Nicotinamide adenine dinucleotide is released from sympathetic nerve terminals via a botulinum neurotoxin A-mediated mechanism in canine mesenteric artery

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Smyth, Lisa M., Leanne T. Breen, and Violeta N. Mutafova-Yambolieva. Nicotinamide adenine dinucleotide is released from sympathetic nerve terminals via a botulinum neurotoxin A-mediated mechanism in canine mesenteric artery. Am J Physiol Heart Circ Physiol 290: H1818–H1825, 2006. First published December 9, 2005; doi:10.1152/ajpheart.01062.2005.—Using high-performance liquid chromatography techniques with fluorescence and electrochemical detection, we found that β-nicotinamide adenine dinucleotide (β-NAD) is released in response to electrical field stimulation (4–16 Hz, 0.3 ms, 15 V, 120 s) along with ATP and norepinephrine (NE) in the canine isolated mesenteric arteries. The release of β-NAD increases with number of pulses/stimulation frequencies. Immunohistochemistry analysis showed dense distribution of tyrosine hydroxylase-like immunoreactivity (TH-LI) and sparse distribution of TH-LI-negative nerve processes, suggesting that these blood vessels are primarily under sympathetic nervous system control with some contribution of other (e.g., sensory) neurons. Exogenous NE (3 μmol/l), α,β-methylene ATP (1 μmol/l), neuropeptide Y (NPY, 0.1 μmol/l), CGRP (0.1 μmol/l), vasoactive intestinal peptide (VIP, 0.1 μmol/l), and substance P (SP, 0.1 μmol/l) had no effect on the basal release of β-NAD, suggesting that the overflow of β-NAD is evoked by neither the sympathetic neurotransmitters NE, ATP, and NPY, nor the neuropeptides CGRP, VIP, and SP. Botulinum neurotoxin A (BoNTA, 0.1 μmol/l) abolished the evoked release of NE, ATP, and β-NAD at 4 Hz, suggesting that at low levels of neural activity, release of these neurotransmitters results from N-ethylmaleimide-sensitive factor attachment protein receptor/synaptosomal-associated protein of 25 kDa-mediated exocytosis. At 16 Hz, however, the evoked release of NE, ATP, and β-NAD was reduced by BoNTA by ∼90, 60, and 80%, respectively, suggesting that at higher levels of neural activity, β-NAD is likely to be released from different populations of synaptic vesicles or different populations of nerve terminals (i.e., sympathetic and sensory terminals).

The splanchnic circulation comprises a substantial portion of the cardiac output and blood volume and is generally assumed to play an important part in overall cardiovascular homeostasis, in addition to providing adequate perfusion in the gastrointestinal system. Disturbances in the neural control of mesenteric arteries in particular might contribute to the pathogenesis of hypertension and local vascular dysfunctions. In fact, any condition that is associated with sympathetic nervous system hyperactivity (i.e., stress, strenuous exercise, and hemorrhage) may manifest disproportionate vasoconstrictive responses in the splanchnic circulation. Therefore, a great deal of effort has been devoted to understanding the mechanisms of autonomic vascular control in the mesentery. The perivascular nerves of the mesenteric vasculature in various species consist primarily of sympathetic postganglionic nerve terminals, in which norepinephrine (NE), ATP, and neuropeptide Y (NPY) are the established cotransmitters (5, 7, 9, 10, 20, 33, 34). In addition, various neuropeptides including CGRP, vasoactive intestinal peptide (VIP), and substance P (SP), primarily released from sensory motorneurons, also contribute to the neural control of the mesenteric circulation (13, 15, 17, 19, 22, 30). The substances that are released by action potentials serve as neurotransmitters, neuromodulators, or both (7, 8, 22). The precise combinations of neurotransmitter and neuromodulator substances in vasomotor pathways vary with species (12). Canine mesenteric arteries, in particular, have been shown to corelease NE and ATP in a tetrodotoxin (TTX)- and guanethidine-sensitive manner (4, 5, 28). The tissue superfusates collected during stimulation of peripheral nerve terminals also contain the ATP metabolites ADP, AMP, and adenosine. Most recently, we found that short-pulse electrical field stimulation (EFS) of the canine mesenteric artery in vitro evokes the release of β-nicotinamide adenine dinucleotide (β-NAD) along with NE, ATP, ADP, AMP, and adenosine (35). The release of β-NAD depends on neural activity and is not a result of smooth muscle contraction. Exogenous β-NAD reduces the EFS-evoked release of NE (35), suggesting that β-NAD may represent a novel extracellular player at the vascular neuroeffector junction in the mesenteric circulation. The source(s) and mechanisms of release of this novel extracellular factor, however, remain to be elucidated. The present study was designed, therefore, to tackle this issue by investigating the role of sympathetic neurotransmitters, neuropeptides, and synaptosomal-associated protein of 25 kDa (SNAP-25)-mediated exocytosis in the evoked release of β-NAD in the canine isolated mesenteric artery devoid of endothelium. In most instances, the release of NE, ATP, and β-NAD was evaluated in parallel. Our results suggest that β-NAD is released on stimulation of postganglionic sympathetic nerve terminals and its release partially depends on SNAP-25-mediated exocytosis. These results introduce β-NAD as a putative neurotransmitter or neuromodulator in the mesenteric circulation.

