Presynaptic Trafficking of Synaptotagmin I Is Regulated by Protein Palmitoylation*

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Protein palmitoylation plays a critical role in sorting and targeting of several proteins to pre- and postsynaptic sites. In this study, we have analyzed the role of palmitoylation in trafficking of synaptotagmin I and its modulation by synaptic activity. We found that palmitoylation of N-terminal cysteines contributed to sorting of synaptotagmin I to an intracellular vesicular compartment at the presynaptic terminal. Presynaptic targeting is a unique feature of N-terminal sequences of synaptotagmin I because the palmitoylated N terminus of synaptotagmin VII failed to localize to presynaptic sites. We also found that palmitate was stably associated with both synaptotagmin I and SNAP-25 and that rapid neuronal depolarization did not affect palmitate turnover on these proteins. However, long-term treatment with drugs that either block synaptic activity or disrupt SNARE complex assembly modulated palmitoylation and accumulation of synaptotagmin I at presynaptic sites. We conclude that palmitoylation is involved in trafficking of specific elements involved in transmitter release and that distinct mechanisms regulate addition and removal of palmitate on select neuronal proteins.

Synaptic transmission requires appropriate protein targeting and assembly of pre- and postsynaptic elements. Protein sorting to distinct domains in polarized cells appears to begin in the Golgi/trans-Golgi network, where proteins can segregate and exit in separate transport vesicles (1). One mechanism that regulates protein trafficking is palmitoylation, a post-translational modification involving the addition of palmitate, a 16-carbon fatty acid, via a labile thioester linkage (2–5). In neuronal cells, palmitoylation is critical for sorting of several synaptic proteins (6). These include the postsynaptic density protein PSD-95, the AMPA1 receptor-binding protein, and the presynaptic proteins GAP-43 (growth-g ssociated protein of 43 kDa) and GAD-65 (7–11). Palmitoylation of the AMPA receptor-binding protein and PSD-95 is essential for clustering at the PSD (8–10), whereas palmitoylation of GAD-65 is important for presynaptic targeting (11, 12).

Acylation of several other axonal proteins as well as proteins associated with neurotransmitter release machinery has been recently reported (5, 6, 13). These include members of the synaptotagmin family that regulate synaptic vesicle trafficking and neurotransmitter release (14, 15). The synaptotagmin family includes 13 members characterized by a unique N-terminal region followed by a transmembrane domain, a cluster of cysteines (the putative palmitoylation site), a variable domain, and two C-terminal C2 domains (15–18). Synaptotagmin I, the most characterized member of the family, is proposed to act as a Ca2+ sensor for regulated exocytosis (19). Other abundant members of the family include synaptotagmins III and VII (15). Interestingly, synaptotagmin I is localized to synaptic vesicles, whereas synaptotagmin VII is localized on the plasma membrane opposite synaptic vesicle docking sites (15). Despite the striking differences in their subcellular distribution, the mechanisms involved in differential sorting of individual members of the synaptotagmin family remain unclear. SNAP-25 (synaptosome-g ssociated protein of 25 kDa) is another palmitoylated protein that is involved in regulated release of neurotransmitters at the presynaptic terminal (20, 21). The palmitoylated cysteines are contained within the linker region between the N- and C-terminal helices involved in association with the core synaptic fusion complex. Recent studies showed that palmitoylation of SNAP-25 relies on the secretory pathway and that acylation of SNAP-25 anchors the protein to the membrane (22, 23). Membrane targeting of SNAP-25 also relies on association with the SNARE protein syntaxin (24, 25).

In addition to the role of palmitate in the regulation of protein targeting to membranes and synaptic sites, palmitoylation is also reversible and dynamically regulated by specific cellular stimuli (26, 27). Recently, we identified palmitate turnover on PSD-95 at the synapse (6). This palmitate cycling occurs in an activity-dependent fashion, and acutely disrupting palmitoylation disperses synaptic clusters of PSD-95. Therefore, we asked whether palmitoylation of synaptotagmin I and SNAP-25, two major elements involved in regulated neurotransmitter release, is dynamic in neuronal cells and whether this process is modulated by synaptic activity.

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¶ The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SNARE, soluble NSF attachment protein receptor; APV, α-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; TTX, tetrodotoxin; GFP, green fluorescent protein; HA, hemagglutinin; GABA, γ-aminobutyric acid.
We here demonstrate that palmitoylation is critical for sorting synaptotagmin I to the intracellular vesicular pool at pre-synaptic contacts. The reduced presynaptic localization of the palmitoylation-deficient mutant correlated with an increase in surface expression of synaptotagmin I and reduced endocytosis, indicating that palmitoylation is required for internalization and sorting to the presynaptic vesicle pool. Pulse-chase experiments using [3H]palmitate in cultured cortical neurons showed that, in contrast with PSD-95 and GAP-43, palmitate turnover was undetectable on synaptotagmin I or SNAP-25 within 6 h of chase with unlabeled palmitate. Significantly, long-term blockade of synaptic activity enhanced the amount of palmitoylated SNAP-25 and synaptotagmin I. In contrast, treatment with botulinum toxin C reduced palmitoylation of synaptotagmin I but not SNAP-25. These findings show that distinct mechanisms regulate addition and removal of palmitate on select neuronal proteins and that this process is regulated by long lasting changes in synaptic activity and association with specific elements of the synaptic vesicle release machinery.

