Release of nicotinamide adenine dinucleotide (β-NAD) upon stimulation of postganglionic nerve terminals in blood vessels and urinary bladder

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Running title: Release of novel nucleotides upon nerve stimulation

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The abbreviations used are: ATP, adenosine 5'-triphosphate; NE, norepinephrine; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; ADO, adenosine; β-NAD, nicotinamide adenine dinucleotide; cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; HPLC, high-pressure liquid chromatography; EFS, electrical field stimulation; TTX, tetrodotoxin; 6-OHDA, 6-hydroxydopamine; NPP, nucleotide pyrophosphatase; ET-1, endothelin-1; U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2α); ANGII, angiotensin II; PMA, phorbol 12-myristate 13-acetate; Ap₆A, diadenosine haxaphosphate; Ap₅A, diadenosine pentaphosphate; Ap₄A, diadenosine tetrphosphate; Ap₃A, diadenosine triphosphate; Ap₂A, diadenosine diphosphate; UTP, uridine 5'-triphosphate; GTP, gianosine 5'-triphosphate; GDP, gianosine 5'-diphosphate; GMP, gianosine 5'-monophosphate; Gp₂G, diguanosine pentaphosphate; Gp₃G, diguanosine tetraphosphate; Gp₄G, diguanosine triphosphate; Gp₅G, diguanosine diphosphate; NAAD, nicotinic acid adenine dinucleotide; NAADP, nicotinic acid adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate; TEAA, tetraethyl acetic acid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; DMSO, dimethylsulfoxide; PS, pre-stimulation; ST, during stimulation sample; ApₙA, diadenosine polyphosphates; HPLC-FLD, HPLC fluorescence detection; eATP, 1,N⁶-etheno-ATP; eADP, 1,N⁶-etheno-ADP; eAMP, 1,N⁶-etheno-AMP; eADO, 1,N⁶-etheno-ADO; eADPR, 1,N⁶-etheno-ADPR; cGDPR, cyclic GDP-ribose; XAC, xanthine amine congener
Summary:

Chemical signaling in autonomic neuromuscular transmission involves agents that function as neurotransmitters and/or neuromodulators. Using high performance liquid chromatography techniques with fluorescence and electrochemical detection we observed that in addition to adenosine 5’-triphosphate and norepinephrine (NE), electrical field stimulation (EFS, 4-16 Hz, 0.1-0.3 ms, 15 V, 60-120 s) of isolated vascular and non-vascular preparations co-releases a previously unidentified compound with apparent nucleotide or nucleoside structure. Extensive screening of more than 25 nucleotides and nucleosides followed by detailed peak identification revealed that nicotinamide adenine dinucleotide (β-NAD) is released in tissue superfusates upon EFS of canine mesenteric artery (CMA), canine urinary bladder (CUB) and murine urinary bladder (MUB) in the amounts of 7.1±0.7, 26.5±4.5, and 15.1±3.2 fmol/mg tissue, respectively. Smaller amounts of the NAD metabolites cyclic adenosine 5’-diphosphoribose (cADPR) and ADPR were also present in the superfusates collected during EFS of CMA (2.5±0.9 and 5.8±0.8 fmol/mg tissue, respectively), CUB (1.8±0.5 and 9.0±6.0 fmol/mg tissue, respectively), and MUB (1.4±0.1 and 6.2±2.4 fmol/mg tissue, respectively). The three nucleotides were also detected in the samples collected before EFS (0.2-1.6 fmol/mg tissue). Exogenous β-NAD, cADPR and ADPR (all 100 nmol/L) reduced the release of NE in CMA at 16 Hz from 27.8±6.0 fmol/mg tissue to 15.5±5.0, 12±3.0 and 10.0±4.0 fmol/mg tissue, respectively. In conclusion, we detected constitutive and nerve-evoked overflow of β-NAD, cADPR, and ADPR in vascular and non-vascular smooth muscles, β-NAD being the prevailing compound. These substances modulate the release of NE, implicating novel nucleotide mechanisms of autonomic nervous system control of smooth muscle.
Introduction

Chemical signaling constitutes a major mechanism in neuroeffector transmission in the central and peripheral nervous systems. Postganglionic nerve terminals, in particular, characteristically release multiple factors upon action potential, a process referred to as plurichemical neurotransmission (1) or co-transmission (2). The evidence is particularly strong for postganglionic sympathetic nerves, which have been shown to co-release adenosine 5’-triphosphate (ATP), NE, and neuropeptide Y, factors that presently fulfill transmitter criteria (3, 4, 5, 6). Postganglionic parasympathetic nerve terminals, on the other hand, release acetylcholine and ATP (2). In addition, neural control of effector cells involves molecules that implement potent excitatory or inhibitory actions at the neuroeffector junction in the absence of stringent evidence for transmitter function. These effects, collectively referred to as neuromodulation, are usually poorly understood, often due to lack of potent and selective antagonists (6); nonetheless, neuromodulation is an important mechanism, as is neurotransmission (7, 8).

We report here that postganglionic nerve terminals in several smooth muscle preparations that are under autonomic nervous system control release novel factor(s) along with NE, ATP and the ATP metabolic products ADP, AMP, and ADO. We hypothesized that this novel factor has a nucleotide/nucleoside structure and acquires fluorescence properties upon etheno-derivatization. Extensive screening revealed that the compound is a member of the β-NAD/cADPR/ADPR family. Further experimentation in canine mesenteric artery and vein, and canine and murine urinary bladders established that all three nucleotides, β-NAD, cADPR and ADPR, are present in tissue superfusates collected during EFS at parameters specific for neural activation, β-NAD being the prevailing nucleotide. Smaller amounts of β-NAD, cADPR and ADPR were found in
superfusates from non-stimulated tissues. Exogenous application of β-NAD and its metabolic products cADPR and ADPR modulate the release of the classic sympathetic neurotransmitter NE in vascular preparations. To our knowledge, release of members of this nucleotide axis at rest or during stimulation of postganglionic nerve terminals has not been reported. Therefore, novel mechanisms of autonomic neural control of vascular and non-vascular smooth muscle functions are implicated.