MATERIALS AND METHODS

Tissue preparations. Sixty-four mongrel dogs of either sex (average 15 kg) were obtained from vendors licensed by the U.S. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Department of Agriculture and used for this study. The use of the laboratory animals for these experiments was approved by the University of Nevada Animal Care and Use Committee. The animals were euthanized with pentobarbital sodium (100 mg/kg iv). The abdomens were opened, and segments of second- and third-order branches of the superior and inferior mesenteric artery (0.5–1 mm in diameter) were dissected out and bathed in oxygenated Krebs solution containing (in mM) 118.5 NaCl, 4.2 KCl, 1.2 MgCl₂, 23.8 NaHCO₃, 1.2 KH₂PO₄, 11.0 dextrose, and 1.8 CaCl₂ (pH 7.4). Vessels were denuded of their endothelium by rubbing with a rough-surface needle and slowly perfusing with distilled water. This procedure successfully removes endothelium while maintaining smooth muscle contractility intact (27).

**Overflow experiments.** The tissue segments were placed in 200-μl water-jacket Brandel superfusion chambers as described previously (3–5, 29, 39). Briefly, after 45 min equilibration, the tissues were subjected to a 15-s “conditioning” stimulation with a train of square wave pulses of 0.1-ms duration and a frequency of 4 Hz. Thirty minutes after the conditioning stimulation, the preparations were subjected to EFS for 120 s with a train of suprathreshold pulses of 0.3 ms at 4 or 16 Hz. These parameters were chosen after verification that NE overflow is abolished by either TTX (0.3–1 μmol/l) or ω-conotoxin GVIA (5 nmol/l). Samples of the superfusion solution were collected before the electrical stimulation (resting overflow) and during the electrical stimulation (nerve-evoked overflow) in ice-cold Eppendorf tubes. Samples were analyzed for ATP and β-NAD content by using HPLC techniques with fluorescence detection as described previously (3, 5, 28, 35). Aliquots of some superfusate samples were processed for NE assay by HPLC techniques with electrochemical detection as described previously (28).

To test the possibility that sympathetic neurotransmitters could evoke β-NAD overflow, tissues were superfused with 15 min with either NE (3 μmol/l), α,β-methylene ATP (α,β-MeATP, 1 μmol/l), or NPY (0.1 μmol/l), and the basal overflow of β-NAD was evaluated in the absence and presence of each substance. To test the possibility that neuropeptides could evoke β-NAD overflow, tissues were superfused with either CGRP (0.1 μmol/l), VIP (0.1 μmol/l), or SP (0.1 μmol/l) for 5 min, and the content of β-NAD in the superfuse samples in the absence and presence of a neuropeptide was evaluated. In another series of experiments, arteries were incubated for 5.5 h at 37°C in a sealed vial containing 250 μl of RPMI-1640 solution, pH 7.4. For botulinum neurotoxin A (BoNTA) treatment, BoNTA was added to the 250 μl of RPMI-1640 solution to make a final concentration of 100 nmol/l. Vessels were washed three times (5 min each) in Krebs solution without BoNTA before being placed in Brandel superfusion chambers, and overflow experiments were performed to analyze the content of NE, ATP, and β-NAD in the tissue superfusates collected before and during EFS at 4 or 16 Hz (0.3 ms, 15 V, for 120 s).

