Botulinum neurotoxin A attenuates release of norepinephrine but not neuropeptide Y from vasoconstrictor neurons

JUDY L. MORRIS, PHILLIP JOBLING, IAN L. GIBBINS

Department of Anatomy and Histology, Centre for Neuroscience, Flinders University, Adelaide, SA 5001, Australia

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Correspondence: Associate Professor Judy L. Morris
Department of Anatomy and Histology, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia
Email: Judy.Morris@flinders.edu.au
Phone and fax: +61 8 8204 4263

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We examined effects of botulinum neurotoxin A (BoNTA) on sympathetic constrictions of the vena cava and uterine artery from guinea-pigs, to test the role of soluble NSF attachment protein receptor (SNARE) proteins in release of the cotransmitters norepinephrine (NE) and neuropeptide Y (NPY). Protein extracts of venae cavae and uterine arteries showed partial cleavage of synaptosomal associated protein of 25 kDa (SNAP-25) after treatment in vitro with BoNTA (50-100nM). The rising phase of isometric contractions of isolated venae cavae to field stimulation at 20Hz, mediated by NE acting on α-adrenoceptors, was reduced significantly by 100nM BoNTA. However, sustained sympathetic contractions mediated by NPY were not affected by BoNTA. In uterine arteries, noradrenergic contractions to 1Hz stimulation were almost abolished by BoNTA, and contractions at 10Hz were reduced by 50-60%. We conclude that SNARE proteins are involved in exocytosis of NE from synaptic vesicles at low frequencies of stimulation, but may not be essential for exocytosis of NPY and NE from large vesicles at high stimulation frequencies.
INTRODUCTION

Sympathetic vasoconstrictor neurons regulate vascular resistance in response to a wide range of physiological demands in order to achieve adequate tissue perfusion whilst maintaining central arterial blood pressure. Many vasoconstrictor neurons fire in short bursts during rest and thus maintain a tonic level of vasoconstriction (9, 10, 39). Increasing demands on the cardiovascular system lead to greater activation of sympathetic vasoconstrictor pathways that is positively correlated with higher plasma levels of the cotransmitters norepinephrine (NE) and neuropeptide Y (NPY) (27, 28, 32). Whereas NE is released at low levels of sympathetic nerve activity, significant NPY release can be detected only after more intense sympathetic activation such as during sustained physical exercise or hemorrhage (27, 28, 32). Both NE and NPY contribute to sympathetic vasoconstriction, although vascular segments constricted by NPY are more limited in distribution throughout the body than those constricted by NE (21). ATP also is a cotransmitter from sympathetic vasoconstrictor neurons (15, 34). ATP often mediates rapid depolarization of vascular smooth muscle, but this leads to smooth muscle contraction only in some blood vessels (11, 21, 22).

The differential release of NE and NPY from sympathetic neurons can be explained by release of NE from small synaptic vesicles at low levels of activation and preferential release of NE and NPY from large vesicles at higher firing frequencies. This interpretation is consistent with results from many biochemical, ultrastructural and functional studies in vivo and in vitro (18, 22). ATP is stored in and released from both small and large synaptic vesicles (3, 36). It follows that low levels of sympathetic activity should release both ATP and NE from small vesicles (see 11). However, there are reports that the release of ATP and NE can be regulated differentially (3). Furthermore, it has been proposed that release of NE occurs primarily from large vesicles in noradrenergic neurons (5). Thus, there is no general agreement about the intracellular compartments from which sympathetic cotransmitters are released and how release of each transmitter is modulated.

Recent studies on proteins involved in synaptic vesicle exocytosis, especially the soluble NSF attachment protein receptor (SNARE) proteins (31, 35), demonstrate clearly that release of co-
transmitters from autonomic neurons can be regulated differentially (24, 29). Botulinum neurotoxins that cleave specific SNARE proteins (2, 8) inhibit release of only some co-transmitters from enteric and pelvic neurons. Our study on vasodilator innervation of the uterine artery found that release of acetylcholine was abolished by treatment with botulinum neurotoxin A (BoNTA) in vitro, whereas peptide release was only partly reduced and nitric oxide release was unaffected. In the current study we set out to test whether release of the peptide cotransmitter, NPY, from vasoconstrictor neurons is regulated differently from the non-peptide transmitter NE. We examined the effects of BoNTA on sympathetic vasoconstrictions of the guinea-pig inferior vena cava that are mediated primarily by NPY (19, 20), and compared this with its effect on release of NE. Furthermore, we extended our previous study on the uterine artery (24) to test the hypothesis that NE release from small vesicles, but not large vesicles, is sensitive to BoNTA treatment.
MATERIALS AND METHODS

Guinea-pigs (Hartley-IMVS, 250-350g body weight) were killed by stunning and exsanguination via the carotid arteries prior to removal of the thoracic portion of the inferior vena cava or the main uterine arteries. These experiments were approved by the Flinders University Animal Welfare Committee.

