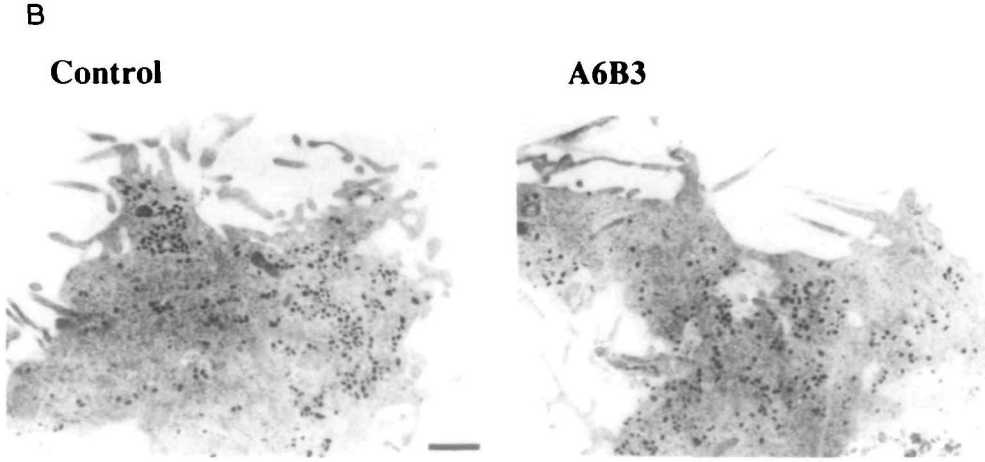


Fig. 1. **Clones stably transfected with antisense RNA of HPC-1/syntaxin 1A.** (A) The amount of HPC-1/syntaxin 1A protein was estimated by densitometric analysis of Western blotting using HPC-1/syntaxin 1A specific monoclonal antibody, 14D8 (*18*). Results are expressed as a percentage of the amount of HPC-1/syntaxin 1A protein in PC12h cells. (B) Clones of the syntaxin 1A reduction appeared to have normal intracellular organelles, especially vesicles, by electron microscopy. Scale bar, 1 μm.

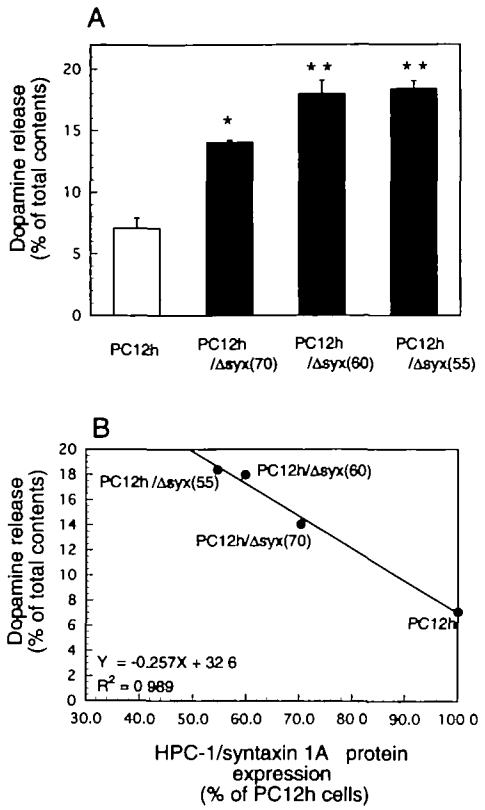


Fig. 2. **Increase of dopamine release from PC12h/⊿syx.** (A) Dopamine release after depolarization of PC12h/⊿syx and PC12h cells. The amount of dopamine released is expressed as a percentage of the total amount of dopamine stored in the cells. Results are expressed as the mean ± SE ($n = 3$–5). One-way ANOVA was used to compare the means of four groups, and the Dunnett's multiple comparison was used for post hoc analysis (**$p < 0.01$, compared with the amount of PC12h). A probability of less than 0.05 was considered to be significant. (B) Correlation between the amount of syntaxin 1A expressed and the amount of dopamine released after depolarization.