**EXPERIMENTAL PROCEDURES**

**Materials and Immunoblotting—**Dithiothreitol, phenylmethylsulfonyl fluoride, paraformaldehyde, bovine neurotoxin A, APV, CNQX, and TTX were obtained from Sigma. Botulinum neurotoxin C was obtained from Calbiochem. Horseradish peroxidase-labeled goat anti-mouse IgG was from Amersham Biosciences. Guinea pig anti-GFP antibody was from Biovendor, Zvonek, Czech Republic; mouse anti-HA antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-syntaxin monoclonal antibody was described previously (28). The following primary antibodies were used: anti-synaptotagmin I monoclonal (Synaptic System, Gottingen, Germany), anti-SNAP-25 (Sternberger Monolocals Inc.), anti-GAP-43 (Chemicon International, Inc., Temecula, CA), anti-PSD-95 (Affinity Bioreagent), and anti-synaptophysin (Sigma). [3H]Palmitic acid was obtained from PerkinElmer Life Sciences. For immunoblotting, primary antibodies were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were diluted in blocking solution containing 1% bovine serum albumin and 0.1% Tween 20 in Tris-buffered saline and incubated with membranes overnight at 4 °C. Labeled bands were visualized using ECL (Amersham Biosciences).

dNA Cloning and Mutagenesis—Construction of synaptotagmin mutants was done on overlap extension PCR. Wild-type and cysteine mutant synaptotagmins I and VII were generated as N-terminal fusions with a GFP or an HA tag and subcloned into XhoI/EcoRI and HindIII/EcoRI of pEGFP-N3 (Invitrogen). N- and C-terminal deletion mutants of synaptotagmin I were generated by PCR and subcloned into XhoI/EcoRI and HindIII/EcoRI of pEGFP-N3. GFP-tagged full-length SNAP-25 and mutants were generated by PCR and subcloned into the HindIII and EcoRI sites in-frame with GFP in a pEGFP-N3 construct. All mutations were verified by DNA sequencing.

**Primary Neuronal Culture, Transfection, and Immunofluorescence—**Neuronal cultures were prepared from embryonic day 18/19 rats. Cortical and striatal neurons were dissociated by enzyme digestion with papain followed by brief mechanical trituration. Cells were plated on poly-D-lysine (Sigma)-pretreated 6- or 24-well plates with glass coverslips (12 mm in diameter) and then maintained in neurobasal medium (Invitrogen) supplemented with B27, penicillin, streptomycin, and 1% glutamine. For analysis of presynaptic targeting, striatal cultures were used since they represent a more homogeneous population of neurons, composed mainly of medium spiny neurons. For transfection of the various constructs used, striatal cultures were transfected using the Effectene lipid-mediated gene transfer kit (QIAGEN Inc.). Coverslips were removed from culture wells and fixed in methanol for 10 min. The cells were fixed with Tris-buffered saline containing 0.1% Triton X-100 and blocked in the same buffer with 3% normal goat serum for 1 h. Primary antibodies were blocked to add blocking solution for 1 h at room temperature, followed by incubation with secondary antibodies conjugated to Cy2 and Cy3 fluorophores (diluted 1:200 in blocking solution) for 1 h at room temperature. Coverslips were then mounted on slides (Frost Plus, Fisher) with Fluoromount-G (Southern Biotechnology), and images were taken under a fluorescence microscope with a ×63 objective affixed to a Zeiss Axiovert inverted microscope.

**Cell Radiolabeling and Immunoprecipitation—**Cortical neurons (4 × 10⁶ cells/well) were labeled for 3 h in neurobasal medium containing 1 μCi/ml [3H]palmitic acid (57 Ci/mmol; PerkinElmer Life Sciences). For metabolic labeling with [3H]palmitate and [3S]methionine, cells were incubated for 3 min with methionine-free neurobasal medium and then for 1 h in medium containing 300 μCi/ml Expre35S (1175 Ci/mmol; PerkinElmer Life Sciences). For pulse-chase experiments using [3H]palmitate and [3S]methionine, cells were incubated for variable times in conditioned neurobasal medium supplemented with 100-fold excess unlabeled palmitate and methionine. For blocking synaptic activity, cells were treated with 100 μM each CNQX and APV, 100 nM TTX, or 10 mM kynurenic acid for 3 days before labeling. Labeled cells were washed with ice-cold phosphate-buffered saline and resuspended in 0.1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% SDS. After extracting the proteins for 5 min at 4 °C, Triton X-100 was added to 1% to neutralize the SDS in a final volume of 0.5 ml. Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C. For immunoprecipitation, samples were incubated with anti-synaptotagmin I, anti-SNAP-25, anti-PSD-95, and anti-GAP-43 monoclonal antibodies for 1 h at 4 °C. After the addition of 20 μl of protein G-Sepharose beads (Amersham Biosciences), samples were incubated for 1 h at 4 °C. Immunoprecipitates were washed three times with buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% Triton X-100. Washed SDS-PAGE sample buffers with 1% SDS were loaded in SDS-PAGE sample buffer with 1% SDS for 2 min, and analyzed by SDS-PAGE. For autoradiography, protein samples were separated by SDS-PAGE and dried under vacuum. For [3S]-labeled samples, gels were exposed overnight to Biomax MS (Eastman Kodak Co.) at −80 °C. For H-labeled samples, gels were exposed to Hyperfilm (Amersham Biosciences) with intensifying screens at −80 °C for 3–20 days.

**Quantitative Measurement of Presynaptic Targeting—**Images were acquired on a Zeiss Axiovert 200 motorized microscope with a monochrome 14-bit Zeiss AxioCam HR CCD camera at native resolution (1300 × 1030 pixels). The exposure time of the camera was adjusted to limit photobleaching and so that the maximum pixel intensity was approximately one-half to three-fourths saturating for cells with low-to-moderate expression levels (as determined by total pixel counts). 14-bit images were scaled to 16 bits and exported into Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada) where custom-written software routines were applied for image analysis. For analysis of axonal clustering of various transfected constructs, images were taken at individually optimized exposure times adjusted to prevent saturation of pixel values in each color channel. Within one image (field of view), usually one transfected cell was present, and the puncta of the axon of that cell were manually outlined. Cell bodies were not included. To quantify puncta numbers, a binary image (“mask”) was created by using image processing (equalization and thresholding). This mask image was then multiplied with the original image using Boolean arithmetic to yield a discrete number of clusters containing the original pixel values.