Immunohistochemical localization of SNAP-25 in the vena cava

Thoracic inferior venae cavae were fixed immediately in Zamboni’s solution at 4°C for 24-72 h, then were processed for cryostat sectioning at 10 µm or as wholemount preparations. Sections and wholemounts were triple-labelled for immunoreactivity (IR) to: tyrosine hydroxylase (TH; antibody AS2-512 raised in a rabbit by Dr J Thibault), neuropeptide Y (NPY; antibody E2210 raised in a sheep by Dr J Oliver and W Blessing) and synaptosomal associated protein of 25 kDa (SNAP-25; mouse monoclonal antibody Sternberger SMI81, Affiniti Research Products, Mamhead, Exeter, UK); calcitonin gene-related peptide (CGRP; antibody 1780 raised in a goat, Arnel, New York, NY) together with TH and SNAP-25 antibodies; or substance P (SP; antibody raise in a rat, Sera-Lab, Sussex, UK) together with CGRP and SNAP-25. Species-specific secondary antibodies used were donkey anti-rabbit IgG or donkey anti-rat IgG conjugated to dichlorotriazinylamino fluorescein (DTAF; dilution 1/100), donkey anti-mouse IgG conjugated to Cy3 (dilution 1/1000) and donkey anti-sheep IgG (which recognizes goat IgG) conjugated to Cy5 (dilution 1/50). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). Wholemounts and sections were examined on an Olympus AX70 microscope fitted with highly discriminating filters that differentiate between the fluorophores used (Chroma Optical, Brattleboro, VT). Digital images were collected using a Hamamatsu Orca cooled CCD camera (Hamamatsu Photonics, Japan) running IPLab Spectrum software (version 3.2; Scanlytics Inc., VA). Images were adjusted for brightness and contrast using Adobe Photoshop v4.0 (Adobe Systems, Mountain View, CA).
In vitro preparations and botulinum toxin treatment

Vessels were placed in HEPES-buffered balanced salt solution (composition in mM: 146 NaCl, 4.7 KCl, 0.6 MgSO₄, 1.6 NaHCO₃, 0.13 NaH₂PO₄, 2.5 CaCl₂, 7.8 glucose, 20 HEPES, 0.1 ascorbic acid) adjusted to pH 7.3 with NaOH. The entire length of each main uterine artery was dissected free from the adjacent uterine vein and adipose tissue. Both uterine arteries or the thoracic inferior vena cava from each animal were then incubated for 2 h at 36°C in a sealed vial containing 250 µl of HEPES buffered solution. For toxin treatment, 50 nM or 100 nM botulinum toxin A (BoNTA; toxicity 17 x 10⁶ mouse LD₅₀/mg; Sigma Chemical Co., Castle Hill, NSW) was added to the 250 µl of HEPES buffered solution. Vessels were washed 3 times (5 min each) in HEPES solution without BoNTA.

Venae cavae were mounted on two parallel wires and were stretched until the equivalent of 1g force was applied. A ring segment 4-5 mm long was dissected from the caudal end of one uterine artery from each animal and was mounted in a myograph for measurement of isometric tension. The remaining segments of uterine arteries from each animal were returned to the sealed vial for incubation at 36°C until conclusion of the pharmacological experiments. Arterial segments were stretched between 2 nichrome wires (25 µm diameter) until the wires were separated by 1-1.25 times the resting internal circumference. Vessels were incubated in HEPES-buffered solution bubbled with 100% O₂ at 36°C and were left to equilibrate for 1 h. For venae cavae, propranolol (0.3 µM) was present in the HEPES solution throughout each experiment to block β-adrenoceptors.

