Differential Roles of Developmentally Distinct SNAP-25 Isoforms in the Neurotransmitter Release Process†

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Received February 21, 2001; Revised Manuscript Received May 30, 2001

ABSTRACT: The role of SNAP-25 (synaptosomal associated protein of 25 kDa) isotypes in the neurotransmitter release process was examined by varying their relative abundance during PC12 cell differentiation induced by nerve growth factor (NGF). Norepinephrine release by NGF-differentiated PC12 cells is more sensitive to type A botulinum toxin (BoNT/A) than by nondifferentiated cells, while both differentiated and nondifferentiated PC12 cells are equally sensitive to type E botulinum toxin (BoNT/E). The differential sensitivity to BoNT/A corresponds to an altered susceptibility of SNAP-25 isotypes to BoNT/A cleavage in vitro, whereas both isotypes are equally vulnerable to cleavage by BoNT/E. Using recombinant SNAP-25 preparations, we show that BoNT/A cleaves SNAP-25b (present in differentiated cells) 2-fold more readily than SNAP-25a (present in both differentiated and nondifferentiated cells). Structural studies using far-ultraviolet circular dichroism (UV−CD) and thermal denaturation suggest a difference in the polypeptide folding as the underlying molecular basis for the differential sensitivity of SNAP-25b and SNAP-25a to BoNT/A cleavage. We propose differential roles for SNAP-25b and SNAP-25a in the neurotransmitter release process since our results suggest that BoNT/A inhibits neurotransmitter release by primarily cleaving SNAP-25b.

SNAP-25, an integral component of the heterotrimeric synaptic SNARE (soluble *N*-ethylmaleimide-sensitive attachment protein receptor) complex, is essential for the regulated vesicle trafficking and exocytosis-mediated neurotransmitter release (*1*). SNAP-25 and two other key members (synaptobrevin and syntaxin) of the SNARE complex are expressed in isotypic forms in neuronal tissues (*1*). However, only SNAP-25 isoforms (SNAP-25b and SNAP-25a) are differentially expressed during neuronal development (*2*).

The functional role of SNAP-25 in neuroexocytosis was first clearly established when it was demonstrated that cleavage by BoNT/A endopeptidase impaired neurotransmitter release (*3*). Intriguingly, BoNT/A, which cleaves SNAP-25 at position 197, is not effective in blocking neurotransmitter release from undifferentiated PC12 cells, whereas BoNT/E, which cleaves SNAP-25 at position 180, is able to completely block the neurotransmitter release from PC12 cells (*4*, *5*). These observations combined with the appearance of the two SNAP-25 isoforms at different developmental stages allowed us to distinguish the roles of the two SNAP-25 isotypes.

The two isotypes of SNAP-25, whose sequences are 96% homologous, are created by alternative splicing of exon 5 of the SNAP-25 gene. Although the subcellular localization and developmental regulation of the two SNAP-25 isotypes have been examined (*2*), little is known about their individual functional roles. In mice, SNAP-25a mRNA is the predominantly expressed isoform during embryonic and early postnatal development, while 7 days after birth SNAP-25a and SNAP-25b mRNA are of equal abundance. SNAP-25b mRNA is the predominant isoform in the adult brain, whereby the SNAP-25b to SNAP-25a ratio is approximately 5 to 1 (*2*). Interestingly, the subcellular localization of the two isotypes differs in NGF-differentiated PC12 cells; whereas SNAP-25b is localized to axon terminals, SNAP-25a is more diffusely located throughout the cell (*2*).

Although SNAP-25b is the predominant species in neurons, nondifferentiated PC12 cells primarily express SNAP-25a, with little SNAP-25b. After NGF-induced differentiation, the relative expression of both SNAP-25 isoforms increases, but the amount of SNAP-25a still exceeds that of SNAP-25b (*2*). Therefore, PC12 cells provide an ideal system to study the differential functioning of the two SNAP-25 isotypes.

† The work was supported, in part, by grants from the NIH to B.R.S. (NS33740) and R.B.L. (3 R15 NS/OD36423-0151) and from the Camille and Henry Dreyfus Foundation to B.R.S.