**Quantification of polarized protein expression of wild-type and mutant synaptotagmins I and VII in axons was performed on 10–15 samples; two to three independent transfections. Cells from each transfection were analyzed (see “Results” for details of the total number of cells analyzed in each experiment). For each cell, the brightness of axonal clusters was determined by calculating the average pixel intensity in the axon versus axonal puncta. These averages were then converted into a ratio of axonal puncta versus axonal background. Data obtained from independent transfections were pooled and analyzed using the two-tailed non-parametric Mann-Whitney U test to compare changes in protein clusters between experimental groups.

For analysis of changes in clustering of endogenous proteins upon treatment with 2-bromopalmitate and glutamate receptor blockers, protein puncta were quantified on at least 15 fields chosen randomly using bright-field from three independent neuronal cultures. After treatment with various drugs, the intensity of the puncta in each field was determined by calculating the average pixel intensity of the puncta at intensities at least twice the background signal. Images acquired from both controls and treated cells were taken at equal exposure times. The values obtained for each treatment were then compared with those obtained for controls performed in parallel at the same time. Data obtained from independent experiments were pooled, and experimental parameters were analyzed to compare changes in protein clusters between experimental groups.

**Surface Labeling of Synaptotagmin I—**Neurons were fixed in 2% paraformaldehyde with 4% sucrose at room temperature for 10 min. Cells were washed with phosphate-buffered saline and then incubated with antibody that recognizes the N-terminal luminal domain of synaptotagmin I (epitope 604.1, Synaptic Systems) at 1:1000 dilution for 1 h at room temperature. Neurons were washed with phosphate-buffered saline.
FIG. 1. Palmitoylation regulates presynaptic targeting of synaptotagmin I. A, five cysteines at the boundary between the cytoplasmic tail and the transmembrane domain of synaptotagmin I are required for palmitoylation. HA-tagged wild-type (WT) synaptotagmin I (SYT I) and the cysteine mutants Cys mut1 (Cys-74, Cys-75, Cys-77, Cys-79, and Cys-82 to Ser) and Cys mut2 (Cys-77, Cys-79, and Cys-82 to Ser) were expressed...
erased saline and incubated for 1 h with Cy3-conjugated secondary antibodies before mounting. Analysis of the intensity of surface expression in axons was determined by calculating the ratio of signal intensity of surface synaptotagmin I in transfected/untransfected cells present in the same field of view. Changes in the amounts of surface synaptotagmin I in 11 cells expressing wild-type and palmitoylation-deficient forms of synaptotagmin I obtained from two independent transfections were then analyzed using the two-tailed non-parametric Mann-Whitney U test.

Endocytosis Assays and Antibody Uptake—PC12 cells were transfected with synaptotagmin I constructs using LipofectAMINE reagent (Invitrogen). 2 days later, cells were washed on ice with solution containing 120 mM NaCl, 2.5 mM MgCl₂, 2 mM CaCl₂, 25 mM HEPES, 30 mM glucose, and 3% bovine serum albumin. Cells were then incubated with primary antibody against the luminal domain of synaptotagmin I for 1 h on ice or at 37 °C. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with anti-HA primary antibody followed by secondary antibodies to visualize total and endocytosed proteins, respectively. Images of HA-stained transfected cells were taken with a Zeiss Meta confocal microscope with a 1.2-numerical aperture water lens with a correction collar (512 × 512 pixel resolution; excitation 1, argon 488 nm at 10%; emission 1, Bandpass Filter 505–530; excitation 2, HeNe 543 nm at 80%; emission 2, Longpass Filter 560 nm). Cells chosen from 5–10 fields of view (HA staining; channel 1) were used for analysis of antibody uptake on channel 2. The intensity of antibody uptake was measured using a line profile across the cell in the middle z-section using Zeiss LSM 5 META Image Examiner.

RESULTS

Palmitoylation Regulates Presynaptic Sorting of Synaptotagmin I—Previous studies showed that protein palmitoylation is important for trafficking of several synaptic proteins, including PSD-95, AMPA receptor-binding protein, and GAD-65 (8–11). Five Cys residues (Cys-74, Cys-75, Cys-77, Cys-79, and Cys-82) present in the junction between the transmembrane and cytoplasmic regions of synaptotagmin I have been shown to be palmitoylated (18). Here, we asked whether palmitoylation contributes to presynaptic targeting of synaptotagmin I. To assess this, we first constructed HA-tagged wild-type and cysteine mutant forms of synaptotagmin I by replacing the five palmitoylated cysteines with Ser residues. Simultaneous mutation of all cysteine residues (Cys mut1) abolished palmitoylation of synaptotagmin I in heterologous cells (Fig. 1A). In contrast, mutation of three cysteines (Cys-77, Cys-79, and Cys-82; Cys mut2) only reduced protein palmitoylation (Fig. 1A), indicating that efficient palmitoylation requires all five cysteines present in this region.