**Sample preparation.** A modified method of Levitt et al. (16) was employed to detect 1,N⁶-etheno-derivatives of the endogenous nucleotides present in the tissue superfusates. Briefly, 100 μl of a citrate phosphate buffer (pH 4.0) were added to 200 μl of the superfuse sample in plastic Eppendorf tubes (Fisher Scientific). 2-Chloroacetaldehyde (10 μl) was added to the samples in a fume hood; the samples were then heated for 40 min at 80°C in a dry bath incubator (Fisher Scientific). With the use of this procedure, endogenous ATP is derivatized to 1,N⁶-etheno-ATP (eATP), whereas endogenous β-NAD is derivatized to 1,N⁶-etheno-ADP-ribose (eADPR) as described previously (3, 35). The reaction was stopped by placing the samples on ice.

**HPLC assay of etheno-nucleotides and etheno-nucleosides.** The HP1100 liquid chromatography module system (Agilent Technologies, Wilmington, DE) was used throughout this study and has been previously described (3). The mobile phase consisted of 0.1 mol/l KH₂PO₄ (pH 6.0) as eluant A; eluant B contained 35% methanol and 65% eluant A. Gradient elution was employed according to the following linear program: time 0, 0% eluant B; 18 min, 100% eluant B. Flow rate was 1 ml/min and run time was 20 min. Column temperature was ambient, whereas the autosampler temperature was 4°C. The fluorescent detector was set to record signals at an excitation wavelength of 230 nm and emission wavelength of 420 nm, which are the optimum conditions for detection of etheno-derivatives of nucleotides and nucleosides as shown previously (3). The amounts of nucleotides in each sample were calculated from calibration curves of nucleotide standards run simultaneously with every set of unknown samples. Results were normalized for sample volume and tissue weight, and the overflow of nucleotides was expressed in femtomoles per milligram of tissue.

**HPLC assay of NE.** The overflow of NE was assayed as described previously (28). Briefly, 115-μl aliquots from the samples were acidified with 3 μl of 1 M perchloric acid to pH 2.6 and injected (70 μl) into an isocratic HP1100 HPLC system equipped with an HP1049A electrochemical detector (Agilent Technologies) and a MD-150 column (ESA, Chelmsford, MA). The mobile phase for separation consisted of the following (in mmol/l): 50 NaH₂PO₄, 0.2 EDTA, 3.0-heptanesulfonic acid, 10 LiCl, and methanol 3% vol/vol in deionized water (pH 2.6). The HPLC systems were controlled and data collected by a HP Kayak XA computer equipped with HP ChemStation (A.06.03) software (Agilent Technologies). The amounts of NE in each sample were calculated from calibration curves of NE standards run simultaneously with every set of unknown samples. Results were normalized for sample volume and tissue weight, and the overflow of NE was expressed in femtomoles per milligram of tissue.

**Immunohistochemistry.** Immunohistochemical analysis was performed on segments of second- and third-order branches of the superior and inferior mesenteric artery. Whole mount preparations were prepared after removal of the surrounding adipose and connective tissue. Segments of artery were opened longitudinally and pinned to the base of a dish filled with Sylgard elastomer (Dow Corning, Midland, MI) with the adventitial side facing upward. Tissues were fixed in paraformaldehyde (4% wt/vol in 0.1 M phosphate buffer for 20 min at 4°C). After fixation, preparations were washed for 3–4 h in PBS (0.01 M, pH 7.4). Tissues were incubated at 4°C overnight in 1% BSA to reduce nonspecific antibody binding. This solution also contained 0.5% Triton X-100 to permeabilize the tissue for easier antibody penetration. For cryostat studies, segments of second- and third-order branches of the canine superior and inferior mesenteric artery were perfused with Krebs solution before being fixed with paraformaldehyde for 30 min at 4°C. After fixation, tissues were washed with PBS and processed through a sucrose gradient (5, 10, and 15% sucrose-PBS; 15 min each) before being immersed in 20% sucrose-PBS overnight. The following day, the tissue was cut into ~2-mm segments and embedded in Tissue-Tek (Miles, Elkhart, IN) before being quickly frozen in liquid nitrogen. Cryostat sections were cut at 8- to 10-μm thicknesses by using a Leica CM3050 cryostat and collected on coated glass slides (Surgipath, Richmond, IL). Sections were preincubated with 1% BSA-PBS for 1 h before being incubated with antibodies.