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10.1021/bi010362z CCC: $20.00
Published on Web 07/13/2001

METHODS

Norepinephrine Release Assay. PC12 cells (passage 19−63) were the gift of Dr. T. F. J. Martin and were grown in either the absence or presence of 40 ng/mL nerve growth factor (NGF; Roche Molecular Biochemicals, Indianapolis, IN) for 7−9 days in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) containing 5% iron-supplemented calf serum, 5% horse serum, 0.4% (w/v) glucose, and 0.37% (w/v) $NaHCO_3$ at 37 °C, in a humidified 5% CO_2 incubator, as described earlier (*5*). NGF-treated cells were grown on collagen I coated culture dishes (Biocoat; Becton Dickinson, Bedford, MA). Cells were observed using light microscopy, and extended processes were noted on the NGF-treated cells. Inhibition of Ca^{2+}-stimulated [^{3}H]norepinephrine ([^{3}H]NE; American Radiolabeled Chemicals, St. Louis, MO) release by BoNT/A and BoNT/E was measured using mechanically permeabilized PC12 cells (>98% permeability to trypan blue) as described earlier (*5*). Maximal release of [^{3}H]NE occurred at 0.3 μM free Ca^{2+} for NGF-treated cells and 1 μM free Ca^{2+} for untreated cells, as seen earlier (*7*).

Cloning of SNAP-25 cDNA and Construction of Recombinant Expression Plasmids. The SNAP-25 cDNA was amplified from the total rat brain mRNA by RT-PCR as described (*6*). The expected size DNA fragment was purified by agarose gel electrophoresis and subsequently cloned into the pCR-TA vector (Invitrogen, CA). Complete DNA sequence analysis of several independent recombinant plasmids revealed that the PCR amplified DNA was a mixture of both SNAP-25a and SNAP-25b cDNAs. The SNAP-25a and SNAP-25b cDNAs were subsequently cloned in-frame to the glutathine *S*-transferase (GST) gene of the pGET-2T plasmid (*6*) to create the recombinant expression plasmids pGEX.SNAP-25a and pGEX.SNAP-25b, respectively.

Expression and Purification of SNAP-25 Isotypes. SNAP-25b and SNAP-25a were expressed as glutathione *S*-transferase (GST) fusion proteins in bacteria. *Escherichia coli* DHαF′ was transformed with either the pGEX.SNAP-25a or pGEX.SNAP-25b recombinant plasmid, and the recombinant GST fusion proteins were overexpressed and purified from these cells using glutathione affinity chromatography as described previously (*6*).

Structural Analysis by Far-UV−CD and Thermal Denaturation. For these analyses, the SNAP-25 isotypes were further purified by cleaving the fusion proteins with human thrombin (Sigma Chemical Co., St. Louis, MO) according to a standard procedure (*8*). After purification, the proteins were extensively dialyzed against 50 mM Tris-HCl/pH 7.5, containing 150 mM NaCl. All spectra were recorded using a Jasco Model 715 spectropolarimeter (Jasco Inc., Easton, NJ). For far-UV−CD, both SNAP-25 isotypes were at 25 °C, with concentrations of 3 μM; spectra were recorded from 180 to 260 nm at a speed of 20 nm/min, with a response time of 8 s and a path length of 1 mm. Two spectra were taken for each sample, and their average was recorded. The protein spectra were obtained after subtracting the buffer spectrum from each of the average recorded SNAP-25 spectra. Thermal denaturation was performed by increasing the temperature of the sample from 20 to 85 °C, with a slope of 2 °C/min, using a Jasco PTC-348W temperature control module, and the far-UV−CD signal was monitored at 222 nm. All of the experiments were repeated at least three times, and representative graphs and calculations are presented in the Results and Discussion section.