Perivascular axons were stimulated via 2 platinum wires lying either side of and parallel to the long axis of the vessel. For the vena cava, trains of 600 pulses of 0.3 ms duration were delivered at 20 Hz every 20 min. For the uterine artery, trains of 200 pulses of 0.3 ms duration at 1 Hz or 10 Hz were delivered every 20 to 30 min. Pulse trains were delivered by a Grass S44 stimulator connected to a low impedance interface (Biomedical Engineering, Flinders Medical Centre). Changes in isometric tension were detected with a Gould-Statham UTC2 transducer or a Grass FT03 transducer and recorded digitally using Chart v4.0 on a PowerLab 4S (AD Instruments, Castle Hill, NSW, Australia) connected to a Power Macintosh 7600/200 (Apple Computer Inc., CA). Changes in tension were calibrated in
grams and the amplitude and area of responses were determined using Chart v4.0 and NIH Image v1.62. The time from the start of the stimulation period to the initial rise in tension (latency) was determined from the Chart records on an expanded time scale. Group data were analyzed using repeated measures analysis of variance (ANOVA) and covariance (ANCOVA) accompanied by preplanned single degree of freedom linear contrasts or Ryan-Einot-Gabriel-Welsch F (REGWF) tests for post-hoc comparisons (SPSS for Macintosh v10.0, SPSS Inc., Chicago, IL), or t-tests (Microsoft Excel v5.0).

Experimental protocols

Protocol 1: effects of 50 nM and 100 nM BoNTA on sympathetic constrictions of the vena cava. Five veins from each of control, 50 nM BoNTA and 100 nM BoNTA groups were stimulated with trains of pulses at 20 Hz five times prior to application of phentolamine (1 µM) then another 3 times after phentolamine. Guanethidine (1 µM) was then added to confirm that the contraction remaining after phentolamine was mediated by sympathetic neurons. After washout of phentolamine and guanethidine, contractions to KCl (0.126 M) were determined. In statistical analyses, the amplitude of the KCl contraction was used as a covariate for amplitude and area of sympathetic contractions. In separate experiments (4 control veins, 2 veins treated with 100 nM BoNTA), the NPY Y$_1$ receptor antagonist 1229U91 (0.3 µM; 21) was added after phentolamine to confirm that remaining contractions were mediated by NPY.

Protocol 2a: effects of 100 nM BoNTA on uterine artery constrictions. In five control and five BoNTA-treated arteries, a priming stimulus at 10 Hz was given, then 3 pairs of stimuli at 1 Hz and 10 Hz to determine the magnitude of smooth muscle contractions. Prazosin (1 µM) was added 30 min prior to another pair of stimuli at 1 Hz and 10 Hz to confirm that contractions were mediated by NE. Prazosin was then washed out and the amplitude of contraction produced by KCl (0.126 M) was determined.

Protocol 2b: effects of 100 nM BoNTA on neurogenic dilations of the uterine artery. To check whether neurogenic dilations mediated by NO and neuropeptides remaining after 50 nM
BoNTA were further inhibited by 100 nM BoNTA, we tested five control and five BoNTA-treated arteries. One pair of stimuli at 1 Hz and 10 Hz was given prior to blockade of sympathetic neurotransmission with guanethidine (1 µM) and precontraction with prostaglandin F₂α (PGF; 3 µM). Two more pairs of stimuli at 1 Hz and 10 Hz were given, one before and one after addition of L-NAME (30 µM) to the superfusate. The area of relaxations after L-NAME was used to quantify the peptide component of vasodilator responses (see 24).

**Drugs used**

Guanethidine sulphate; N-nitro-L-arginine methyl ester hydrochloride (L-NAME); prazosin hydrochloride; propranolol hydrochloride (all from Sigma-Aldrich; Castle Hill, NSW, Australia); phentolamine hydrochloride (Regitine; Ciba-Geigy, Pendle Hill, NSW, Australia); prostaglandin F₂α (PGF; Sapphire Bioscience, Crows Nest, NSW, Australia); and the Y₁ receptor antagonist 1229U91 (synthesized and kindly provided by Dr. Roger Murphy, Dr Michael Lew and Prof James Angus, Department of Pharmacology, University of Melbourne).

**Immunoblotting for SNAP-25**

After pharmacological experimentation, the thoracic inferior vena cava from 15 animals (5 control, 5 treated with 50 nM BoNTA, 5 treated with 100 nM BoNTA) and all segments of uterine arteries from each of 20 animals (10 control, 10 BoNTA treated), were weighed, frozen in liquid nitrogen and stored at -70°C. Vena cavae also were removed from 3 animals and frozen without prior in vitro incubation. Proteins were extracted by homogenization in a solution of 0.1% sodium dodecyl sulphate (SDS), 20 mM Tris, 150 mM NaCl, 1 mM EDTA with broad spectrum protease inhibitor (Roche Diagnostics, Castle Hill, NSW, Australia), followed by centrifugation at 100,000 g for 1 h. Total protein concentration of the supernatant was determined by Lowry assay using bovine serum albumen for standards. Samples were concentrated by freeze drying, and mixed 1:1 with reducing buffer containing dithiothreitol before boiling at 100°C for 5 min. Six µg protein (venae cavae) or fifteen µg protein (uterine arteries) from each sample was loaded onto 12.5% polyacrylamide gels.
alongside BioRad Precision protein standards (BioRad Laboratories, Regents Park, NSW, Australia), and electrophoresis (SDS-PAGE) was performed using a BioRad MiniProtean III. All fifteen samples of vena cava were loaded in triplets from control, 50 nM BoNTA and 100 nM BoNTA groups across two gels. The ten samples of uterine artery were loaded on two gels with equal numbers of control and treated samples on each gel.