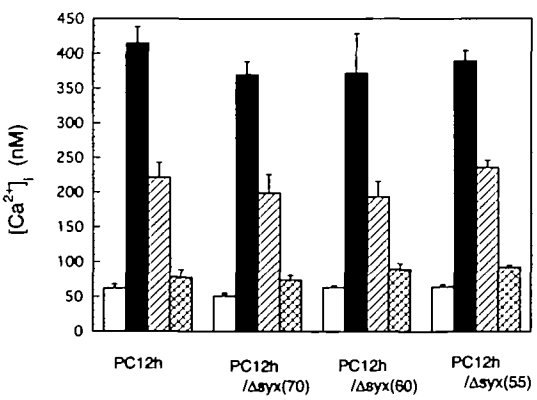


Fig. 3. **Cytosolic concentrations of free Ca^{2+} ($[Ca^{2+}]_i$) in PC12h and PC12h/⊿syx.** The $[Ca^{2+}]_i$ was measured in normal solution (open bar), high-K^+ solution (transient phase, solid bar; sustained phase, hatched bar) and high-K^+ containing 10 μM nifedipine (cross-hatched bar). Data were obtained every 5 s and expressed as the means ± SEM for 30 s from approximately 100 cells.

clones, PC12h/⊿syx(55), PC12h/⊿syx(60), and PC12h/⊿syx(70), expressed approximately 55, 60, and 70% of the syntaxin 1A protein of PC12h cells, respectively (Fig. 1A). The expression of syntaxin 1B, the isoform of syntaxin 1A but a distinct gene product from syntaxin 1A, was the same as that of PC12h cells (data not shown). The intracellular organelles of the clones, especially the vesicles, appeared normal by electron microscopy (Fig. 1B). The dopamine content and neurite extension induced by NGF treatment were also unchanged (data not shown).

To investigate the effect of syntaxin 1A reduction on exocytosis, the dopamine release from PC12h/⊿syx was measured. To rule out the possibility that stable expression of antisense mRNA affected the expression of the secretagogue receptor and second signal machinery, high K^+ buffer (60 mM) stimulation was selected. The dopamine release from PC12h/⊿syx was significantly higher than from PC12h cells (Fig. 2A). Furthermore, there was a strong inverse correlation between the expression of syntaxin 1A and the amount of dopamine released ($r^2 = 0.989$) (Fig. 2B).

These data demonstrated that the reduction of HPC-1/syntaxin 1A stimulated the exocytosis of dopamine. To determine whether HPC-1/syntaxin 1A reduction modifies Ca^{2+} channel activity, $[Ca^{2+}]_i$ was measured. None of the