To determine whether palmitoylation is involved in presynaptic targeting of synaptotagmin I, we compared the localization of the HA-tagged wild-type and palmitoylation-deficient (Cys mut1) forms of synaptotagmin I in cultured striatal neurons. We found that wild-type synaptotagmin I was targeted to axons with a prominent punctate pattern (Fig. 1B, upper left panel). However, the palmitoylation-deficient mutant (Cys mut1) exhibited mainly a diffuse staining in axons (Fig. 1B, upper right panel). Quantification of the mean ratio of axonal puncta relative to axonal background revealed that the average intensity of the axonal puncta of wild-type synaptotagmin I was 5.38 ± 1.16 (mean ± S.D., n = 14) (Fig. 1C). In comparison, the mean ratio of puncta to background intensity in axons of the cysteine mutant was 2.70 ± 0.64 (mean ± S.D., n = 18) or 50% of the wild-type form. Similar results were obtained using the GFP-tagged wild-type and cysteine mutant forms of synaptotagmin I (Fig. 1B, lower panels) (data not shown). The reduced presynaptic targeting of palmitoylation-deficient synaptotagmin I indicates that palmitoylation contributes to sorting of synaptotagmin I to presynaptic terminals.

Consistent with the punctate localization in axons, wild-type synaptotagmin I co-localized with the presynaptic vesicle marker synaptophysin (Fig. 1D). The reduced synaptic targeting of palmitoylation-deficient synaptotagmin I may have resulted either from inefficient sorting to presynaptic vesicles or from enhanced protein degradation due to misfolding and instability of the mutated protein. To differentiate between these possibilities, we utilized pulse-chase experiments with [35S]methionine to determine whether the half-life of palmitoylation-deficient synaptotagmin I is altered. This analysis revealed no significant difference in the half-life of the wild-type and cysteine mutant forms of synaptotagmin I (Supplemental Fig. 1), indicating that inefficient sorting rather than enhanced degradation may have contributed to the altered trafficking of palmitoylation-deficient synaptotagmin I.

We next addressed whether palmitoylation regulates trafficking and presynaptic targeting of SNAP-25. Strikingly, palmitoylation-deficient SNAP-25 showed reduced accumulation in a perinuclear compartment resembling the cis-Golgi (Supplemental Fig. 2). Moreover, wild-type but not cysteine mutant SNAP-25 exhibited punctate pattern in the axons; however, the enrichment of SNAP-25 in the axonal puncta was not significantly different from axonal background, and the contribution of palmitoylation to presynaptic localization of SNAP-25 could not be assessed (Supplemental Fig. 2) (data not shown).

Recent studies showed that N-glycosylation is important for sorting synaptotagmin I from the plasma membrane to an intracellular vesicular compartment (29). To assess whether palmitoylation plays a role in sorting synaptotagmin I to an intracellular presynaptic compartment, we compared the surface localization of both the wild-type and cysteine mutant forms of synaptotagmin I in non-permeabilized neurons using antibody that recognizes the N-terminal luminal domain of synaptotagmin I (Fig. 2). Consistent with the previous study by Han et al. (29), we found that surface expression of synaptotagmin I was modestly enhanced in neurons expressing GFP-tagged wild-type synaptotagmin I (134 ± 6%, mean ± S.D., n = 11). Remarkably, GFP-positive axonal puncta were devoid of surface synaptotagmin I, indicating the sequestration of GFP-tagged synaptotagmin I to an intracellular presynaptic vesicle pool. In contrast, a significant increase in surface expression of synaptotagmin I was observed in neurons expressing the palmitoylation-deficient mutant (181 ± 8%, mean ± S.D., n = 11).

The correlation between the increase in surface expression and the lack of accumulation of palmitoylation-deficient synaptotagmin I at presynaptic terminals indicates that palmitoylation is involved in protein internalization and sorting to the presynaptic vesicle pool. To test this possibility, we utilized PC12 cells to assess whether endocytosis of the cysteine mutant is altered. For this analysis, PC12 cells were first transfected with the HA-tagged wild-type and palmitoylation-deficient forms of synaptotagmin I. 2 days post-transfection, cells
were incubated for 1 h at 0 or 37 °C with antibody that recognizes the luminal domain of synaptotagmin I. Cells were then fixed, permeabilized with 0.1% Triton X-100, and incubated with anti-HA primary antibody followed by secondary antibodies to visualize total and endocytosed proteins, respectively. Consistent with the previous finding by Han et al. (29), wild-type synaptotagmin I was not detectable on the cell surface at both 0 °C (n = 7) and 37 °C (n = 20), but rather in an intracellular pool that could be observed upon uptake of surface antibodies at 37 °C (Fig. 3). In contrast, efficient surface labeling was observed in cells expressing the palmitoylation-deficient mutant at both 0 °C (n = 10) and 37 °C (n = 22). These

**FIG. 2.** Palmitoylation of synaptotagmin I regulates surface expression and sorting to an intracellular presynaptic compartment. 

_A_, neurons were transfected with GFP-tagged wild-type (WT) synaptotagmin I (SYT I) or palmitoylation-deficient mutant Cys mut1 (green). Neurons were fixed without permeabilization and then stained for synaptotagmin I (surface synaptotagmin I; red) with antibody that recognizes the luminal domain of synaptotagmin I (epitope 604.1). Higher magnification micrographs are shown to the right. The images show that the GFP-positive axonal puncta of GFP-tagged wild-type synaptotagmin I were present mainly in an intracellular pool. In contrast, the GFP-tagged Cys mut1 signal was detected on the axonal surface. Scale bars = 10 μm. _B_, quantitative analysis showed enhanced surface staining in the axons of neurons expressing GFP-tagged Cys mut1 but not in those expressing GFP-tagged wild-type synaptotagmin I. **, p < 0.001; ***, p < 0.0001.
results demonstrate that palmitoylation contributes to proper sorting and sequestering of synaptotagmin I from the plasma membrane to an intracellular vesicular pool in PC12 cells and to presynaptic terminals in neurons.