Table 1: Effect of Botulinum Neurotoxins A and E on Release of Norepinephrine from PC12 Cells[a]

	% inhibition by	
	1×10^{-7} M BoNT/A	1×10^{-7} M BoNT/E
NGF-treated PC12 cells	43.0 ± 13.1 ($n = 5$)	97.4 ± 15.2 ($n = 6$)
control PC12 cells	14.7 ± 5.4 ($n = 5$)	79.3 ± 10.8 ($n = 6$)

[a] Results are from five to six experiments performed in triplicate. Numbers represent mean and standard deviation of the average inhibition during each experiment.

Cleavage of SNAP-25b and SNAP-25a by BoNT/A and -E. GST−SNAP-25 fusion proteins (20 μM) were incubated with 20 nM reduced BoNT/A or 20 nM BoNT/E (each pretreated with 20 mM DTT for 30 min at 37 °C) for 0, 10, 30, and 60 min at 37 °C. The cleavage product then was detected by Western blot analysis as described previously (*6*) using a polyclonal antibody raised against the 12 C-terminal amino acid residues of SNAP-25 (Stressgen Biotechnologies Corp., Victoria, Canada).

RESULTS AND DISCUSSION

We first tested the ability of BoNT/A and BoNT/E to inhibit neurotransmitter release from differentiated and nondifferentiated PC12 cells. In NGF-treated PC12 cells, BoNT/A was about three times more effective in blocking neurotransmitter release than in the nondifferentiated PC12 (Table 1). BoNT/E did not show any differential effect on differentiated and nondifferentiated PC12 cells. These results are consistent with findings from Banerjee et al. (*7*).

To determine if the difference in BoNT/A activity on PC12 cells was related to a difference in the effectiveness of BoNT/A to cleave the two isotypes of SNAP-25, we incubated 20 μM purified SNAP-25 with 20 nM reduced BoNT/A at 37 °C for various time periods and subsequently quantified the percent cleaved by Western blot analysis (Figure 1A). After 60 min incubation, SNAP-25b was cleaved 52 ± 2.5% ($n = 3$), while SNAP-25a was only cleaved 23 ± 5.7% ($n = 3$). A similar experiment was performed using 20 nM BoNT/E. After an incubation time of 60 min, SNAP-25b was cleaved 61 ± 1.4% ($n = 2$), and SNAP-25a was cleaved 60 ± 3.5% ($n = 2$) (Figure 1B). These results indicate that while BoNT/E cleaved both SNAP-25b and SNAP-25a equally, BoNT/A preferentially cleaved SNAP-25b.

To elucidate the structural basis of their differential cleavage by BoNT/A, we first analyzed the secondary structures of recombinant SNAP-25b and SNAP-25a by far-UV−CD (Figure 2). Differences in the far-UV−CD signal at 222 nm for the two SNAP-25 isotypes suggested that SNAP-25b has more α-helical structure than SNAP-25a. In addition, the far-UV−CD signal at 208 nm indicated that SNAP-25a possesses more random coil structure than SNAP-25b. Two recent structural studies proposed a parallel four-stranded coiled coil structure for the SNARE complex, in which SNAP-25 has two α-helical domains (*9*, *10*). The amino-terminal α-helical domain of SNAP-25 overlaps with the region where the two SNAP-25 isotypes differ in their amino acid sequence (Figure 3). Therefore, our observed