**Experimental Procedures**

*Tissue preparations:*

Mongrel dogs of either sex (averaging 15 kg), male guinea-pigs (400-500 g), male Wistar rats (200-250 g), male New Zealand rabbits (3-5 kg), and male C57BL6 mice (15-20 g) were obtained from vendors licensed by the United States Department of Agriculture. The use of the laboratory animals for these experiments was approved by the University of Nevada’s Animal Care and Use Committee. The animals were euthanized either with pentobarbitone sodium, 100 mg kg\(^{-1}\) intravenously (dogs) or an overdose of CO\(_2\) (guinea-pigs, rats, mice). The abdomens were opened and segments of second and third order branches of the canine superior and inferior mesenteric artery (0.5-1 mm in diameter) and vein (0.7-1.2 mm in diameter), or canine urinary bladder, or first and second order branches of guinea-pig inferior mesenteric artery (0.2-0.4 mm in diameter) and vein (0.3-0.5 mm in diameter), or vasa deferentia from rats and guinea-pigs, or murine urinary bladder were dissected out and bathed in oxygenated Krebs solution containing (mmol/L): 118.5 NaCl; 4.2 KCl; 1.2 MgCl\(_2\); 23.8 NaHCO\(_3\); 1.2 KH\(_2\)PO\(_4\); 11.0 dextrose; 1.8 CaCl\(_2\) (pH 7.4). In some experiments the tail arteries of rats (~ 100 mm artery length) and mice (~ 50 mm artery length) were dissected out and bathed in oxygenated Krebs solution. The urothelium was removed from the urinary bladders. All vascular preparations were
rubbed with rough-surface needle and perfused with distilled water to remove the endothelium. This procedure has been shown to successfully remove endothelium while smooth muscle contractility remains intact (9).

Overflow experiments:

The tissue segments were placed in 200-µl water-jacket BRANDEL superfusion chambers as described previously (10, 11, 12, 13). 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. Previous experiments have shown that the transmitter overflow evoked by stimulations subsequent to the conditioning stimulation is more consistent than in the absence of a conditioning stimulation. Thirty minutes after the conditioning stimulation the preparations were subjected to EFS for 60-120 s with a train of supra-threshold pulses of 0.1-0.3 ms at 4-16 Hz depending on the tissue. The EFS parameters were chosen after preliminary optimization experiments and verification that NE overflow is abolished by either tetrodotoxin (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 (electrically evoked overflow) in ice-cold test tubes. Samples were analyzed for nucleotide/nucleoside contents by HPLC techniques with fluorescence detection as described previously (12, 13, 14). Aliquots of some superfusate samples were processed for NE assay by HPLC techniques with electrochemical detection as described previously (15).

A more detailed characterization of the EFS-evoked overflow of nucleotides, nucleosides (i.e., ADO), and NE was carried out in canine isolated mesenteric arteries. In some experiments the tissue segments were superfused with either TTX (0.3-1 µmol/L) or ω-conotoxin GVIA (5
nmol/L) for 30 min before EFS to block neuronal fast Na\(^+\) channels and N-type Ca\(^{2+}\) channels, respectively. Other tissue segments were treated with guanethidine (3-10 \(\mu\)mol/L) for 1.5 h to inhibit action potential-induced activation of sympathetic nerve terminals. In some experiments tissues were treated with 6-OHDA using a method modified from Aprigliano and Hermsmeyer (16) to “denervate” adrenergic neurotransmission. Briefly, a modified Krebs-Ringer solution that lacked NaHCO\(_3\) and NaH\(_2\)PO\(_4\) was prepared and the pH was adjusted to 4.9 using 20 \(\mu\)mol/L glutathione. 6-OHDA was dissolved in this solution at a concentration of 300 \(\mu\)g/ml. The tissue was placed in the 6-OHDA solution for 10 min. The tissue was then placed in regular oxygenated Krebs for 30 min, and then subsequently placed in the 6-OHDA solution for an additional 10 min. The tissue was then loaded in the chambers and equilibrated as usual. Samples were collected after 2 hr 15 min perfusion with regular Krebs. Parallel “no-6OHDA” time-controls were carried out, as well. In some experiments NE content of the “pre-stimulation” and “during-stimulation” samples was also determined in the absence or presence of exogenous nucleotides (e.g., \(\beta\)-NAD, cADPR, ADPR, \(\alpha\)-NAD, NAAD, NGD, cGDPR, 8-Br-cADPR, and ADO, 100 nmol/L). To test the possibility that contraction of the smooth muscle is the primary cause of nucleotide/nucleoside overflow, tissues were perfused either with ET-1 (0.05 \(\mu\)mol/L), U46619 (1 \(\mu\)mol/L), ANGII (0.1 \(\mu\)mol/L) or PMA (0.1 \(\mu\)mol/L) for 20 min.

**Sample Preparation**

A method modified from Levitt et al. (17), which originally describes a procedure for detection of \(1,N^6\)-etheno-derivatives of ATP, ADP, AMP and ADO, was employed. Briefly, 100 \(\mu\)l of a citrate phosphate buffer (pH 4.0) was added to 200 \(\mu\)l of the superfusate sample in a borosilicate glass culture tube (Fisher Scientific, USA). Chloroacetaldehyde was synthesized...
according to a method modified from Secrist et al. (18) and Levitt et al. (17) and described previously (14). Ten µl of 2-chloroacetaldehyde was added to the samples in a fume hood; the culture tubes were covered with glass marbles, and the samples were heated for 40 min at 80°C in a dry bath incubator (Fisher Scientific, USA) to produce 1,N6-etheno-nucleotides and 1,N6-etheno-nucleosides.

Chemical substances with nucleotide/nucleoside structure were subjected to etheno-derivatization and subsequent HPLC analysis. The following chemicals were tested: adenine compounds (i.e., ATP, ADP, AMP, ADO, diadenosine polyphosphates, namely Ap6A, Ap5A, Ap4A, Ap3A, Ap2A), pyridine compounds (i.e., UTP), guanidine/guanosine compounds (i.e., GTP, GDP, GMP, diguanosine polyphosphates, namely Gp5G, Gp4G, Gp3G, Gp2G), cyclic nucleotides (cAMP, cGMP, cADPR), NAAD, NAADP, α-NAD, β-NAD, NADP, and ADPR.

In some experiments authentic nucleotides were subjected to etheno-derivatization with 2-chloroacetaldehyde at 80°C and the samples were desalted as described previously (14). The nucleotides were then eluted with a binary system consisting of eluent A (water) and eluent B (100 % acetonitrile) by a gradient according to the following linear program: time 0, 0 % eluent B; 40 min, 80 % eluent B; 80 min, 80 % eluent B. The flow rate was 0.8 ml/min. The absorbing fraction was collected. The fraction was then lyophilized and kept at –16°C until processed for mass spectra verification of the etheno-nucleotide.