Protein bands were transferred to nitrocellulose and were preincubated (1 h) in phosphate buffered saline (PBS) containing 3% w/v skim milk powder before exposure for 18 h at 4°C to Sternberger SMI81 mouse monoclonal antibody (1/2,000), recognizing the NH2-terminus of SNAP-25. Immunoreactive protein bands were detected by incubation with a sheep anti-mouse IgG conjugated to horse radish peroxidase (1/3,000; AMRAD Biotech, Boronia, VIC, Australia) for 1 h at room temperature, followed by the enhanced chemiluminescence reaction (NEN Renaissance kit, NEL101; NEN Life Science Products, Boston, MA), and were recorded on Kodak X-Omat Blue XB-1 film using a range of exposure times for each membrane. Films were scanned and digitized using a Microtek Scanmaker 4 with exposures ensuring that the maximum intensity levels fell within the gray-scale range. The area and optical density of the immunoreactive bands were determined using NIH Image. Files were saved in TIFF format and imported into Adobe Photoshop v4.0 for compilation of figures. Equal loading of total protein for all samples on the same gel was confirmed by subsequent staining of nitrocellulose membranes with amido black.
RESULTS

SNAP-25 immunoreactivity in the inferior vena cava

The thoracic inferior vena cava is innervated by a plexus of sympathetic axons with immunoreactivity for TH and NPY, that are arranged circumferentially around the vein (26). Most of these axons contained moderate to strong immunoreactivity for SNAP-25 (Fig. 1). Low to moderate levels of SNAP-25 immunoreactivity also was localized in some sensory axons with immunoreactivity for substance P and calcitonin gene-related peptide (7). Schwann cells in the perivascular axon bundles had SNAP-25 immunoreactivity, giving the bundles a smooth, ribbon-like appearance (Fig. 1).

Western blots of protein extracts from freshly frozen venae cavae showed a single band of immunoreactivity for SNAP-25 at 25 kDa. Similarly, protein extracts from venae cavae incubated in vitro without BoNTA showed a single band at 25 kDa. After incubation of veins with 100 nM BoNTA, protein extracts showed a distinct band of SNAP-25 immunoreactivity at 24 kDa, representing the major cleavage product of SNAP-25, in addition to the band at 25 kDa (Fig. 2). BoNTA treatment produced a significant decrease in integrated density of the 25 kDa band, and an increase in the 24 kDa band that was statistically significant at 100 nM BoNTA (Fig. 3).

Effects of BoNTA on sympathetic constrictions of the inferior vena cava

The initial response to stimulation of sympathetic axons in the vena cava is a vasodilatation mediated by norepinephrine acting on β-adrenoceptors, followed by an α2-adrenoceptor mediated constriction. With long pulse trains (200-600 pulses) of higher frequencies of stimulation (> 2Hz) these responses were followed by a long-lasting constriction mediated by NPY (19, 20). In the present study, we compared the relative effects of BoNTA treatment on the α2-adrenoceptor mediated contraction and the NPY-mediated contraction in the presence of propranolol to block β-adrenoceptors. We used trains of 600 pulses at 20 Hz to maximize the NPY component (Protocol 1). In control preparations, the maximum amplitude and area of sympathetic contractions increased
progressively with each stimulation. The area of contractions was enhanced further after addition of the non-selective $\alpha$-adrenoceptor antagonist, phentolamine (1 µM; Figs. 4,5), indicating a presynaptic inhibitory effect mediated by neurally-release NE (20). The latency of contractions also was increased significantly after phentolamine treatment (Figs. 4,5), showing that the initial phase of sympathetic contraction was mediated by NE. The contraction remaining after phentolamine was abolished by the NPY Y$_1$ receptor antagonist 1229U91 (0.3 µM, Fig. 4A), or by guanethidine (1 µM, Fig. 4B), confirming that it was mediated by NPY released from sympathetic neurons. We used the area of the response remaining after phentolamine as an index of the magnitude of the NPY-mediated contractions (see 20). After blockade of sympathetic contractions a small relaxation sometimes was apparent during the stimulation period (Fig. 4).