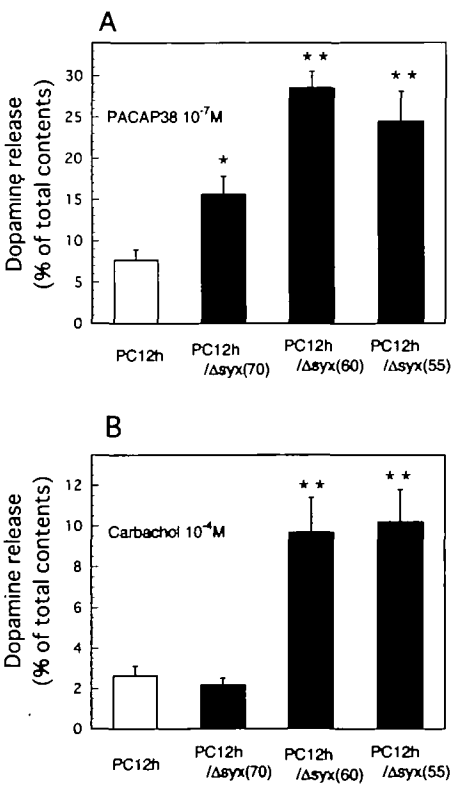


Fig. 4. **Increase of dopamine release from PC12h/⊿syx compared to PC12h cells by two secretagogues.** (A) Dopamine release by the peptidergic secretagogue PACAP (10^{-7} M) was measured as described in Fig. 2. Cells were preincubated in HBSS-BSA for 10 min, then PACAP was added. (B) Dopamine release by the cholinergic secretagogue carbachol (10^{-4} M). Results are expressed as the mean ± SEM ($n = 3$–5). One-way ANOVA was used to compare the means of four groups, and Dunnett's multiple comparison was used for post hoc analysis. *$p < 0.05$, **$p < 0.01$, compared with the value of PC12h cells. A probability of less than 0.05 was considered to be significant.

PC12h/⊿syx clones produced an increase in either basal $[Ca^{2+}]_i$ before stimulation or maximal $[Ca^{2+}]_i$ after high K^+ stimulation (Fig. 3). In addition, there was no difference between PC12h and PC12h/⊿syx clones in sustained phase $[Ca^{2+}]_i$ after high K^+ loading. This sustained phase $[Ca^{2+}]_i$ was decreased to the basal level by nifedipine (Fig. 3).

To examine the effects of the HPC-1/syntaxin 1A reduction on physiological exocytosis, two native secretagogues, PACAP (pituitary adenylate cyclase activating polypeptide, peptidergic; *21*) and carbachol (cholinergic), were used. PC12h/⊿syx(55) and PC12h/⊿syx(60) released significantly higher amounts of dopamine than PC12h cells when stimulated by PACAP or carbachol (Fig. 4). These results were the same as that of high K^+ stimulation. PC12h/⊿syx(70) also had enhanced dopamine release with PACAP compared to PC12h. The amount of dopamine released from PC12h/⊿syx(70) by carbachol stimulation, however, did not differ from that of PC12h.

DISCUSSION

Three clones were made of HPC-1/syntaxin 1A-reduced PC12h. The intracellular organelles, especially vesicles, appeared normal by electron microscopy (Fig. 1, A and B). Dopamine release from PC12h/⊿syx was, however, significantly higher than from PC12h (Fig. 2A), and there was a strong inverse correlation between the expression of syntaxin 1A and the amount of dopamine released (Fig. 2B). These results indicate that syntaxin 1A suppresses exocytosis.

In the SNARE hypothesis, synaptic vesicles dock to a SNARE complex (7S complex) on the plasma membrane, then the 7S complex assembles into a stable multimeric complex (20S complex) with α-SNAP and NSF. Upon ATP hydrolysis, the 20S complex dissociates (priming step) and membrane fusion proceeds (*1-3*). In the dissociation of the 20S complex by NSF following ATP hydrolysis, syntaxin 1A, particularly its amino terminal regions (*22*), is involved. If this ATP hydrolysis step is the rate-limiting factor of the priming step, syntaxin 1 could act as a stabilizer of the 20S complex and suppress the fusion of vesicles and the plasma membrane. Recently, it was reported that only v-SNARE (VAMP) and t-SNARE (syntaxin 1A and SNAP25), but not NSF and SNAP, could lead to spontaneous fusion of the docked membranes at physiological temperature (*23*). Consequently, syntaxin 1A is likely to have a negative role on exocytosis until ATP hydrolysis occurs, possibly in cooperation with a Ca^{2+} entry. Our results might be explained by the observation that the reduction of syntaxin 1A hastens the priming step with destabilization of the 20S complex and then increases exocytosis. More recently, it was reported that the overexpression of syntaxin 1A suppressed the release of coexpressed GH from COS-1 (*10*) and PC12 (*11*).