*HPLC assay of etheno-nucleotides and etheno-nucleosides*

The liquid chromatographic system used throughout this study was an HP1100 LC module system (Agilent Technologies, Wilmington, DE) as described previously (14). The mobile phase comprised 0.1 mol/L KH$_2$PO$_4$ (pH 6.0) as eluent A; eluent B consisted of 35 %
methanol and 65 % eluent A. Gradient elution was employed according to the following linear program: time 0, 0 % eluent B; 18 min, 100 % eluent B. Flow rate was 1 ml/min, run time 20 min and post-run time 5 min. Column temperature was ambient while 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 (14). The non-derivatized compounds were detected at an excitation wavelength of 270 nm and emission wavelength of 410 nm according to preliminary optimization of the HPLC application.

Fraction Collection and Sample Concentration

To identify the compound that is released during EFS in canine mesenteric arteries, superfusate samples from 12 chambers containing ~65 mg tissue per chamber were placed together in two 2-ml Eppendorf tubes containing the pre-stimulation samples and the samples collected during EFS, respectively. The two resulting samples were further concentrated by Speed Vacuum (Savant SVC100, Thermo Electron Corp., Westmont, IL) to 1 ml volume. Seven hundred and fifty µl of each concentrated sample were injected into the HPLC system and 400 µl-fractions corresponding to the retention times of cADPR (7.0-7.4 min, “7.2-min fraction”), ADPR (8.3-8.7 min, “8.5-min fraction”), and β–NAD (10.3-10.7 min, “10.5-min fraction”) were collected in borosilicate culture tubes containing 160 µl citric buffer. The exact retention times for the three nucleotides were determined by injecting β-NAD, cADPR and ADPR standards (40 nmol/injection) in the same sequence prior to the concentrated superfusate samples. The fractions were further subjected to etheno-derivatization with 17 µl 2-chloroacetaldehyde as described in HPLC Assay of Etheno-nucleotides and Etheno-nucleosides. The derivatized
samples were injected into the HPLC and analyzed for 1,N\textsuperscript{6}-etheno-ADPR content. To identify the compound that is released in urinary bladder, superfusate samples from 16-20 chambers containing either canine detrusor strips (~70 mg/chamber) or mouse bladder detrusors (2 bladders averaging ~15 mg per chamber) were processed as described for canine mesenteric artery segments. The experiments were performed in triplicate with each species.

In another series of experiments, superfusate samples (either PS or during EFS) form 24 chambers containing canine mesenteric artery strips were first put together and then concentrated with Speed Vacuum, and processed through the HPLC. The 400-µl HPLC fractions corresponding to cADPR (“7.2-min fraction”), ADPR (“8.5-min fraction”), and β-NAD (“10.5-min fraction”) of superfusate samples were mixed with 400 µl Krebs solution to add Mg\textsuperscript{2+}. The fractions were then again concentrated via Speed vacuum to 600 µl. NPP (E.C.3.6.1.9), 0.2 U, was added to each sample and the samples were incubated with the enzyme for 10 min at 26\textdegree C. The reaction was stopped with 275 µl ice-cold citric buffer. The samples were then etheno-derivatized with 30 µl 2-chloroacetaldehyde for 40 min at 80\textdegree C and processed again through the HPLC to re-verify availability of cADPR, ADPR and β-NAD in the 7.2-min fraction, 8.5-min fraction and 10.5-min fraction, respectively, by assessing the content of 1,N\textsuperscript{6}-etheno-AMP in each sample.

HPLC assay of NE

The overflow of NE was assayed as described previously (15). Briefly, 115-µl aliquots from the samples were acidified with 3 µl 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, Wilmington, DE, USA) and a MD-150 column (ESA Inc., Chelmsford, MA,
USA). The mobile phase for separation consisted of the following (mmol/L): 50 Na₂PO₄; 0.2 EDTA; 3.0 l-heptanesulfonic acid, 10 LiCl, and methanol 3 % v/v 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 from Agilent Technologies (Wilmington, DE, USA). 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 fmol/mg tissue.

**MALDI-MS identification**

The chemical identities of the molecules in the absorbing fraction from reversed-phase HPLC-FLD of ADPR standards, β-NAD standards, and cADPR standards subjected to etheno-derivatization were validated by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Lyophilized samples were reconstituted with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in 70 % acetonitrile : 2 % TFA. One μl was then loaded on a sample spot. Mass spectra were acquired on a Bruker Proflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in linear mode, with delayed ion extraction.

**Degradation of β-NAD and cADPR in contact with tissue**

Canine mesenteric artery segments were loaded in a BRANDEL superfusion system as described in *Overflow Experiments*. Following a 45-min equilibration period the tissues were superfused either with β-NAD (0.2 mmol/L), cADPR (0.2 mmol/L) or NGD (0.2 mmol/L). A 200-μl sample from the beaker containing the substrate (no tissue present) was collected (S1).
The superfusion was stopped and 2 min later the content of the chamber containing the tissue was drained and collected (S2). All samples were collected in ice-cold test tubes and the reaction was stopped with liquid N₂. The samples were then processed for nucleotide detection by a reverse phase HPLC technique in conjunction with fluorescence detection as described above. Ecto-enzyme activity was determined by measuring the amount of substrate that had decreased in the S2 as compared to S1.

**Force development**

Ring preparations (5 mm long) of canine isolated mesenteric artery were mounted in 3 ml organ baths by inserting two stainless steel triangle mounts into the lumen and force displacements were further investigated as described previously (9). A resting force of 1 g was applied to the arterial segment. This was found to stretch vessels to near the optimum length for tension development. In all experiments tissues were initially equilibrated for 1 hour followed with at least 3 alternating 3 min exposures every 15 minutes to KCl (70 mmol/L) and NE (1 μmol/L) in order to establish viability and equilibrate the tissue.

Single applications of ET-1 (50 nmol/L), ANGII (100 nmol/L), U46619 (1 μmol/L), or PMA (100 nmol/L) were applied to the preparations in order to verify that at these concentrations the chemicals induce robust vasoconstrictions. Once this was validated, the effects of the above mentioned vasoconstrictors on the resting overflow of nucleotides were measured.

**Drugs**
TTX, guanethidine, 6-OHDA, ω-conotoxin GVIA, ET-1, U46619, ANGII, PMA, cADPR, ATP, ADP, AMP, ADO, Ap6A, Ap5A, Ap4A, Ap3A, Ap2A, UTP, GTP, GDP, GMP, Gp5G, Gp4G, Gp3G, cyclic AMP, cyclic GMP, NAAD, α-NAD, β-NAD, NGD, NADP, and NPP were all purchased from Sigma Chemical company (Saint Louis, MO, USA). ADPR was purchased from Calbiochem (San Diego, CA, USA) and cADPR was purchased from Biolog (San Diego, CA). Gp2G was a gift from Dr. Antonio Sillero. All drugs were initially dissolved in redistilled water with the exception of PMA and U46619, which were initially dissolved in DMSO, and further diluted in Krebs solution (final concentration of 0.1% DMSO). 6-OHDA was dissolved in Krebs solution which lacked NaHCO3 and NaH2PO4 and contained 20 µM glutathione.