BoNTA at 100 nM (but not 50 nM) produced a significant increase in latency of contractions (Figs. 4,5). However, even in the presence of BoNTA, phentolamine was able to increase further the latency of the contractile responses (Figs. 4,5). BoNTA had no effect on the calculated area of sympathetic contractile responses, either before or after phentolamine (Fig. 6). Similar to control preparations, the contractions of BoNTA-treated veins remaining after phentolamine were abolished by the NPY Y$_1$ receptor antagonist 1229U91 (0.3 µM) or by guanethidine (Fig. 4C). Furthermore, BoNTA did not affect the increase in area of responses following phentolamine treatment (Fig. 6), nor did it affect the amplitude of contractions produced by KCl (ANOVA: $F_{(2,12)} = 0.83$, $P = 0.5$). These results demonstrate that BoNTA significantly reduced the NE-mediated contraction, but did not abolish it. In contrast, BoNTA did not reduce the NPY-mediated component of sympathetic contractions, nor did it reduce significantly the presynaptic inhibitory effect of neurally-released NE on NPY-mediated contractions.

**Effects of 100 nM BoNTA on sympathetic constrictions of the uterine artery**

Western blotting of protein extracts from uterine arteries confirmed that 100 nM BoNTA produced significant cleavage of SNAP-25 within the time course of our experiments in vitro. There
was a significant reduction in density of the band at 25 kDa (whether expressed as absolute density or integrated density) accompanied by appearance of a band at 24 kDa (Fig. 7).

Smooth muscle contractions produced by stimulation with trains of 200 pulses were significantly larger at 10 Hz than 1 Hz in both control and BoNTA treated uterine arteries (Protocol 2a). BoNTA produced a significant reduction in the amplitude of sympathetic contractions at both 1 Hz and 10 Hz in response to the second and third pairs of stimuli, but not the first pair (Fig. 8). Furthermore, contractions were attenuated by BoNTA significantly more at 1 Hz (80-90% reduction in amplitude) than at 10 Hz (50-60% reduction in amplitude; see Fig. 8 for details of statistical analysis). This pattern of BoNTA reduction in sympathetic contractions, particularly the rundown with repeated stimulation, was very similar to that produced by 50 nM BoNTA (cf. 24). Prazosin (1 µM) abolished the contractions at 1 Hz and 10 Hz (Fig. 8), confirming that these responses were mediated by NE (23). BoNTA at 100 nM did not affect the amplitude of contractions produced by KCl (t-test: \( t(8) = 0.27, P = 0.4 \)).

As sympathetic contractions were examined in preparations where the autonomic vasodilator neurons unavoidably were stimulated simultaneously (see 23), we checked how the dilations were affected by 100 nM BoNTA (Protocol 2b). The effects of 100 nM BoNTA were qualitatively and quantitatively similar to the effects of 50 nM BoNTA we observed previously (24). The initial dilation that reached peak during the stimulation period, mediated by neuronal NO, was not affected by 100 nM BoNTA (repeated measures ANOVA: \( F_{(1,8)} = 2.1, P = 0.2 \)). However, the peptide-mediated dilation was reduced approximately 50% by 100 nM BoNTA (repeated measures ANOVA: \( F_{(1,8)} = 11.0, P = 0.01 \)), which was similar to the reduction produced by 50 nM BoNTA (24).
DISCUSSION

Cleavage of SNAP-25 in sympathetic axons by botulinum neurotoxin A

SNAP-25 is present in the sympathetic axons innervating the vena cava and uterine artery (24, 25). Furthermore, BoNTA can cleave SNAP-25 in these vessels. This was demonstrated by a reduction in area or density of the protein band for intact SNAP-25 and appearance of a lower molecular weight band representing the major 197 amino acid cleavage product, as has been reported previously (2, 14, 24, 33). We compared the relative amount of protein cleavage produced by the two concentrations of BoNTA. However, the densities of the 24kDa and 25 kDa bands did not always vary in a reciprocal manner, as would be predicted if all of the cleaved SNAP-25 appeared in the 24 kDa band. This may be due to cleavage of both the 25 kDa and 24 kDa proteins by endogenous proteases in the isolated blood vessels.