For examination of SNAP-25-like immunoreactivity (SNAP-25-LI) in whole mount preparations, tissues were incubated for 24–48 h at 4°C with a monoclonal antibody raised against SNAP-25 protein (1:100; catalog number 111011, Synaptic Systems, Götingen, Germany). SNAP-25-LI was detected by Alexa 594 secondary antibody (1:1,000 in PBS, rabbit anti-mouse, 1 h, room temperature; Molecular Probes, Carlsbad, CA, red fluorescence).

For double-label immunostaining of Protein Gene Product (PGP 9.5) and tyrosine hydroxylase (TH) in both whole mount preparations and cryostat sections, tissues were first incubated with rabbit PGP 9.5 primary antibody (1:1,000 in PBS, 24–48 h at 4°C, catalog number RA-95101, Ultraclone, Isle of Wight, UK). The tissues were subsequently washed in PBS for several hours. PGP 9.5-LI was detected by Alexa 594 secondary antibody (1:1,000 in PBS, goat anti-rabbit, 1 h, room temperature, Molecular Probes, red fluorescence). The tissues were processed for NE assay by HPLC techniques with electrochemical detection as described previously (28).

For examination of SNAP-25-like immunoreactivity (SNAP-25-LI) in whole mount preparations, tissues were incubated for 24–48 h at 4°C with a monoclonal antibody raised against SNAP-25 protein (1:100; catalog number 111011, Synaptic Systems, Götingen, Germany). SNAP-25-LI was detected by Alexa 594 secondary antibody (1:1,000 in PBS, rabbit anti-mouse, 1 h, room temperature; Molecular Probes, Carlsbad, CA, red fluorescence).
were then washed overnight in PBS with subsequent addition of sheep TH primary antibody (1:400 in PBS for 24–48 h at 4°C, catalog number A-2027; Signal Transduction Products, San Clemente, CA). The tissues were then washed again overnight in PBS at 4°C, and TH-LI was detected by Alexa 488 secondary antibody (1:1,000 in PBS, donkey anti-sheep, 1 h, room temperature, Molecular Probes, green fluorescence).

For double-label immunostaining of TH and SNAP-25 in cryostat preparations, tissues were first incubated with sheep TH primary antibody as specified in the previous paragraph, followed by donkey anti-sheep Alexa 488 secondary antibody for 1 h at room temperature. The preparations were then washed in PBS for several hours before being incubated with a monoclonal SNAP-25 primary antibody (1:100; Synaptic Systems). SNAP-25-LI was detected by Alexa 594 secondary antibody (1:1,000 in PBS, rabbit anti-mouse, 1 h, room temperature).

Control preparations were prepared by omitting either primary or secondary antibodies from the incubation solutions. After being washed with PBS, specimens were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA). Tissues were examined with a Zeiss LSM 510 Meta confocal microscope (Zeiss) with an excitation wavelength appropriate for Alexa 488 or Alexa 594. Confocal micrographs of Z-series scans of 10–15 optical sections through a depth of 3–40 μm were taken. Final images were constructed with Zeiss LSM Meta software and reconstructed with the use of Adobe Photoshop 7.0.

Chemicals. Norepinephrine hydrochloride, β-NAD, α,β-MeATP, and SP were all purchased from Sigma Chemical (St. Louis, MO). NPY was purchased from Bachem Biosciences (King of Prussia, PA). CGRP was purchased from Tocris Cookson (Ellisville, MO). BoNTA was purchased from List Biological Laboratories (Campbell, CA). All drugs were initially dissolved in double-distilled water, except BoNTA, which was dissolved in BSA (1 mg/ml).

Statistics. Data are presented as means ± SE. Means were compared by one-way ANOVA (Graph Pad Prism version 3, Graph Pad Software). A probability value of P < 0.05 was considered significant.