Statistics

Data are presented as means ± s. e. mean. Means were compared by analysis of variance (one-way and two-way ANOVA) (GraphPadPrism v. 3, GraphPad Software, Inc.). A probability value of less than 0.05 was considered significant.

Results

EFS evokes overflow of nucleotides, nucleosides, and NE

Fig. 1 shows typical results from canine isolated mesenteric artery subjected to EFS at 4, 8, and 16 Hz. In aliquots from samples collected before EFS (pre-stimulation, PS) and analyzed with an electrochemical detector, no spontaneous release of NE was detected (Fig. 1A, PS). However, in samples collected during EFS, increasing amounts of NE were observed with the
increase in the EFS frequency (Fig. 1A, 4, 8, 16 Hz). In aliquots subjected to etheno-
derivatization and analyzed with a fluorescence detector, peaks that correspond to $1,N^\text{6}$-etheno-
ATP (eATP, elution time $\sim 9.9$ min), $1,N^\text{6}$-etheno-ADP (eADP, elution time $\sim 10.8$ min), $1,N^\text{6}$-
etheno-AMP (eAMP, elution time $\sim 12.6$ min), and $1,N^\text{6}$-etheno-ADO (eADO, elution time $\sim 16.6$
min) were observed (Fig. 1B). The overflow of all four purines was also enhanced when the
stimulation frequencies were increased. In chromatograms from samples subjected to etheno-
derivatization, an additional peak with elution time of $11.2\pm0.2$ min and unknown nature was
observed (Fig. 1B, shown as “?”). Likewise, the area of this peak increased with the increase in
stimulation frequency. Besides the canine mesenteric artery, a peak with the same elution time
was detected in several other autonomically innervated vascular (Fig. 2A) and non-vascular (Fig.
2B) smooth muscle preparations upon EFS, including canine isolated mesenteric vein, guinea-
pig isolated mesenteric artery and vein, rat and murine tail artery, canine, guinea-pig, rabbit and
murine urinary bladder, and rat vas deferens. We have also detected this peak in chromatograms
from tissue superfusate samples of stimulated rat tail artery, rat and guinea-pig vas deferens and
monkey urinary bladder (unpublished observations). Therefore, release of this factor is not
restricted to one species or one nerve-smooth muscle preparation and may have a universal role.
The compound that elutes at $\sim 11.2$ min is subjected to changes by agents affecting
neurotransmitter release (see below) and, therefore, might represent a novel factor in the
autonomic nervous system control. The present study was designed, therefore, to identify this
novel factor or the chemical group to which this compound belongs.

Given the fact that the 11.2-min peak can only be observed after etheno-derivatization of
the superfusate samples, we hypothesized that this compound has a nucleotide/nucleoside
structure.
**Screening of 1,N\(^6\)-etheno-derivatives of nucleotides and nucleosides**

The general approach was to subject authentic compounds with nucleotide or nucleoside structures, for which important intracellular or extracellular functions are well-established, to etheno-derivatization according to an identical procedure as for tissue superfusates (discussed in Methods). Thus, uridine, guanidine and adenine nucleotides or nucleosides, as well as some compounds with di-nucleotide strictures, were tested. Table 1 shows the retention times of the compounds after subjecting various nucleotides, nucleotides and dinucleotides to ethenoderivatization. Neither UTP nor guanidine derivatives (i.e., GTP, GDP, GMP, cyclic GMP), nor diguanosine polyphosphates (Gp\(_n\)G) eluted at ~11.2 as the peak of interest (Table 1, Group I). Likewise, adenine nucleotides including adenine phosphates, cAMP and Ap\(_n\)A differ significantly from ~11.2 min under our experimental conditions (Table 1, Group II). Next, we tested some nicotinamide adenine nucleotides including NADP, NAAD, and NAADP that have prominent intracellular functions with regard to Ca\(^{2+}\) handling. However, neither NAAD, nor NAADP, nor NADP had elution times of ~11.2 min (Table 1, Group III).

\(\beta\)-NAD and its metabolic products cADPR and ADPR were also considered potential candidates, taking into account their nucleotide structure, potent intracellular and potential extracellular roles (19, 20). While the elution time of the authentic ADPR was ~8.2 min (Fig. 3A), the derivatized product of ADPR (presumably etheno-ADPR) eluted at ~11.2 min (Fig. 3B), suggesting that the compound from the tissue superfusates having undergone derivatization with 2-chloroacetaldehyde is likely to be 1,N\(^6\)-etheno-ADPR. Indeed, MALDI-MS analysis of the converted product showed a major positive ion of 583 mass units, which is 24 mass units higher than the 559 mass units measured for the positive ion of ADPR (Fig. 3C, D). The mass
difference is consistent with 1,N\textsuperscript{6}-etheno-ADPR being larger than ADPR and confirms that the experimental procedure of etheno-derivatization indeed produces 1,N\textsuperscript{6}-etheno-derivatives from the authentic nucleotide compounds.

We further tested two precursors of ADPR, \(\beta\)-NAD and cADPR. HPLC-FLD analysis showed that cADPR (with elution time of \(~\sim 7.2\) min when non-derivatized) was converted with 2-chloroacetaldehyde to the fluorescent 1,N\textsuperscript{6}-etheno-ADPR within 40 min at 80\(^\circ\)C, pH 4 (Fig. 4A). The compound was separated by reverse phase HPLC and detected as one peak at 11.2±0.2 min. The retention time is identical to one, which corresponds to 1,N\textsuperscript{6}-etheno-ADPR (as seen in Fig. 3B). The structure of the nucleotide produced by derivatization of cADPR at 80\(^\circ\)C has been determined by MALDI-MS as 1,N\textsuperscript{6}-etheno-ADPR with a major positive ion of 583 mass units.