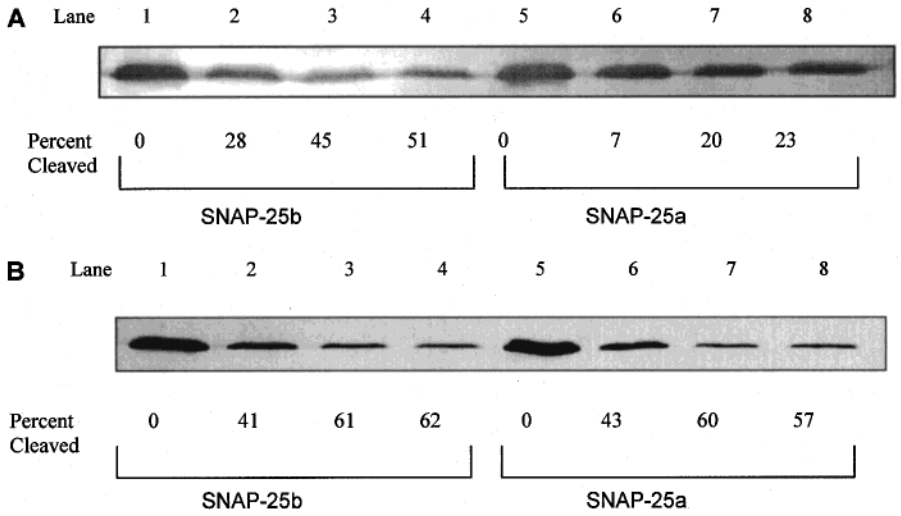

FIGURE 1: BoNT/A and BoNT/E cleavage of SNAP-25b and SNAP-25a, monitored by Western blotting. All bands shown correspond to proteins with a molecular mass of 50 kDa, which was determined by comparison to a prestained protein ladder (Bio-Rad). (A) Lanes 1, 2, 3, and 4 contained 20 μM SNAP-25b and 20 nM reduced BoNT/A, which had been incubated at 37 °C for 0, 10, 30, and 60 min, respectively. Lanes 5, 6, 7, and 8 contained 20 μM SNAP-25a and 20 nM reduced BoNT/A, which had been incubated at 37 °C for 0, 10, 30, and 60 min, respectively. (B) Lanes 1, 2, 3, and 4 contained 20 μM SNAP-25b and 30 nM reduced BoNT/E, which had been incubated at 37 °C for 0, 10, 30, and 60 min, respectively. Lanes 5, 6, 7, and 8 contained 20 μM SNAP-25a and 30 nM reduced BoNT/E, which had been incubated at 37 °C for 0, 10, 30, and 60 min, respectively.

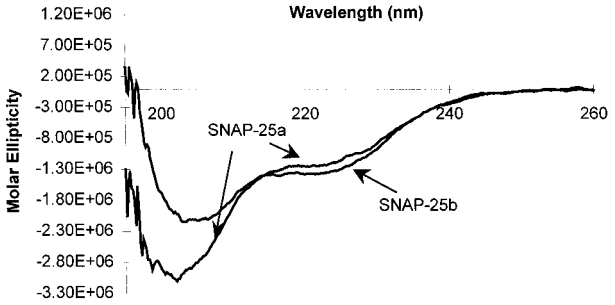

FIGURE 2: Far-ultraviolet circular dichroism (far-UV−CD) spectra of SNAP-25b and SNAP-25a (labeled with arrows). Note that SNAP-25b had a larger signal at 222 nm, while SNAP-25a had a larger signal at 208 nm.

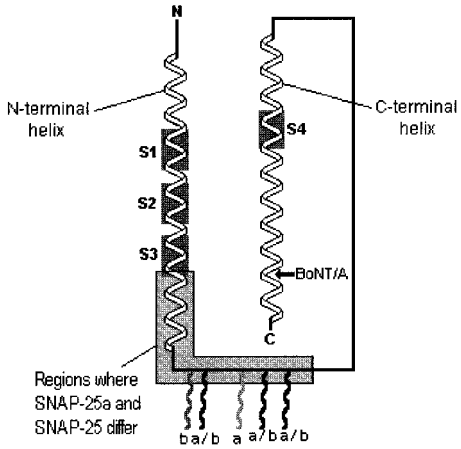

FIGURE 3: Schematic representation of SNAP-25 isotypes during SNARE complex formation. The N-terminal α-helical region of SNAP-25 contains three SNARE motifs, S1(L^{21}−R^{31}), S2(L^{35}−R^{45}), and S3(M^{49}−R^{59}), while the C-terminal α-helical region contains one SNARE motif, S4(E^{145}−G^{155}). Note that S3 overlaps the sequences where SNAP-25b and SNAP-25a differ (designated by the gray box outlined in black; amplified and out of proportion). Each SNAP-25 isotype also contains four palmitoylated cysteines, which are represented by b for SNAP-25b, a for SNAP-25a, and a/b for those common to both isotypes. The cleavage site of BoNT/A (between $Q^{197}$and R^{198}) is also shown.

difference in α-helical content may be related to the variant peptide segment of the two isotypes.