**The Palmitoylated N-terminal Domain of Synaptotagmin I but Not Synaptotagmin VII Targets to Presynaptic Sites—**We next generated constructs containing either the N- or C-terminal region of synaptotagmin I to define sequences sufficient for presynaptic targeting. For this analysis, we generated two deletion constructs, one including amino acids 1–98 (SYT I NT) and the other containing amino acids 72–421 (SYT I CT). Since palmitoylation was required for efficient presynaptic targeting of the full-length protein, deletion constructs were designed to include the palmitoylation motif (Fig. 4A). Metabolic labeling experiments showed that both N- and C-terminal sequences were palmitoylated (Fig. 4B). Quantification of the ratio of

**FIG. 3.** Blocking palmitoylation diminishes endocytosis of synaptotagmin I. A, PC12 cells were transfected with HA-tagged wild-type (WT) synaptotagmin I (SYT I) or Cys mut1. 2 days later, cells were incubated with primary antibody against the luminal domain of synaptotagmin I for 1 h either at 37 °C or on ice (0 °C). Cells were then fixed, permeabilized, and stained with anti-HA antibody before labeling with secondary antibodies (Alexa 488 for HA and Alexa 568 for synaptotagmin I). B, the results obtained from measuring the intensity of antibody uptake using a line profile across the cell are summarized. *, p < 0.01.
axonal puncta intensity to axonal background revealed that N-terminal but not C-terminal sequences of synaptotagmin I were efficiently targeted to presynaptic sites (Fig. 4, C–E). The average intensity of the puncta in axons relative to axonal background for SYT I NT was 5.31 ± 1.16 (mean ± S.D., n = 12). In contrast, the mean ratio of axonal puncta in neurons expressing SYT I CT was 1.94 ± 0.62 (n = 9). These results show that palmitoylation is essential for sorting to axonal terminals, but that additional N-terminal sequences of synaptotagmin I are required for presynaptic targeting.

Synaptotagmin VII is a closely related member in the family that contains three conserved cysteines that are possible sites for palmitoylation (15). Indeed, our analysis showed that full-length synaptotagmin VII was palmitoylated and that its palmitoylation relies on Cys-35, Cys-38, and Cys-41 (Supplemental Fig. 3). Moreover, synaptotagmin VII puncta were detected in axons (3.82 ± 0.51, n = 7), although the average intensity of these puncta was less than that observed for synaptotagmin I (Fig. 4E and Supplemental Fig. 3). Conservation of the palmitoylation motif suggests that palmitoylation represents a general mechanism involved in sorting members of the synaptotagmin family to axonal terminals. Hence, we analyzed whether the N-terminal sequences of synaptotagmin VII (SYT VII NT) containing the palmitoylated cysteines are sufficient for presynaptic targeting. This analysis revealed that, in contrast with SYT I NT, the average intensity of the puncta in axons for SYT VII NT was 2.17 ± 0.35 (n = 9) (Fig. 4, D and E).

Thus, alternative mechanisms are involved in regulating presynaptic sorting of individual members of the synaptotagmin family.

Previous functional studies indicated that calcium- and membrane-dependent assembly of synaptotagmin I into multimeric structures regulates membrane dynamics during exocytosis and/or endocytosis (14, 15, 30–34). Furthermore, calcium-independent multimerization of synaptotagmin I requires the palmitoylated cysteine cluster (32, 33, 35). Consistent with these findings, we found that palmitoylation was involved in synaptotagmin I multimerization and that multimers of endogenous synaptotagmin I were also palmitoylated (Supplemental Fig. 4). These results indicate that palmitoylation-dependent multimerization may also contribute to appropriate sorting of synaptotagmin I to presynaptic sites.

Stable Palmitoylation of Synaptotagmin I and SNAP−25 in Cortical Neurons—Protein palmitoylation is a reversible process, with a half-life of palmitate on certain proteins ranging from 90 to 120 min (3, 5). With such a rapid turnover, palmitoylation may also be important for regulating protein function. Therefore, we examined whether the addition and/or removal of palmitate on synaptotagmin I and SNAP−25 is dynamic at the presynaptic terminal. Assessment of synaptotagmin I and SNAP−25 palmitoylation was monitored by metabolic labeling and pulse-chase experiments (Fig. 5). Cortical neurons were first labeled with [3H]palmitate for 3 h and then chased with unlabeled palmitate for different time periods. Samples were analyzed by autoradiography of immunoprecipitated products. Surprisingly, we found no detectable change in the amounts of palmitoylated synaptotagmin I and SNAP−25 up to 6 h post-chase with unlabeled palmitate (Fig. 5A). Moreover, labeling with [35S]methionine showed that newly synthesized synaptotagmin I and SNAP−25 were also stable during the 6-h chase period. In contrast, the half-life of palmitate on the postsynaptic protein PSD−95 was ~3 h (Fig. 5C).

The decrease in palmitoylation of PSD−95 could have resulted from a decrease in palmitoylation or an increase in protein turnover. However, labeling with [35S]methionine showed that newly synthesized PSD−95 protein was stable during the chase period. Therefore, a decrease in PSD−95 palmitoylation is most likely due to a loss of palmitate association rather than a change in protein synthesis. We next assessed whether treatment with 2-bromopalmitate, an agent that blocks protein re-palmitoylation, alters synaptic localization of PSD−95 and synaptotagmin I (Supplemental Fig. 5). Quantitative analysis showed that this treatment significantly reduced the intensity of PSD−95 puncta; however, no change in the intensity of synaptotagmin I puncta was observed. The lack of change in the localization of synaptotagmin I is consistent with the slow turnover rate of palmitate on synaptotagmin I but not PSD−95.