Authentic \(\beta\)-NAD, without undergoing derivatization, eluted at 10.5±0.2 min (Fig. 4B). After derivatization of \(\beta\)-NAD at 80\(^\circ\)C and pH 4, two peaks were observed: the major peak eluted at \(~\sim 11.2\) min and a smaller peak eluted at 12.8±0.2 min. On the basis of the elution times of standard 1,N\textsuperscript{6}-etheno-NAD (Sigma) and 1,N\textsuperscript{6}-etheno-ADPR (Fig. 3B) the first peak was identified as 1,N\textsuperscript{6}-etheno-ADPR and the second peak was identified as 1,N\textsuperscript{6}-etheno-NAD. When derivatization was carried out at room temperature for 48 h, the major product of \(\beta\)-NAD was 1,N\textsuperscript{6}-etheno-NAD. The molecular masses of both products were also determined by mass spectrometry. Thus, MALDI-MS of authentic \(\beta\)-NAD derivatized at 80\(^\circ\)C showed a major peak with positive ion of 583 mass units (as for 1,N\textsuperscript{6}-etheno-ADPR) and a second peak with positive ion of 688 mass units (as for 1,N\textsuperscript{6}-etheno-NAD). It is clear, therefore, that both \(\beta\)-NAD and cADPR are unstable at high temperature and acidic conditions and form ADPR, which is further derivatized to 1,N\textsuperscript{6}-etheno-ADPR. To test again this assumption we examined the effects of either citric buffer (pH 4.0) or perchloric acid (pH 2.0) on either \(\beta\)-NAD or cADPR at 23\(^\circ\)C and
at 80°C for 40 min in the absence of 2-chloroacetaldehyde. β-NAD eluted at 10.5 min when was dissolved in citric buffer and left at room temperature, whereas β-NAD at 80°C was partially degraded to ADPR (elution time 8.2 min) and nicotinamide (elution time 12.0 min) (data not shown). Under the same experimental conditions, cADPR (elution time 7.2 min) was entirely converted to ADPR (elution time 8.3 min), suggesting that cADPR was completely unstable under acidic conditions and high temperature. In parallel controls, ADPR (50 nmol) remained unchanged at acidic conditions and 80°C. Identical results were obtained when the three nucleotides were incubated with perchloric acid (pH 2.0) (data not shown). Thus, ADPR is the major product of both β-NAD and cADPR at acidic conditions and 80°C. We conclude, therefore that all three nucleotides, namely β-NAD, cADPR and ADPR, when reacted with 2-chloroacetaldehyde at 80°C for 40 min at pH 4.0 form 1,N⁶-etheno-ADPR (Fig. 5). Hence, the source of 1,N⁶-etheno-ADPR in the tissue superfusate samples subjected to etheno-derivatization may be either ADPR itself or either of its precursors, β-NAD and cADPR.

Identification of released nucleotide in tissue superfusates

We then carried out experiments to identify which one, β-NAD, cADPR or ADPR, is the nucleotide released upon EFS in the canine mesenteric artery and vein, canine urinary bladder, and murine urinary bladder. We based our approach on the finding that each of the three nucleotides has a different elution time, but after reacting with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min, each nucleotide forms 1,N⁶-etheno-ADPR that elutes at ~11.2 min. Thus, if an HPLC fraction of the tissue superfusate does not produce a peak at 11.2 min after reacting with 2-chloroacetaldehyde at 80°C for 40 min, then this fraction contains neither β-NAD, nor cADPR or ADPR. In contrast, if a fraction collected at a retention time specific for either β-NAD,
cADPR or ADPR produces 1,\(\text{N}^6\)-etheno-ADPR (and hence a peak at ~11.2 min) after etheno-derivatization then this fraction contains the corresponding nucleotide. As described in Methods, superfusate samples collected before (PS) and during EFS (ST) of 12-20 perfusion chambers were combined, concentrated and injected in the HPLC system. Fractions corresponding to the retention times of cADPR (7.2-min fraction), ADPR (8.5-min fraction) and \(\beta\)-NAD (10.5-min fraction) were then collected, etheno-derivatized and re-injected in the HPLC system. Fig. 6A, B, C (left column of the figure) show representative chromatograms from an experiment with canine mesenteric artery. In the 7.2-min fraction of the PS sample a peak at ~11.2 min was seen (Fig. 6A, upper panel), suggesting that cADPR was present in the tissue superfusate at resting (no EFS) conditions. A peak of slightly greater size was seen in the samples collected during EFS (Fig. 6A, bottom panel). Fig. 6B shows the 8.5-min fraction (presumably containing ADPR) in PS and ST samples. A peak at ~11.2 min was seen in both the PS and ST samples, the peak in the ST sample was much greater than in the PS sample. Finally, Fig. 6C shows the 10.5-min fraction (presumably containing \(\beta\)-NAD) in the PS and ST samples subjected to etheno-derivatization: a peak at 11.2 min is seen in both PS and ST, suggesting that some amount of \(\beta\)-NAD is released at rest; however, additional \(\beta\)-NAD is released during EFS. Similar results were obtained in canine urinary bladder (Fig. 6D, E, F – right column of the figure), murine urinary bladder (Supplemental materials-Fig.1), and canine mesenteric vein (data not shown). To provide negative controls for these studies we collected random fractions at retention times different from 7.2, 8.5 and 10.5 min (i.e., the 5-min fraction and 13-min fraction). No peak with retention time of 11.2 min was seen in either the 5-min or the 13-min fraction (data not shown). To provide positive controls, samples containing standards of cADPR, ADPR and \(\beta\)-NAD were injected in the HPLC system; then, the 7.2-min fraction was collected from the cADPR standard,
the 8.5-min fraction was collected from the ADPR standard, and the 10.5-min fraction was collected from the β-NAD standard. These fractions were then etheno-derivatized and analyzed with the HPLC-FLD. In all three samples a single peak of 1,N\(^6\)-etheno-ADPR at 11.2 min was observed (data not shown). Further, fractions from superfusate samples with retention times corresponding to authentic ATP, ADP, AMP, and ADO were also collected, etheno-derivatized and re-injected in the HPLC: peaks corresponding to eATP, eADP, eAMP and eADO were detected in the related samples as expected (Supplemental materials-Fig. 2), supporting the validity of our experimental approach. Finally, the 7.2-min, 8.5-min and 10.5-min HPLC fractions from tissue superfusates were treated with NPP to cleave mononucleotides and then etheno-derivatized at 80°C for 40 min. As anticipated, the 7.2-min fraction still contained 1,N\(^6\)-etheno-ADPR (formed from cADPR that was present in the superfusate and is resistant to NPP), whereas 1,N\(^6\)-etheno-AMP (the major product of NPP hydrolysis of β-NAD and ADPR) was the only nucleotide detected in both the 8.5-min fraction and the 10.5-min fraction (Supplemental materials – Fig. 3). The 7.2-min fraction also had some amounts of 1,N\(^6\)-etheno-AMP, which is a product of ATP released upon EFS and present in the 7.2-min fraction. This series of experiments re-verified that, indeed the 7.2-min HPLC fractions contained cADPR, whereas the 8.5-min and 10.5-min fractions contained ADPR and β-NAD, respectively. The results from the peak identification experiments in canine mesenteric artery and vein as well as canine and murine urinary bladders were normalized to tissue weight and injection volume and compared to standard curves of 1,N\(^6\)-etheno-ADPR produced from equal concentrations of β-NAD, cADPR, and ADPR standards (Table 2, n=3, 12-20 chambers per experiment). Thus, all three nucleotides, β-NAD, cADPR, and ADPR, are present in both PS and ST samples; however, β-NAD is the
primary nucleotide released during EFS of the canine mesenteric artery and vein, canine urinary bladder, and murine urinary bladder.