RESULTS

Canine mesenteric arteries are densely innervated by sympathetic nerves. Whole mount preparation of mesenteric artery labeled for PGP 9.5-LI, a pan-neuronal marker, revealed a dense network of nerve fibers within the arterial wall (Fig. 1A). Arteries labeled for TH-LI, the rate-limiting enzyme involved in the synthesis of NE, also revealed a network of neural fibers with a density similar to PGP 9.5-LI (Fig. 1B). Double labeling for PGP 9.5-LI and TH-LI revealed that the majority of the nerve fibers that contained PGP 9.5-LI also contained TH-LI (Fig. 1C); a small population of nerve fibers that contained PGP 9.5-LI did not contain TH-LI. Cryostat sections of mesenteric artery revealed that the PGP 9.5-LI and TH-LI were located within a dense layer at the level of the adventitia (Fig. 1, D–F). The majority of the nerve fibers showed colocalization of PGP 9.5-LI and TH-LI.
Release of β-NAD increases with the number of stimuli. Figure 2 shows typical chromatograms from superfusate samples collected before and during EFS of canine mesenteric artery subjected to EFS with 0.3-ms pulse width for 120 s. Thus the tissues were subjected to 480 and 1,920 pulses at 4 Hz and 16 Hz, respectively. In samples subjected to etheno-derivatization and analyzed with a fluorescence detector, peaks that correspond to eATP (elution time ~9.9 min), 1,N\(^6\)-etheno-ADP (eADP, elution time ~10.8 min), eADPR (elution time ~11.2 min), 1,N\(^6\)-etheno-AMP (eAMP, elution time ~12.6 min), and 1,N\(^6\)-etheno-adenosine (eADO, elution time ~16.6 min) were observed. As shown before (3), eATP, eADP, eAMP, and eADO correspond to ATP, ADP, AMP, and ADO, respectively. However, the eADPR peak reflects primarily the content of β-NAD in the superfusate samples (35). Therefore, the peak at 11.2 min was referred to as β-NAD throughout this study. The overflow of β-NAD evoked by 4 and 16 Hz was 2.8 ± 1.1 (n = 5) and 16.8 ± 2.4 (n = 8) fmol/mg tissue, respectively. We have previously shown that the release of β-NAD at 16 Hz in the canine mesenteric artery was significantly reduced by either TTX (0.3–1 μM) or guanethidine (3 μmol/l) (35), suggesting that either TTX or β-NAD was released from sympathetic nerve terminals or factors that are released from these terminals evoke the release of β-NAD.

![Chromatograms](image)

**Fig. 2.** Electrical field stimulation (EFS; 0.3 ms, 15 V, 120 s) evokes frequency-dependent overflow of β-NAD in canine isolated mesenteric artery. Original chromatograms are shown from HPLC-FLD analysis of β-NAD in tissues superfusates collected before (PS, prestimulation) and during nerve stimulation at 4 and 16 Hz (0.3 ms, 120 s). Aliquots were derivatized with 2-chloroacet-aldehyde (80°C, pH 4, 40 min) to 1,N\(^6\)-etheno (e)-nucleotides (i.e., eATP, eADP, eAMP) and 1,N\(^6\)-etheno-adenosine (eADO). At both frequencies of stimulation, eADP-ribose (eADPR) with elution time of ~11.2 min is also observed. eADPR reflects overflow of β-NAD (35). Amounts of all purines are increased with stimulation frequency.

**Fig. 3.** Exogenous neurotransmitters and neuropeptides do not evoke β-NAD overflow in canine mesenteric arteries. Exogenous application of norepinephrine (NE; 3 μmol/l), α,β-methylene ATP (α,β-MeATP, 1 μmol/l), or neuropeptide Y (NPY, 0.1 μmol/l) (A) or CGRP, vasoactive intestinal peptide (VIP), and substance P (SP) (all 0.1 μmol/l) (B) does not significantly change basal overflow of β-NAD. Data represent means ± SE from 4–6 experiments.

Sym pathetic neurotransmitters NE, ATP, and NPY do not evoke overflow of β-NAD. To investigate whether the classic sympathetic neurotransmitters NE, ATP, and NPY were responsible for the release of β-NAD during electrical stimulation, these transmitters or their analogues were exogenously applied to the mesenteric arteries. Figure 3A shows results from canine isolated mesenteric arteries incubated with either NE (3 μmol/l), α,β-MeATP (1 μmol/l), or NPY (0.1 μmol/l). The basal overflow of β-NAD in arteries incubated with NE, α,β-MeATP, or NPY did not differ significantly from control tissues, suggesting that the overflow of β-NAD is not induced by NE, ATP, or NPY and hence is not mediated by activation of α-adrenoceptors, purinergic receptors, or NPY receptors.

Neuropeptides CGRP, VIP, and SP do not evoke overflow of β-NAD. To investigate whether neuropeptides were responsible for the release of β-NAD during electrical stimulation, CGRP, VIP, and SP were exogenously applied to the mesenteric arteries. CGRP (0.1 μmol/l), VIP (0.1 μmol/l), or SP (0.1 μmol/l) did not evoke release of β-NAD in the superfusate samples (Fig. 3B), suggesting that these neuropeptides (which may have been released during EFS) could not account for the release of β-NAD in this blood vessel.