To determine whether the differential turnover rate of palmitate is due to differences in protein localization (presynaptic versus postsynaptic), we examined the turnover of palmitate on GAP−43, another axonally localized protein. We found that the half-life of palmitate on GAP−43 was ~3 h, similar to that of PSD−95 (Fig. 5D). These results suggest that the stable association of palmitate with synaptotagmin I and SNAP−25 is a unique feature of proteins associated with the SNARE complex.

Long-term Blockade of Synaptic Activity Modulates Palmitoylation of SNAP−25 and Synaptotagmin I—Activity-regulated addition and removal of palmitate on several neuronal and non-neuronal proteins have been proposed to provide a mechanism for rapid modulation of function and/or trafficking of several molecules (6). To test whether acute activation of vesicular neurotransmitter release triggers fast turnover of palmitate, we assessed the levels of palmitoylated synaptotagmin I, SNAP−25, and PSD−95 after acute treatment with 90 mM KCl, which triggers neuronal depolarization and rapid neurotransmitter release from all presynaptic terminals. For these experiments, cortical neurons were labeled with [3H]palmitate for 3 h and then treated either with vehicle or with 90 mM KCl for 15 min before harvesting. Synaptotagmin I, SNAP−25, and PSD−95 were immunoprecipitated and subjected to Western blot analysis and autoradiography. To assess changes in the fraction of palmitoylated proteins after these treatments, the levels of [3H]palmitate-labeled products were normalized to the amounts of immunoprecipitated proteins. In agreement with our previous work (6), increased neuronal activity induced rapid de-palmitoylation of PSD−95 (Fig. 6, A and B). However, no change in the levels of palmitoylated SNAP−25 and synaptotagmin I was observed. These results demonstrate that acute induction of neuronal activity induces rapid de-palmitoylation of specific acylated neuronal proteins.

To examine whether a prolonged change in synaptic activity could modulate palmitoylation of synaptotagmin I, we treated cortical neurons with 40 μM bicuculline, an agent that enhances excitatory synaptic activity by blocking the inhibitory GABAergic transmission. However, this treatment did not result in a significant change in palmitoylation of synaptotagmin I (data not shown). As an alternative approach, long-term blockade of synaptic activity was used to assess changes in protein palmitoylation. For this analysis, neurons were exposed to several agents that block synaptic transmission before labeling with [3H]palmitate (Fig. 6, C–E). These include the glutamate receptor blockers CNQX (AMPA receptor-specific), APV (N-methyl-d-aspartic acid receptor-specific), and kynurenic acid (a non-selective ionotropic glutamate receptor blocker). Treatment with the Na+ channel blocker TTX was also performed to block action potential-dependent neurotransmitter release. In these experiments, cultured cortical neurons were treated with 100 μM each CNQX and APV, 100 nM TTX, or 10 mM kynurenate for 3 days. Next, cells were labeled with [3H]palmitate for 3 h, and the effects on palmitoylation of
FIG. 4. N-terminal sequences of synaptotagmin I are sufficient for presynaptic targeting. A, the key features of the constructs used are summarized. SYT I WT, wild-type synaptotagmin I; TM, transmembrane domain. B, GFP-tagged SYT I NT, SYT VII NT, and SYT I CT were expressed in COS cells and labeled for 3 h with [3H]palmitate. Proteins were then immunoprecipitated with anti-GFP antibody and subjected to Western blotting and autoradiography. C, cultured neurons were transfected with the indicated constructs and immunostained for microtubule-associated protein-2 (MAP2; red). SYT I NT (but not SYT VII NT or SYT I CT; green) was targeted to clusters in axons (arrowheads). D, the graph summarizes the changes in the intensity of the axonal puncta of GFP-tagged full-length wild-type synaptotagmin I (SYT I WT) and synaptotagmin VII (SYT VII WT) and the truncated forms of these proteins. **, p < 0.001. E, the axonal puncta of SYT I NT co-localized with the presynaptic marker synaptophysin (arrowheads). Scale bars = 10 μm.
synaptotagmin I and SNAP-25 were analyzed by autoradiography. As shown in Fig. 6, all treatments induced significant increases in the levels of palmitoylated synaptotagmin I and SNAP-25. The increase in the pool of palmitoylated synaptotagmin I also correlated with the enhanced intensity of endogenous synaptotagmin I puncta upon treatment with 100 μM each CNQX and APV (Fig. 6, F and G). Similar results were obtained in cultured hippocampal neurons (data not shown).

It is important to note that treatments that enhanced neuronal activity did not have reverse effects on palmitoylation of synaptotagmin I. This is possibly due to a high basal level of activity in our culture system, which may have masked the detection of changes in the levels of palmitoylated proteins upon further enhancement of neuronal activity.

Next, we assessed changes in palmitoylation upon treatment with drugs that cleave SNARE proteins and block neurotransmission. For this analysis, cultured cortical neurons were treated either with 10 nM botulinum toxin A, which specifically cleaves SNAP-25, or with 300 nM botulinum toxin C, which cleaves both SNAP-25 and syntaxin. In agreement with the changes observed with activity blockers, treatment with botulinum toxin A resulted in enhanced palmitoylation of both SNAP-25 and synaptotagmin I (Fig. 6). In contrast, treatment with botulinum toxin C, which disrupts the association of synaptotagmin I with both syntaxin and SNAP-25, dramatically reduced palmitoylation of synaptotagmin I but not SNAP-25 (Fig. 7). These opposite effects are intriguing and suggest that stability of palmitate on synaptotagmin I is modulated by association with specific elements of the SNARE complex.