Degradation of β-NAD and cADPR in the canine isolated mesenteric artery

To test the possibility that cADPR and ADPR may be formed from β-NAD, we superfused canine mesenteric artery segments with either authentic β-NAD (a substrate for NAD glycohydrolase and ADP-ribosyl cyclase), cADPR (a substrate for cADPR hydrolase) or NGD (a substrate for ADP-ribosyl cyclase) for 2 min and measured the substrate decrease in the superfusate samples. Indeed, all three substrates were reduced while in contact with tissue. Thus, β-NAD was reduced from 0.26±0.007 (S1) to 0.12±0.03 (S2) nmol/mg tissue (n=3, P<0.05), cADPR was reduced from 0.19±0.02 (S1) to 0.13±0.01 (S2) nmol/mg tissue (n=3, P<0.05), and NGD was reduced from 0.48±0.01 (S1) to 0.32±0.03 (S2) nmol/mg tissue (n=3, P<0.05). In the experiments with NGD as a substrate, an increase in the product cGDPR was also observed; thus cGDPR was increased from 0.42±0.01 (S1) to 1.20±0.09 (S2) nmol/mg tissue (n=3, P<0.05). Representative chromatograms are shown in Supplemental materials – Fig. 4. These results suggest that at least part of cADPR and ADPR that are detected in the tissue superfusates may be formed from β-NAD that is released upon EFS.

Contraction of the canine isolated mesenteric artery does not evoke overflow of β-NAD, cADPR or ADPR

To test the hypothesis that β-NAD/cADPR/ADPR are released upon contraction of the smooth muscle, we applied several agents that induce either receptor-mediated- (i.e., ET-1, ANGII, U46619) or non-receptor mediated- (i.e., PMA) vasoconstriction. ET-1 (50 nmol/L),
ANGII (100 nmol/L), U46002 (100 nmol/L) and PMA (100 nmol/L) potently contracted the canine mesenteric artery preparations, causing 34±2.5, 41±4, 36±4.5, 10±1.8 mN (n=3) force displacement, respectively. This represents approximately 50-200 % of the force developed by 70 mmol/L KCl. The amount of 1,N^6-etheno-ADPR in samples collected in the absence of EFS (resting overflow) was 0.6±0.2 fmol/mg tissue in controls (n=9) and 0.8±0.3 (n=5, P>0.05), 0.9±0.5 (n=5, P>0.05), 0.6±0.4 (n=5, P>0.05) and 0.5±0.1 (n=5, P>0.05) fmol/mg tissue in the presence of ET-1, ANGII, U46619, and PMA, respectively.

EFS-evoked overflow of ADPR in canine isolated mesenteric artery depends on stimulation frequency

The content of both 1,N^6-etheno-ADPR (Fig. 7A) and NE (Fig. 7B) is reduced by chemical denervation with either TTX or guanethidine, or after disrupting the adrenergic nerve terminals with 6-OHDA. These findings taken together suggest that the release of β-NAD depends on the degree of neural activity. Finally, ω-conotoxin GVIA, a specific and selective N-type Ca^{2+} channel blocker (21), abolished the release of both β-NAD and NE (Fig. 7A,B) suggesting that Ca^{2+} entry via N-type voltage-operated Ca^{2+} channels is necessary for the release of both NE and β-NAD.

Exogenous β-NAD, cADPR and ADPR modulate the release of NE

To test the hypothesis that ADPR and the other members of the β-NAD/cADPR/ADPR axis modulate neurotransmitter release, we studied the effects of exogenously applied nucleotides on the EFS-evoked release of NE in canine mesenteric artery. The EFS (16Hz)-evoked release of NE was reduced from 27.8±6.0 fmol/mg tissue (n=28) in controls to 15.5±5.0
(n=8), 12±3.0 (n=7) and 10.0±4.0 (n=6) fmol/mg tissue in the presence of 100 nmol/L β-NAD, cADPR and ADPR, respectively (P<0.05). We further tested whether the inhibitory effects of 100 nmol/L NAD, ADPR and cADPR on the release of NE is shared by other nucleotides with similar structures. The EFS-evoked release of NE was 37.7±5.28 (n=3), 31.2±9.6 (n=3), 21.4±1.5 (n=4), 25.0±1.8 (n=4), and 34.1±6.8 (n=4) fmol/mg tissue in the presence of 100 nmol/L α-NAD, NAAD, NGD, cGDPR, and 8-Br-cADPR, respectively (P>0.05). If ADO, a metabolite of β-NAD, cADPR and ADPR, is responsible for the inhibitory effects of these nucleotides on NE release, then exogenous ADO should also decrease the release of NE. However, ADO increased the EFS-evoked NE overflow to 42.8±3.2 (n=5, P<0.05) at a concentration of 100 μmol/L, suggesting that β-NAD, cADPR, and ADPR themselves and not ADO are responsible for the inhibition of NE release in the canine mesenteric artery.

Discussion

ATP and NE serve as sympathetic neurotransmitters and neuromodulators (2, 4, 5, 22). Once released, ATP is rapidly converted to ADP, AMP and ADO by membrane-bound (23, 24, 25) and perhaps soluble or releasable nucleotidases (26, 27). The amounts of these nucleotides and ADO in superfusate samples from small tissue preparations are usually below the threshold of most detection methods. One particularly useful approach of attaining detectable ranges of these endogenous compounds is their chemical conversion to 1,N6-etheno-derivatives (10, 12, 13, 14, 17). This maneuver increases approximately 1,000,000 fold the fluorescence coefficient of tested nucleotides and nucleosides. The present study confirms previous studies of ours and others that upon short-duration pulse stimulation of postganglionic nerve terminals, a cocktail
containing ATP, ADP, AMP, ADO, and NE is released (10, 11, 12, 13). The factors are present in different proportions depending on tissue type, parameters of EFS, and kinetics of neurotransmitter removal; their release clearly depends on the frequency of EFS. The current investigation demonstrates the presence of additional compounds in the tissue superfusates. The overflow (release) of these factors is associated with the degree of neural activity. They appear to be released in numerous vascular and non-vascular neuromuscular preparations and implicate involvement of novel mechanisms in the autonomic nervous system control. The present study was aimed at chemical identification and initial functional characterization of these factors. The general strategy was to identify: (i) the chemical structure; (ii) possible source, and (iii) potential role of this factor(s) at the neuromuscular junction in vascular and non-vascular smooth muscles.