To further distinguish the molecular folding patterns of the two isotypes, thermal denaturation analyses were carried out on purified recombinant SNAP-25b and SNAP-25a (Figure 4). Although the two isotypes were at the same molar concentration (3 μM), their thermal denaturation profiles showed that SNAP-25b had a higher UV−CD signal at 222 nm than SNAP-25a before denaturation. At 85 °C SNAP-25a appeared to have a larger CD signal at 222 nm than SNAP-25b. The T_m's for SNAP-25b and SNAP-25a, calculated from their thermal denaturation curves, were 51.6 and 52.2 °C, respectively. Furthermore, both proteins renatured to their original structures after equilibrating back to 20 °C (data not shown), indicating that the transitions were reversible. Thermodynamic parameters derived from the thermal denaturation curves indicated that ΔH and ΔS for SNAP-25a were approximately 33% higher than those for SNAP-25b (Table 2) (*6*, *11*). The free energy change (ΔG) for the unfolding of SNAP-25a was 62% higher compared to that of SNAP-25b (Table 2), suggesting that the unfolding transition for SNAP-25b is more spontaneous than for SNAP-25a. These results indicate a higher degree of flexibility in the polypeptide folding of SNAP-25b. This observation is supported by both a smaller ΔH and the expected higher entropy (as reflected by smaller ΔS in Table 2) of SNAP-25b at room temperature. The higher flexibility could play a significant role in the interaction of SNAP-25b with the BoNT/A light chain. Such a favorable interaction is perhaps responsible for the 2-fold higher cleavage of SNAP-25b by BoNT/A compared to SNAP-25a. Alternatively, the two SNAP-25 isotypes may differ in their ability to interact with other components of the SNARE complex, and cleavage of a noninteracting SNAP-25 isotype would not affect neurotransmitter release. Since our structural studies show that SNAP-25b has more α-helical structure and more flexible polypeptide folding than SNAP-25a, we propose that con-

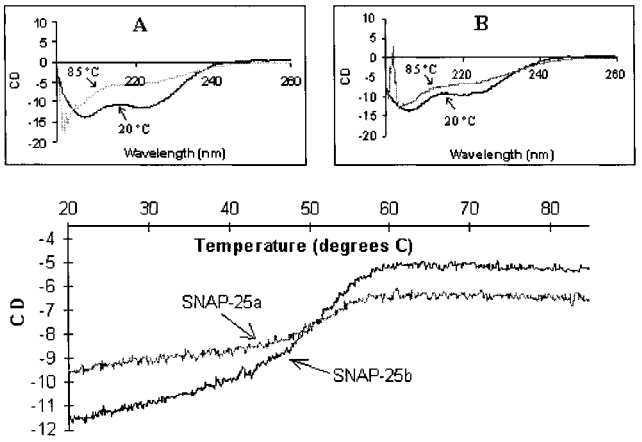

FIGURE 4: Thermal denaturation profiles of SNAP-25b and SNAP-25a, monitored by the far-UV−CD signal at 222 nm (the main figure at the bottom). On the top, inset A shows spectra for SNAP-25b at 20 °C (black) and 85 °C (gray), and inset B shows spectra for SNAP-25a at 20 °C (black) and 85 °C (gray).

Table 2: Thermodynamic Parameters for the Denaturation Curve of SNAP-25b and SNAP-25a As Calculated Previously[a]

	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG_{298} (kJ mol^{-1})
SNAP-25b	273	840	22.5
SNAP-25a	364	1119	36.4

[a] See refs *6* and *11*.

formational differences between the two SNAP-25 isotypes are the underlying cause for their differential susceptibility to BoNT/A cleavage and for their differential role in the neurotransmitter release.