Inhibition of SNAP-25 by BoNTA reduces the EFS-evoked release of β-NAD. We then carried out experiments to investigate whether disruption of SNAP-25 after treatment in vitro...
with BoNTA (100 nmol/l) will affect the EFS-evoked release of β-NAD. Indeed, SNAP-25-LI showed neural network-like distribution in a whole mount preparation of canine mesenteric artery (Fig. 4A). Cryostat sections revealed that SNAP-25 was located within the adventitial layer of the mesenteric artery (Fig. 4B) and was largely colocalized with TH-LI (Fig. 4, C and D) in this blood vessel. Incubation of artery segments with RPMI-1640 for 5.5 h did not significantly affect the EFS-evoked release of NE, ATP, and β-NAD. However, incubation of artery segments with BoNTA (100 nmol/l for 5.5 h) caused significant reduction of the EFS (16 Hz, 0.3 ms, 120 s)-evoked release of β-NAD and ATP (Fig. 5, A, D, and F) as well as of NE (Fig. 5, B and C) in this blood vessel (n = 6; P < 0.05), suggesting that disruption of SNAP-25 by BoNTA significantly affects the EFS-evoked release of NE, ATP, and β-NAD. Thus, in BoNTA-treated tissue segments, the evoked release of NE, ATP, and β-NAD was reduced by ~90, 60, and 80%, respectively. The evoked release of NE, ATP, and β-NAD at 4 Hz of EFS (0.3 ms, 120 s) was abolished after BoNTA treatment from 5.9 ± 0.8, 1.0 ± 0.1, and 2.8 ± 1.1 fmol/mg tissue NE, ATP, and β-NAD, respectively, to undetectable amounts of the three compounds (n = 3).

**DISCUSSION**

As aforementioned, the purinergic/adrenergic cotransmission is particularly important for the neural control of the splanchnic circulation (e.g., Refs. 4, 5, 9, 28, 33, 37). Most recently, we reported that in addition to NE, ATP, and the ATP metabolites ADP, AMP, and adenosine, the tissue superfusates collected during stimulation of postganglionic nerve terminals in canine mesenteric artery contain β-NAD along with small amounts of the β-NAD metabolites ADPR and cyclic ADPR (35). Exogenous β-NAD (100 nmol/l) inhibits the evoked release of NE in this blood vessel (35), although the full range of pre- and postjunctional actions of extracellular β-NAD at the vascular neuroeffector junction remains to be elucidated. Our previous work (35) showed that activation of sympathetic nerve terminals is critical for the release of β-NAD during stimulation of this blood vessel. On the basis of these observations, we hypothesized that either β-NAD is released from sympathetic nerve terminals or factors released on stimulation of these terminals evoke the overflow of β-NAD from neuronal or nonneuronal sources. The present study was designed, therefore, to expand on these initial observations and investigate whether the release of β-NAD is evoked by the three major sympathetic neurotransmitters NE, ATP, and NPY. In addition, we investigated whether neuropeptides that may be released from sensory nerve afferents or autonomic nerve terminals (e.g., CGRP, SP, and VIP) would induce the release of β-NAD. Finally, we investigated whether the evoked release of β-NAD is through stimulus-dependent exocytosis of synaptic vesicles associated with SNAP-25.

Consistent with previous investigations in peripheral blood vessels, double-labeling immunofluorescence and confocal microscopy revealed that the majority of nerve terminals at the adventitial layer of the canine mesenteric artery contain TH-LI and hence are adrenergic/sympathetic in nature. A small portion of the PGP 9.5-positive nerve fibers was negative for TH-LI and hence are adrenergic/sympathetic in nature. A small portion of the PGP 9.5-positive nerve fibers was negative for TH-LI and hence are adrenergic/sympathetic in nature. A small portion of the PGP 9.5-positive nerve fibers was negative for TH-LI and hence are adrenergic/sympathetic in nature.
Likewise, exogenous CGRP, VIP, or SP (factors that are commonly released from sensory fibers) did not evoke \( \beta \)-NAD overflow. Therefore, in the canine mesenteric artery, the release of \( \beta \)-NAD is not induced by the action of the classic sympathetic cotransmitters NE, ATP, and NPY or by the action of the neuropeptides CGRP, VIP, and SP.