We assumed a nucleotide/nucleoside structure for this compound since it can only be detected after etheno-derivatization of the superfusate samples, presumably acquiring fluorescence properties. The general approach was to test whether nucleotides or nucleosides with established extracellular or intracellular functions, after undergoing an identical experimental procedure to the one the tissue superfusates had undergone (i.e., reaction with 2-chloroacetaldehyde at 80°C, pH 4.0), would elute at ~11.2 min when analyzed with the HPLC-FLD technique. More than 25 compounds with nucleotide/nucleoside structures were investigated. However, neither uridine compounds (i.e., UTP) nor guanidine compounds (i.e. GTP, GDP, GMP, cyclic GMP, GpG), nor some adenine compounds, including cAMP, ApA, NADP, NAAD, NAADP, co-eluted with the peak of interest. Interestingly, β-NAD, a coenzyme for cellular oxidation-reduction reactions, did give a major peak with elution time of ~11.2 min. Likewise, etheno-derivatization at 80°C of the two previously described derivatives of β-NAD, cADPR and ADPR (20, 28) also produced fluorescent signals with the same elution time of 11.2
min. MALDI-MS analysis revealed that 1,\(N^6\)-etheno-ADPR is the major product after etheno-derivatization at 80°C of all three: \(\beta\)-NAD, cADPR and ADPR. It appears, therefore, that both \(\beta\)-NAD and cADPR undergo initial chemical conversion to ADPR, which is then derivatized to 1,\(N^6\)-etheno-ADPR. This is in agreement with previous reports that cADPR undergoes spontaneous hydrolysis at acidic conditions (20) and that this process is accelerated at higher temperature (29). In the present study we also verified that at 80°C and acidic conditions both \(\beta\)-NAD and cADPR are quickly hydrolyzed to ADPR, which then in the presence of 2-chloroacetaldehyde is converted to 1,\(N^6\)-etheno-ADPR. At our routine reaction conditions, therefore, we cannot distinguish whether one or more nucleotides from the \(\beta\)-NAD/cADPR/ADPR family are released upon EFS, before being chemically converted \textit{in vitro} into the detectable fluorescent product 1,\(N^6\)-etheno-ADPR. Methods utilized to measure endogenous concentrations of cADPR and \(\beta\)-NAD in cell extracts including radioimmuno (30, 31), HPLC-UV (29) and cycling (32) assays cannot be utilized for detecting the nucleotides in the tissue superfusates investigated in the present study because these methods: 1) do not always discriminate between cADPR and ADPR, and 2) have insufficient sensitivity (i.e., nanomolar range) for detecting endogenous nucleotides in small tissue superfusates. Instead, our approach to identify the nucleotide that is released in tissue superfusates was based on the following reasoning: (i) the sensitivity of the FLD detection of nucleotides is increased when nucleotides are subjected to etheno-derivatization, so that small amounts of released nucleotides that usually go undetected (as is the case with neurotransmitters) could be measured, (ii) authentic \(\beta\)-NAD, cADPR and ADPR are well separated and elute at distinctly different times under our HPLC conditions, and (iii) each of the three nucleotides produces 1,\(N^6\)-etheno-ADPR when reacts with 2-chloroacetaldehyde at 80°C, pH 4.0, for 40 min; a finding that was also confirmed with
MALDI-MS analysis. To obtain greater amounts of released endogenous nucleotides we collected multiple samples and concentrated them by speed vacuum; the HPLC fractions corresponding to authentic β-NAD, cADPR and ADPR were then collected, etheno-derivatized and re-injected in the HPLC for 1, N6-etheno-ADPR analysis. We determined that superfusate samples collected in the absence of EFS (PS samples) contain small amounts of β-NAD, cADPR, and ADPR. Higher amounts of the three nucleotides were present in the tissue superfusates collected during EFS; the primary nucleotide in all tissues tested was β-NAD. The presence of cADPR, ADPR and β-NAD in the HPLC fractions from tissue superfusates was further validated by treating the fractions with NPP, an enzyme that hydrolyzes ADPR and β-NAD but not cADPR to AMP. These are particularly intriguing observations, clearly suggesting that in numerous smooth muscle preparations β-NAD is released upon stimulation of postganglionic nerve terminals.

β-NAD is converted into ADPR by NAD glycohydrolase and into cADPR by ADP-ribosyl cyclase, whereas cADPR can further be degraded to ADPR by cADPR hydrolase (28). Therefore, at least part of cADPR and ADPR that are present in tissue superfusates might be produced from the released β-NAD. In the present study we found that the concentrations of exogenous β-NAD, cADPR, as well as the ADP-ribosyl cyclase substrate NGD, are reduced in contact with artery segments, suggesting that indeed cADPR and ADPR can be produced from β-NAD in this system. It also appears that β-NAD is constitutively released since this compound together with small amounts of cADPR and ADPR are also present in tissue superfusates collected in the absence of nerve stimulation.

Next, we were interested to find whence β-NAD might originate. Our approach was to apply analogies to well-known factors at the neuromuscular junction such as NE and ATP. It is
generally accepted that NE originates exclusively from sympathetic neurons. ATP, however, may originate from either neuronal or extraneuronal sources. The endothelium is considered to be the major extraneuronal source of ATP (32, 33). However, the present study employed endothelium-denuded vessels ruling out this cell type as a major source for β-NAD under our experimental conditions. Besides the endothelium, it has been suggested that ATP can originate from the smooth muscle cells upon contraction (34). In the present study, however, the content of 1,N⁶-etheno-ADPR in tissue superfusates was not increased during contraction of the vessels with several contractile agents including the most potent vasoconstrictor yet described, ET-1. We conclude, therefore, that the contraction of the vascular smooth muscle per se does not cause release of neither β-NAD, cADPR nor ADPR in the canine mesenteric artery.