HPC-1/syntaxin 1A bound to N-type Ca^{2+} channels (*5*), and PC12 expressed both N- and L-type Ca^{2+} channels (*24*). Coexpression of syntaxin 1A with N-type Ca^{2+} channels in *Xenopus* oocytes inhibits the channels (*25*). Recently, it was demonstrated that syntaxin 1A also modifies the L-type Ca^{2+} channel in the *Xenopus* oocyte (*26*). If the observed inhibitory effect of syntaxin 1A is attributable to the modification of Ca^{2+} channel activity, PC12h/⊿syx would be expected to increase $[Ca^{2+}]_i$ when depolarized. There was no difference, however, between PC12h and PC12h/⊿syx in either basal $[Ca^{2+}]_i$ or evoked $[Ca^{2+}]_i$ after high K^+ loading (Fig. 3). Additionally, the sustained phase $[Ca^{2+}]_i$ after high K^+ stimulation was also similar in PC12h and PC12h/⊿syx clones. This sustained phase $[Ca^{2+}]_i$ was decreased to the basal level by nifedipine, indicating involvement of the L-type Ca^{2+} channel during this phase (Fig. 3). These results are consistent with the report by Avidor *et al.* (*27*), demonstrating that dopamine release is mediated by L-type Ca^{2+} channels in PC12 cells. Moreover, it was reported that syntaxin 1A produces no effect on L-type Ca^{2+} channels (*8, 25*). Consequently, the reduction of syntaxin 1A activated the exocytosis without affecting $[Ca^{2+}]_i$ regulation, particularly L-type Ca^{2+} channel activity.

In the case of native secretagogue stimulation, PC12h/⊿syx(55) and PC12h/⊿syx(60) released significantly higher amounts of dopamine than PC12h following PACAP and carbachol stimulation, as they did in the case of high K^+ stimulation (Fig. 4). This indicates that the reduction of HPC-1/syntaxin 1A in these two clones would affect only the exocytotic steps without altering the G-protein-coupled second messenger system, because these two secretagogues are known to be mediated by G-proteins (*19, 28*). PC12h/⊿syx(70) also showed enhanced dopamine release with PACAP compared to PC12h. Carbachol stimulation of PC12h/⊿syx(70), however, released the same amount of dopamine as PC12h. The difference between PACAP and carbachol in stimulation of PC12h/⊿syx(70) might be due to a difference between them in potency. Similar results were reported by Fujita *et al.* (*11*). They showed that overexpression of syntaxin 1A inhibited the high K^+-induced release of coexpressed GH from PC12 cells, but not low K^+-induced release.

In *Drosophila*, electrophysiologic recordings from syntaxin 1A hypomorph mutants (30% of wild-type syntaxin 1A levels) indicate an 80% reduction of evoked synaptic transmission (*29*). The results are in contrast to those obtained in our experiment. If the exocytosis is upregulated in the syntaxin 1A hypomorph mutants because of disinhibition by HPC-1/syntaxin 1A reduction, however, synaptic vesicles might have been depleted during the development of the neuromuscular junction. Additionally, the homologue of syntaxin 1A in *Drosophila* was also similar to syntaxin 1B (to rat syntaxin 1A, 82% similarity, 70% identity; to rat syntaxin 1B, 82% similarity, 68% identity). This homologue was associated with not only the presynaptic membrane but also with synaptic vesicles in *Drosophila* (*29*). In PC12, the syntaxin isoform associated with chromaffin granules was syntaxin 1B (*30*). Consequently, the syntaxin 1A homologue of *Drosophila* might also function as syntaxin 1B; and, the reduction might severely disrupt the exocytosis machinery. We previously found that overexpression of syntaxin 1A inhibited insulin release in the mouse β cell line βTC3, but syntaxin 1B did not (*15*). The functional redundancy and diversity of the syntaxin isoforms, particularly syntaxin 1A and 1B in PC12, remain to be definitively established.

In the syntaxin 1A-deficient *Drosophila*, which demonstrated embryonic lethality, the ultrastructure of embryonic synapses was indistinguishable from the wild type, and vesicle fusion could be triggered by hyperosmotic saline (*31*). This observation indicates that syntaxin 1A might

have a role not only in vesicle trafficking but also in downstream docking. In conclusion, the results of this study indicate that syntaxin 1A has more than one function and exerts a suppressive effect on the exocytosis process.

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