Even at the highest concentration of BoNTA used here (100 nM), SNAP-25 cleavage was incomplete. The partial cleavage of SNAP-25 is consistent with previous findings in intact cells and tissues, and may be explained by pools of SNAP-25 in Schwann cells and non-terminal axons (24, 33), in recycling synaptic vesicles (38) or bound in the core complex (8, 31, 33), that are not accessible to BoNTA (Fig. 1). Nevertheless, maximal inhibition of neurotransmission can occur at a time when protein cleavage is barely detectable with Western blotting (12, 14). This may be due in part to functional antagonism of neurotransmission by the 197 amino acid N-terminus of SNAP-25 remaining after BoNTA cleavage (14). Although a fraction of the SNAP-25 cleavage we detected in tissue extracts of the uterine artery was likely to have been in non-sympathetic axons (24), our functional studies showed clearly that 100 nM BoNTA attenuated some of the effects of sympathetic nerve stimulation in both the uterine artery and vena cava (cf. 1). Thus, BoNTA must have cleaved a pool of SNAP-25 responsible for transmitter release from the terminal axons of sympathetic neurons in the vena cava and uterine artery.
Release of neuropeptide Y is insensitive to botulinum neurotoxin A

NPY is synthesized by sympathetic neurons innervating most blood vessels (18, 22). Whereas NE can be synthesized and stored in both small and large vesicles at sympathetic nerve terminals, supply of NPY to the nerve terminals is dependent on axonal transport of large, dense-cored vesicles from the cell body. Electron microscopic studies of sympathetic axon varicosities have shown that the large dense-cored vesicles are out-numbered many fold by small vesicles, and are not commonly located adjacent to the neuroeffector junctions where small vesicles are clustered (6, 17). Nevertheless, NPY can be released from sympathetic axons in response to trains of stimuli delivered at higher frequencies to produce vasoconstriction directly, or to enhance constrictions produced by NE (18, 22).

The sympathetic vasoconstriction of the guinea-pig inferior vena cava has a particularly pronounced component mediated by NPY (19, 20). The NPY component predominates over the noradrenergic component, which is apparent only during the rising phase of the response. The NPY component is apparent at stimulation frequencies as low as 2 Hz, but is more prominent with stimulation frequencies of 10 to 20 Hz (20). The complete blockade of sympathetic contractions of the vena cava by a combination of adrenoeceptor and Y₁ receptor antagonists confirms that ATP does not contribute to sympathetic constriction of this large capacitance vessel. In the current study, BoNTA up to a concentration of 100 nM did not affect the NPY component of sympathetic vasoconstriction of the vena cava in response to trains of pulses at 20 Hz. Since BoNTA reduced neurogenic contractions mediated by NE in the same vessels, BoNTA clearly was gaining access to SNAP-25 in the sympathetic nerve terminals. As NPY can be released only from large synaptic vesicles, we conclude that our regime of BoNTA treatment has not affected exocytosis from large vesicles in sympathetic varicosities in the vena cava. This suggests that SNAP-25 and the SNARE core complex may not be essential for exocytosis from large vesicles in these sympathetic neurons. In contrast, the peptide component of vasodilation in the uterine artery was reduced by half after incubation with 50 nM BoNTA (24). However, 100 nM BoNTA did not further reduce this peptide component, despite evidence for more cleavage of total tissue SNAP-25. This result adds further support to the proposal
that regulation of exocytosis from large peptide-containing vesicles is fundamentally different from regulation of exocytosis from small vesicles (16, 37).

**Release of norepinephrine is only partly blocked by botulinum neurotoxin A**

In both the vena cava and the uterine artery, BoNTA inhibited some of the effects of sympathetic nerve stimulation mediated by NE. In the uterine artery, constrictions produced by stimulation at 1 Hz were reduced substantially after BoNTA treatment. This inhibition increased with each period of stimulation, demonstrating increased accessibility of SNAP-25 to BoNTA cleavage during vesicle recycling and uncoupling of SNAP-25 from the core complex (8). BoNTA also reduced constrictions produced by stimulation at 10 Hz, although these responses were never abolished. Furthermore, treatment with 100 nM BoNTA did not reduce constrictions more than did 50 nM BoNTA (cf. 24). These results are consistent with the hypothesis that, at 1 Hz stimulation, NE is released primarily from a compartment that is sensitive to BoNTA, while at 10 Hz NE is released from two different compartments, one of which is insensitive to BoNTA. Similarly, the simplest interpretation of our results in the vena cava is that NE is released from two different compartments during stimulation at 20 Hz, one compartment containing NE but not NPY that is sensitive to BoNTA, and a second compartment containing both NPY and NE that is insensitive to BoNTA.

Our results are consistent with release of NE almost exclusively from one compartment at low stimulation frequencies and from two different compartments with higher stimulation frequencies (see 36; Fig. 9). It is highly likely that these two compartments correspond to the populations of small and large synaptic vesicles, although we cannot discount the possibility that at low stimulation frequencies NE (but not NPY) is released from a population of large vesicles that is more sensitive to BoNTA than the large vesicles containing both NPY and NE. As ATP does not contribute directly to sympathetic constrictions of either the vena cava (20) or uterine artery (23), our study monitoring smooth muscle contraction cannot detect the effects of BoNTA on release of this co-transmitter.