**DISCUSSION**

In this study, we have demonstrated that palmitoylation contributed to targeting of synaptotagmin I to the presynaptic terminal. Unlike synaptotagmin I, the palmitoylated N terminus of synaptotagmin VII was not sufficient for targeting to presynaptic sites. Another striking finding depicted by our analysis of several acylated proteins is that acute changes in synaptic activity differentially regulated palmitate turnover on various neuronal proteins at different time scales.

Our analysis revealed that both synaptotagmins I and VII were targeted to presynaptic sites, although synaptotagmin VII sorting was less efficient. These results are in contrast with the recent study showing robust synaptotagmin VII sorting to presynaptic sites in hippocampal cultures (36). The inefficient presynaptic targeting of synaptotagmin VII observed here is most likely due to differences in the type and density of cultures used.

We also found that N-terminal sequences of synaptotagmin I but not synaptotagmin VII were sufficient for presynaptic targeting, implying that specific presynaptic targeting motifs may be present only in a subset of members of the synaptotagmin family. In agreement with these results, recent elegant studies by Han et al. (29) showed that N-terminal sequences of synaptotagmin I but not synaptotagmin VII are critical for vesicular

**FIG. 5. Stable association of palmitate with synaptotagmin I and SNAP-25 in cortical neurons.** Cortical neurons were labeled either with [3H]palmitate for 3 h or with [35S]methionine for 1 h and then chased for 0, 1, 4, and 6 h with unlabeled palmitate and methionine, respectively. A–D, synaptotagmin I (SYT I), SNAP-25, PSD-95, and GAP-43, respectively, were immunoprecipitated and subjected to SDS-PAGE and autoradiography. E, the graph summarizes the changes in the half-life of palmitate on synaptotagmin I, SNAP-25, PSD-95, and GAP-43. *, p < 0.01; **, p < 0.001.
FIG. 6. Prolonged blockade of synaptic activity modulates palmitoylation of synaptotagmin I and SNAP-25. A, cortical neurons were labeled with [3H]palmitate for 3 h and then treated with 90 mM KCl for 15 min before harvest. Synaptotagmin I (SYT I), SNAP-25, and PSD-95 were immunoprecipitated and subjected to Western blotting (WB) and autoradiography. B, the graph summarizes the quantitative changes in the intensity of palmitate labeling of synaptotagmin I, SNAP-25, and PSD-95 after KCl treatment. C, prolonged treatment (3 days) with 100 μM each CNQX and APV or with 100 μM kynurenate (Kyn) enhanced the amounts of [3H]palmitate-labeled synaptotagmin I and SNAP-25. D, similar effects were obtained upon treatment with 100 nM TTX or 10 nM botulinum toxin A (BoNT/A). E, the graph summarizes the changes in the intensity of palmitate labeling of synaptotagmin I and SNAP-25 after these treatments. F, treatment with CNQX and APV enhanced presynaptic accumulation of synaptotagmin I. Scale bar = 10 μm. G, shown are the results from the quantitative analysis of the changes in the intensity of synaptotagmin I puncta after this treatment. *, p < 0.01; **, p < 0.001; ***, p < 0.0001.
targeting in PC12 cells. This study further illustrated that N-glycosylation of synaptotagmin I is the signal required for protein internalization and for redirecting synaptotagmin I from the plasma membrane to an intracellular vesicular pool (29). Palmitoylation has been also shown to regulate internalization of many integral membrane proteins such as G-protein-coupled receptors (6). Indeed, the results presented here show that palmitoylation-deficient synaptotagmin I failed to internalize to a vesicular pool in PC12 cells. We also found that axonal surface expression of palmitoylation-deficient synaptotagmin I was enhanced compared with that of wild-type synaptotagmin I. The enhanced surface expression of the palmitoylation-deficient mutant also correlated with a failure to accumulate at presynaptic sites. Hence, palmitoylation may assist in N-glycosylation in protein internalization and ultimately in the association of synaptotagmin I with synaptic vesicles.

In contrast with our findings, a study by Krasnov and Enikolopov (37) revealed that a structural element located near the C-terminal domain of synaptotagmin I is necessary for protein targeting to neurite terminals in PC12 cells. These differences may be due to the existence of alternative mechanisms for sorting proteins to neurite terminals in PC12 cells and to presynaptic terminals in neurons. It is also possible that each of the general transport machineries interacts with distinct species of synaptic vesicle cargo.
Protein sorting through the Golgi is another pathway involved in sorting molecules destined to presynaptic terminals. A combination of palmitoylation and Golgi-mediated trafficking is required for presynaptic targeting of GAD-65 (11). Here, we found that palmitoylation-deficient SNAP-25 failed to localize to a perinuclear region that resembles the cis-Golgi. Thus, sorting to the Golgi may be important for the initial targeting of SNAP-25 to a vesicular compartment, a process that is potentially involved in pre-assembly of SNAP-25 with other SNARE proteins destined to presynaptic sites. In contrast, synaptotagmin I is a transmembrane protein that traffics through the Golgi, and its palmitoylation is not required for sorting to the Golgi.

Synaptotagmin I is a calcium-sensing protein that regulates fusion pore dynamics. Functional studies indicated that calcium-triggered oligomerization of synaptotagmin I is a critical step in excitation-secretion coupling (32–34, 38). In contrast, calcium-independent oligomerization occurs through N-terminal sequences and involves the palmitoylated N-terminal cysteines (30, 33, 35). Our data are consistent with the analysis of purified synaptosomal preparations obtained from cortical neurons, which demonstrated that synaptotagmin dimers are palmitoylated (16). These results resemble the palmitate-mediated multimerization of PSD-95, a process that is required for PSD-95 clustering at the synapse (39). Since multimerization of synaptotagmin I is required for efficient neurotransmitter release (38), it is possible that palmitoylation-mediated multimerization may also enable synaptotagmin I clustering in response to rapid increases in Ca\(^{2+}\) ions during neurotransmitter release.