How might the differences in conformation and expression of the two SNAP-25 isotypes lead to the observed BoNT/A sensitivities in differentiated and nondifferentiated PC12 cells? The variant region of the two SNAP-25 isotypes overlaps with a SNARE motif (S3; Figure 3) which could be required for recognizing and binding to an α-helical region of BoNT/A (*12*). Interestingly, one recent study has shown that SNAP-25 cleavage by BoNT/A is much more sensitive to the presence of intact SNARE motifs (S1−S3) than is the cleavage by BoNT/E (*13*). The differential sensitivity of neurotransmitter release in differentiated and nondifferentiated PC12 cells to BoNT/A can be reasonably explained by structural differences of the S3 SNARE motif in SNAP-25b and SNAP-25a, allowing BoNT/A to bind and cleave SNAP-25b more readily than SNAP-25a. At the same time, interaction with BoNT/E may not be affected. It is also possible that higher α-helical content and structural flexibility in SNAP-25b makes it more predisposed to form the SNARE complex than SNAP-25a in the same manner as its increased flexibility makes it more susceptible to BoNT/A cleavage.

To support this hypothesis, expression of both SNAP-25 isotypes was induced by treating PC12 cells with NGF. In differentiated PC12 cells SNAP-25a is expressed in larger amount than SNAP-25b (*2*). Because of its higher level of expression and diffused localization throughout the PC12 cell body, SNAP-25a was first hypothesized to be the SNAP-25 isotype responsible for neurite outgrowth in PC12 cells (*14*). Assuming that SNAP-25a plays a role in both neurotransmitter release and neurite growth, the function of SNAP-25a may change upon differentiation. If SNAP-25a is predominantly used for vesicle fusion to increase the area of plasma membrane during neurite growth in NGF-treated PC12 cells, the SNAP-25a-mediated norepinephrine release would be greatly reduced. With the SNAP-25a-mediated exocytosis reduced, the SNAP-25b portion of exocytosis accounts for a greater percentage of SNAP-25-mediated exocytosis. This, in part, is supported by the fact that, although SNAP-25 is abundant, we notice a decrease in maximal norepinephrine release from PC12 cells upon NGF-induced differentiation from 48.9 ± 10.3% for control to 26.6 ± 5.7% in NGF-treated cells (the average of mean values for nine experiments each). These results, in conjunction with previous work (*2*, *14*), suggest that SNAP-25b is likely to be the SNAP-25 isoform primarily responsible for the exocytic release of neurotransmitters from the differentiated PC12 cells.

Changes in PC12 cells due to NGF exposure are not limited to differential expression of the two SNAP-25 isoforms (*15*, *16*). In fact, NGF treatment induces phosphorylation of SNAP-25 in PC12 cells (*17*), in addition to altering expression of SNAP-25 isoforms. Thus, an alternative explanation for the effects of NGF on exocytosis and BoNT sensitivity is due to phosphorylation of SNAP-25. However, the increased phosphorylation of SNAP-25 due to NGF treatment peaks at 36−48 h of treatment then decreases rapidly by 72 h. PC12 cells in the experiments described here were treated with NGF for 7−9 days, decreasing the potential role for phosphorylation effects. Independent of any possible effect phosphorylation may have on BoNT sensitivity on exocytosis in NGF cells, the data presented here demonstrate clear differences in sensitivity to BoNT by the two isoforms.

Although the physiological significance of the developmentally distinct expression of the two SNAP-25 isotypes is not fully understood, our findings are relevant to biomedical research and to the medical community. As SNAP-25b becomes the predominant form of SNAP-25 in peripheral nervous tissue, animals may become more susceptible to BoNT/A toxicity. Correlation of developmental expression of SNAP-25 isoforms specifically in cholinergic neurons with inhibition of exocytosis by BoNT/A could address this hypothesis. The differences in SNAP-25 isoforms in both secondary structure and susceptibility to BoNT/A, and their roles in neurotransmitter release, may provide the first molecular and physiological clues as to the roles of SNAP-25, not only in neuroexocytosis but also in neurotrophic action.

ACKNOWLEDGMENT

We thank Melissa Mahlen for excellent technical assistance.

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BI010362Z