Previous studies on transmitter spillover from the splenic nerve and vas deferens have concluded that NE is released only from large vesicles (5). One obvious reason for the discrepancy
between these studies and our own conclusion is that quantities of NE and NPY sufficient to be
detected biochemically in the vascular lumen following nerve stimulation (5) may not be an accurate
reflection of the level of each transmitter close to release sites. Nevertheless, spontaneous release of
NE from sympathetic nerve terminals in the rat mesenteric artery also can occur from large vesicles
(4). It is possible that NE released in response to sympathetic nerve stimulation at low frequencies
occurs from a pool of vesicles different from that involved in spontaneous release of ATP. These
possibilities need to be tested directly to clarify the degree of heterogeneity of synaptic vesicles and
their mechanisms of exocytosis in sympathetic vasoconstrictor neurons.

Conclusions

This study has demonstrated that NE released from sympathetic vasoconstrictor neurons at
low stimulation frequencies results from SNARE protein-mediated exocytosis from one population of
synaptic vesicles. Our results also are consistent with the conclusion that the cotransmitters NE and
NPY are released from a different population of vesicles at higher stimulation frequencies via a
mechanism that is relatively insensitive to BoNTA. It is likely that these two populations correspond
to small and large vesicles, respectively. It is possible that exocytosis from large peptide-containing
vesicles in sympathetic neurons may not involve any of the SNARE proteins forming the core
complex, including syntaxin and synaptobrevin as well as SNAP-25. Alternatively, different isoforms
of the SNARE proteins might be involved in exocytosis from small versus large vesicles (16).
Furthermore, the calcium sensitivity of transmitter release from small and large vesicles is likely to
differ, and may involve different calcium channels (16) or regulatory proteins such as calcium-
dependent activator protein for secretion (CAPS), which is associated selectively with large vesicles
(37). This is consistent with release of peptide transmitters from large vesicles at sites distant from
release of small vesicles (13, 30). Our results emphasize that modulation of sympathetic
vasoconstrictor neurons by endogenous or pharmaceutical agents during intense activation by
physiological or pathophysiological stimuli is likely to have differential effects on release of
sympathetic cotransmitters.
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REFERENCES


FIGURE LEGENDS

Fig. 1. Immunoreactivity for SNAP-25 in vasoconstrictor axons in the vena cava. Wholemount of the thoracic portion of the inferior vena cava triple-labelled for immunoreactivity to tyrosine hydroxylase (TH), neuropeptide Y (NPY) and synaptosomal-associated protein of 25 kDa (SNAP-25). Most axon varicosities with TH and NPY also have immunoreactivity for SNAP-25 (arrows). In addition, some axon bundles (b) have SNAP-25 immunoreactivity in structures with a ribbon-like appearance that are likely to be Schwann cells. Scale bar = 20 µm.

Fig. 2. Western blots show cleavage of SNAP-25 in vena cava by BoNTA. Protein extracts from one representative sample of vena cava from each of control, 50 nM BoNTA and 100 nM BoNTA groups after SDS-PAGE and immunoblotting for SNAP-25 using an NH₂-terminally directed antiserum. The 25 kDa immunoreactive band is reduced in area or density after BoNTA and an additional band at 24 kDa is apparent after 100 nM BoNTA.

Fig. 3. Cleavage of SNAP-25 in vena cava by BoNTA. Group data showing the mean ± SE of integrated density of SNAP-25 immunoreactive bands at 25 kDa and 24 kDa on Western blots of protein extract from 5 control venae cavae, 5 veins treated with 50 nM BoNTA and 5 veins treated with 100 nM BoNTA. Repeated measures ANOVA showed that BoNTA had a significant effect on integrated density of both protein bands ($F_{(2,12)} = 4.1$, $P=0.4$) and there was a significant interaction between BoNTA and band ($F_{(2,12)} = 11.7$, $P=0.002$). Post-hoc comparisons showed a significant reduction in integrated density of the 25 kDa band by both 50 and 100 nM BoNTA and a significant increase in integrated density of the 24 kDa only after 100 nM BoNTA.
Fig. 4. Effect of BoNTA on sympathetic contractions of the vena cava. Representative traces of isometric tension from control venae cavae (A, B) and a vena cava treated with 100 nM BoNTA (C) showing prolonged contractions produced by field stimulation with trains of 600 pulses at 20 Hz, at bars, before and after phentolamine (Phent, 1 µM). Contractions remaining after phentolamine are abolished by the NPY Y_4 receptor antagonist 1229U91 (0.3 µM; A) or by guanethidine (Guan, 1 µM; B, C). D, Traces of contractions in B and C before phentolamine with an expanded time scale and normalized peak amplitude, demonstrating inhibition of the rising phase of sympathetic contractions by BoNTA.