SNAP-25 is another palmitoylated protein involved in regulating vesicle exocytosis. Palmitoylation of SNAP-25 is required for membrane targeting; however, the formation of an SDS-resistant SNARE complex is independent of palmitoylation (22, 40). These findings indicate that palmitoylation of SNAP-25 is not required for assembly and disassembly of the SNARE complex but rather for stable membrane anchoring. In contrast, other findings showed that cysteine mutant SNAP-25 is resistant against the action of \(\alpha\)-SNAP/N-ethylmaleimide-sensitive factor (41). In this study, we could not assess the relevance of palmitoylation in presynaptic targeting since overexpressed SNAP-25 was diffusely expressed in cultured neurons. The failure of transfected SNAP-25 to properly target to nerve terminals suggests that endogenous SNAP-25 is present in saturation at presynaptic sites, thus limiting the association of exogenous SNAP-25 with presynaptic membranes. Hence, it remains controversial whether palmitoylation contributes to presynaptic targeting and assembly of SNAP-25 with SNARE proteins.

To study the possible connection between palmitoylation and activity-dependent changes in synaptic transmission, we have analyzed changes in protein palmitoylation after inducing neuronal depolarization. In contrast with the rapid turnover rate of palmitate on PSD-95, our analysis showed that palmitate was stably associated with synaptotagmin I and SNAP-25 and that palmitate turnover was insensitive to rapid presynaptic depolarization. These results are in agreement with those obtained from analysis of palmitate turnover on synaptotagmin I and SNAP-25 in PC12 cells (18). The stable association of palmitate with synaptotagmin I and SNAP-25 combined with the lack of change in protein palmitoylation upon rapid synaptic membrane depolarization indicate that palmitoylation most likely serves as a structural signal for proper targeting and assembly of elements involved in neurotransmitter release.

Prolonged treatment with agents that block either neurotransmitter release or excitatory synaptic transmission enhanced palmitoylation of both synaptotagmin I and SNAP-25. The postsynaptic effects are most likely due to dampening neuronal excitability and reduction of neurotransmitter release. However, one cannot rule out the existence of a retrograde signaling pathway that directly regulates the efficiency of presynaptic vesicular release upon manipulation of postsynaptic elements. It is also possible that long-term blockade of synaptic transmission may have altered the activity of enzymes that regulate addition and removal of palmitate or the accessibility of modified proteins to these enzymes. Alternatively, blocking activity may have indirectly altered protein palmitoylation by increasing the pool of proteins available for palmitoylation through enhanced protein synthesis, reduced protein degradation, or increased trafficking to presynaptic terminals. Consistent with this, presynaptic accumulation of synaptotagmin I was enhanced by treatments that block synaptic activity. These changes may have been triggered to compensate for the reduction in neuronal activity.

SNARE proteins form an SDS-resistant complex of a four-helix bundle contributed by vesicle-associated membrane protein/synaptobrevin, syntaxin, and SNAP-25 (42). This complex requires \(N\)-ethylmaleimide-sensitive factor-mediated ATP hydrolysis to be dissociated, and the energy released by the formation of this complex drives the fusion reaction, which leads to transmitter release (42). Recently, two studies have shown that palmitoylation of SNAP-25 is stimulated by binding to syntaxin \textit{in vitro} (24, 25). These results imply that, at nerve terminals, palmitoylation of SNAP-25 is rapidly regulated upon association and dissociation of the SNARE complex and that fatty acylation may play a role during docking and fusion of synaptic vesicles. However, our findings show that the amounts of palmitoylated SNAP-25 were not altered upon rapid induction of transmitter release, suggesting that association with SNARE proteins does not regulate SNAP-25 palmitoylation \textit{in vivo}.

Interaction of target SNAREs with synaptotagmin I is essential for Ca\(^{2+}\)-dependent vesicle fusion with the plasma membrane (43). This process can be manipulated by specific toxins such as botulinum toxins A, C, and E, which cleave SNAP-25 and other SNARE proteins. Our data show that treatment with botulinum toxin A, a drug that blocks transmitter release by specifically cleaving SNAP-25, enhanced palmitoylation of synaptotagmin I and SNAP-25. These results are consistent with the effects observed with other activity blockers. In contrast, treatment with botulinum toxin C reduced palmitoylation of synaptotagmin I. These results are surprising since both drugs cleave SNAP-25 and block neurotransmitter release. However, treatment with botulinum toxin C also resulted in the cleavage of syntaxin. Thus, the differential effects of these toxins suggest that disruption of coupling to syntaxin rather changes in neuronal activity might have altered the stability of palmitate groups on synaptotagmin I upon botulinum toxin C treatment.

It is intriguing that several other components of the synaptic vesicle machinery, including the cysteine string protein, \(\alpha\)-SNAP, and vesicle-associated membrane protein, are also palmitoylated (6). These proteins are involved in targeting of synaptic vesicles to the sites of neurotransmitter release. Further studies are required to define the role of palmitoylation in synaptic targeting and assembly of these proteins during cycles of neurotransmitter release.

In conclusion, our results demonstrate that rapid palmitate turnover is selectively regulated on a subset of neuronal proteins and that this process can be differentially manipulated by synaptic activity. Synaptotagmin I palmitoylation appears to be subject to two modes of regulation, one that relies on long-lasting blockade of neuronal activity and one that depends on...
association with specific elements of the SNARE complex. In addition to the role of palmitoylation in proper sorting to presynaptic sites, it is possible that acylation is involved in regulating the dynamics of vesicular release. By perturbing the lipid bilayer, palmitate may also facilitate opening of a fusion pore and assist in transmitter release.

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