The EFS-evoked release of \( \beta \)-NAD in the canine mesenteric artery increases with the number of stimuli (the present study), as does the EFS-evoked release of NE (5, 28, 35) and of ATP (5, 35). The release of all three, NE, ATP, and \( \beta \)-NAD, is significantly reduced by TTX and guanethidine. Furthermore, the evoked release of both NE and \( \beta \)-NAD is significantly reduced by 6-hydroxydopamine and the selective blocker of neuronal Cav2.2 (N-type) voltage-operated Ca\(^{2+}\) channels (VOCC) \( \omega \)-conotoxin GVIA (35). We conclude, therefore, that in the canine mesenteric artery, \( \beta \)-NAD is primarily released from postganglionic sympathetic nerve terminals via a mechanism that depends on the influx of Ca\(^{2+}\) through neuronal VOCC.

The VOCC of the nerve terminal are intimately related to one group of proteins, the soluble \( N \)-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are involved in the process of synaptic vesicle mobilization (11, 18, 36) in response to the rise in intracellular Ca\(^{2+}\) after an action potential to generate transmitter release (18, 32). The target membrane-associated SNARE protein SNAP-25 in particular is associated with the synaptic plasma membrane and is believed to be primarily involved in exocytosis from small vesicles but not large vesicles (24). SNAREs, including SNAP-25, may not be expressed uniformly in all populations of...
neurons (1, 26, 31). In the canine mesenteric artery, SNAP-25 appears entirely colocalized with TH-LI, suggesting that SNAP-25 may be particularly important for the release of neurotransmitters from the postganglionic sympathetic nerve terminals in this blood vessel. BoNTA cleaves and inactivates SNAP-25 (2). NE and ATP are stored in and released from both small and large synaptic vesicles (6, 38). It has been proposed that the release of neurotransmitters at low frequencies of stimulation represents exocytosis from small vesicles, whereas at higher frequencies of stimulation, large vesicles also contribute to the neurotransmitter release (23).

The regime of BoNTA treatment used in the present study was based on previous functional studies (14, 21, 23, 24) of autonomic neurons showing that BoNTA incubated in the nanomolar range with whole tissues for several hours at 37°C produced substantial reduction or abolition of neurotransmission from some types of neurons. In the present study, the release of NE, ATP, and β-NAD was abolished by BoNTA at a low number of electrical stimuli, suggesting that at low levels of activation, NE, ATP, and β-NAD are released preferentially from a population of synaptic vesicles that are associated with SNAP-25. This population of synaptic vesicles likely represents the small vesicles (24, 25). At a higher number of electrical stimuli, however, the evoked release of NE, ATP, and β-NAD was reduced by ~90, 60, and 80%, respectively. Thus, at high levels of activation, ~20% of β-NAD remained unaffected by BoNTA. Several possible explanations may be considered: 1) BoNTA did not completely inhibit the SNAP-25-mediated exocytosis; 2) the evoked release of β-NAD at 4 Hz was abolished by BoNTA because of the relatively low number of electrical stimuli, whereas increasing the number of stimuli resulted only in partial block of β-NAD+ release; or 3) the component of β-NAD+ release that remained unaffected by BoNTA may originate either from a second population of synaptic vesicles insensitive to BoNTA (e.g., large vesicles) or from a population of nerve terminals that do not contain SNAP-25 (e.g., sensory nerves) (25). Further studies are warranted to define the exact mechanism underlying the small component of β-NAD+ release that was insensitive to BoNTA.

In conclusion, this study has demonstrated that β-NAD released on activation of sympathetic nerve terminals in the canine isolated mesenteric artery is due to direct release of β-NAD from nerve terminals rather than evoked by the sympathetic cotransmitters NE, ATP, and NPY or the neuropeptides CGRP, VIP, and SP. The release of β-NAD, NE, and ATP from sympathetic postganglionic nerve terminals at a low level of nerve activation may result from SNAP-25-associated synaptic vesicles. At higher levels of neural activity, β-NAD may be released from both BoNTA-sensitive and BoNTA-insensitive stores. At higher levels of nerve activation, extracellular β-NAD appears released in greater amounts together with the excitatory neurotransmitters NE and ATP to perhaps prevent extreme excitation. Therefore, β-NAD constitutes a novel extracellular factor that may contribute to autonomic neurovascular control in the mesentery.

GRANTS

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