Fig. 5. BoNTA at 100 nM increased latency of sympathetic contractions. Group data showing mean ± SE (n=5) of latency of sympathetic contractions before and after phentolamine (1 µM) for control veins and veins treated with 50 or 100 nM BoNTA. Data shown for responses to 3rd to 8th trains of stimuli (S3 – S8). Repeated measures ANOVA showed a small overall effect of BoNTA on latency (F(2,11) = 3.5, P=0.06), but contrast analysis showed a highly significant increase in latency by 100 nM BoNTA compared with controls (P = 0.02). Phentolamine produced a significant increase in latency (F(2,11) = 9.1, P=0.01) but no interaction between BoNTA and phentolamine (F(2,11) = 0.1, P=0.9).

Fig. 6. BoNTA does not reduce sympathetic contractions produced by neurally-released NPY. Group raw data showing mean ± SE (n=5) of magnitude of sympathetic contractions before and after phentolamine (1 µM), expressed as area under the traces, for control veins and veins treated with 50 or 100 nM BoNTA. Data shown for responses to 3rd to 8th trains of stimuli (S3 – S8). Repeated measures ANCOVA (with amplitude of KCl contraction as a covariate) showed a significant increase in contractions after phentolamine (F(1,11) = 6.2, P=0.03), no effect of BoNTA (F(2,11) = 0.5, P=0.6) and no interaction between phentolamine and BoNTA (F(2,11) = 0.9, P=0.5).
Fig. 7. Cleavage of SNAP-25 in uterine artery by 100 nM BoNTA. Group data showing mean ± SE of integrated density of SNAP-25 immunoreactive bands at 25 kDa and 24 kDa for 10 control uterine arteries and 10 arteries treated with 100 nM BoNTA.

Fig. 8. BoNTA at 100 nM reduced sympathetic contractions of uterine artery. Group data showing mean ± SE for the peak amplitude of sympathetic contractions of uterine arteries produced by trains of 200 pulses at 1 Hz or 10 Hz in 5 control arteries and 5 arteries after treatment with 100 nM BoNTA. Three pairs of stimuli were applied before prazosin and one pair after addition of prazosin (1 µM). Repeated measures ANOVA showed a significant interaction between number of stimulus pairs and BoNTA (F(2,16) = 22.7, P=0.001). Significant 3-way interaction between BoNTA, frequency and number of stimulus pairs (F(2,16) = 9.3, P=0.002). Single degree of freedom contrasts showed that BoNTA significantly reduced responses to the 2nd and 3rd pair of stimuli compared with the 1st pair, and the effect was greater at 1 Hz than at 10 Hz (F(1,8) = 11, P=0.001).

Fig. 9. Model of selective involvement of SNARE proteins in exocytosis from a subpopulation of NE-containing synaptic vesicles. Our results are consistent with the conclusion that BoNTA has blocked transmitter release from one population of synaptic vesicles containing NE but not NPY. In contrast, BoNTA has not blocked exocytosis from vesicles containing NPY and NE. These two populations of vesicles are highly likely to be small and large synaptic vesicles, respectively. The lack of effect of BoNTA on the large peptide-containing vesicles could be due to the lack of involvement of SNAP-25 and other SNARE proteins in exocytosis from large vesicles, or regulation of SNARE proteins by mechanisms that are fundamentally different from those regulating small vesicle exocytosis.
Figure 3

SNAP-25 immunoreactivity

Integrated optical density

Control  50 nM BoNTA  100 nM BoNTA

□ 25 kDa

■ 24 kDa
Figure 4

A  CONTROL

200 mg

Phent

1229U91

2 min

B  CONTROL

300 mg

Phent

Guan

C  BoNTA 100nM

300 mg

Phent

Guan

D  Control

30 s

BoNTA
Figure 5

Latency of contractions (s)

- Control
- 50 nM BoNTA
- 100 nM BoNTA

S3, S4, S5, S6, S7, S8

+ Phentolamine 1μM
Figure 6

Area of contractions (x10^3 g.s.)
- Control
- 50 nM BoNTA
- 100 nM BoNTA

S3  S4  S5  S6  S7  S8

+ Phentolamine 1μM