In canine isolated mesenteric artery preparations, the amount of 1,N⁶-etheno-ADPR in samples collected during EFS at 16 Hz exceeded the amount of 1,N⁶-etheno-ADPR in samples collected during 4 Hz, as did the EFS-evoked overflow of NE. In addition, the content of 1,N⁶-etheno-ADPR was reduced by chemical denervation with TTX, guanethidine, or 6-OHDA indicating that the release of β-NAD was action potential-induced. The release of NE was inhibited upon action of these agents, as well. Finally, ω-conotoxin GVIA, a specific and selective blocker of neuronal N-type Ca²⁺ channels, abolished the release of both β-NAD and NE. Thus, release of β-NAD depends on the degree of neural activity, on the influx of extracellular Ca²⁺ via N-type Ca²⁺ channels, and generally parallels the release of NE and may, therefore, originate from sympathetic nerve terminals. The release of β-NAD in non-vascular preparations (i.e., murine urinary bladder) also depends on neural activity (our unpublished observations). Further studies are needed to resolve the exact origin and detailed characteristics
of β-NAD release; however, it is clear that the release of this nucleotide shows a steep correlation with the activity of peripheral nerve terminals in various smooth muscle preparations.

We then asked what the role of these nucleotides might be at the vascular neuromuscular junction. The β-NAD/cADPR/ADPR system has well-known intracellular functions. For example, β-NAD serves as a coenzyme for cellular oxidation-reduction reactions and a precursor of ADPR in the posttranslational modification of proteins, cADPR shows potent Ca2+-releasing activity from ryanodine-sensitive stores in a wide variety of cells, and ADPR modulates the function of membrane ion channels in addition to serving a well-known role in posttranslational modification of proteins (reviewed in 28, 35). This system, therefore, appears to be involved in the regulation of cell functions related to intracellular Ca2+ handling and membrane ion channel activity and, hence, might be an important factor in the smooth muscle neuroeffector process. Besides the aforementioned functions that β-NAD, cADPR and ADPR play as important intracellular constituents, it is likely that these molecules also play extracellular roles. Interestingly, in the present study exogenous application of β-NAD, cADPR and ADPR (100 µmol/L) reduced the release of NE. At present it is not clear whether the inhibitory effect of β-NAD we observed is mediated by β-NAD itself or by its metabolic products ADPR and cADPR. It is also unclear whether these effects are mediated by other nucleotide metabolites such as ATP and ADO. In the present study we showed that other nucleotides with similar structures including α-NAD, NAAD, NGD, cGDPR, and 8-Br-cADPR (100 µmol/L) did not significantly reduce the release of NE. Interestingly, exogenous ADO, which is a major metabolite of β-NAD, cADPR and ADPR, even facilitated the release of NE in the canine mesenteric artery possibly via facilitatory adenosine A2 receptors as shown in other systems (36, 37). Although the mechanisms of action are far from clear, the present study suggests that the β-
NAD/cADPR/ADPR axis may have a novel role in neuromodulation. Further work will be necessary to resolve the full range of extracellular functions and the mechanisms of extracellular action of β-NAD and its metabolites at the smooth muscle neuroeffector junction.

In conclusion, we have identified constitutive and nerve-evoked release of β-NAD and its metabolic products cADPR and ADPR in numerous smooth muscle preparations. These nucleotides appear to regulate the release of NE at the vascular neuroeffector junction. Therefore, the β-NAD/cADPR/ADPR system constitutes a novel pathway in the autonomic nervous system control.

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Table 1. Average elution times (min) of uridine and guanidine nucleotides (Group I), adenine nucleotides (Group II) and adenine dinucleotides (Group III) subjected to etheno-derivatization with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min and analyzed by HPLC-FLD technique.

| Group I | UTP | GTP | GDP | GMP | cGMP | Gp₂G | Gp₄G | Gp₃G | Gp₂G |
|---------|-----|-----|-----|-----|------|------|------|------|------|
|         | 3.6 | 7.2 | 8.1 | 9.2 | 14.5 | 8.3  | 8.8  | 9.2  | 9.6  |

| Group II | ATP | ADP | 5'AMP | ADO | Ap₆A | Ap₅A | Ap₄A | Ap₃A | Ap₂A | cAMP | 3'AMP |
|----------|-----|-----|-------|-----|------|------|------|------|------|------|-------|
|          | 9.9 | 10.8| 12.6  | 16.6| 12.5 | 12.8 | 13.1 | 13.6 | 13.3 | 17.7 | 14.4  |

| Group III | NAAD | NAADP | NADP | αNAD |
|-----------|------|-------|------|------|
|           | 11.6 | 9.0   | 7.4  | 10.4 |

Table 2. Content of $\beta$-NAD, ADPR and cADPR (fmol/mg tissues) in HPLC fractions derivatized to 1,N$^6$-etheno-ADPR at 80°C, pH 4.0 for 40 min. The fractions are from concentrated superfusate samples collected before (PS) and during EFS at 16 Hz, 0.1-0.3 ms, for 60 s (ST) of murine urinary bladder (MUB), canine urinary bladder (CUB), canine isolated mesenteric artery (CMA), and canine mesenteric vein (CMV). Data represent mean±SEM from 3 experiments. Asterisks denote significant difference between PS and ST samples (P<0.05).

|       | NAD   | ADPR   | cADPR  |
|-------|-------|--------|--------|
|       | PS    | ST     | PS     | ST     | PS     | ST     |
| MUB   | 1.58±0.19 | 15.1±3.20* | 1.40±0.59 | 6.17±2.40* | 0.40±0.40 | 1.41±0.10* |
| CUB   | 0.60±0.20 | 26.5±4.50* | 0.40±0.08 | 9.00±6.00* | 0.24±0.20 | 1.86±0.50* |
| CMA   | 0.50±0.22 | 7.10±0.70* | 0.42±0.10 | 5.80±0.80* | 0.20±0.08 | 2.50±0.90* |
| CMV   | 1.29±0.07 | 6.80±0.10* | 0.50±0.07 | 2.40±0.10* | 0.10±0.10 | 0.72±0.10* |
Figure legends:

Fig. 1: EFS (0.1 ms, 15 V, 120 s) evokes frequency-dependent overflow of NE and adenine nucleotides (ATP, ADP, AMP, ADO) in canine isolated mesenteric artery. Original chromatograms are shown from HPLC-ECD analysis of catecholamines (A) and HPLC-FLD analysis of purines (B) in tissue superfusate samples collected before (pre-stimulation, PS) and during EFS (ST) at 4, 8 and 16 Hz. Aliquots were derivatized with 2-chloroacetaldehyde (80°C, pH 4, 40 min) to 1,N⁶-etheno (e)-nucleotides (i.e., eATP, eADP, eAMP) and 1,N⁶-etheno-(e)ADO. At all frequencies of stimulation an additional peak with unknown identity (marked “?”) with elution time of ~11.2 min is observed. Scales apply to all chromatograms.

Fig. 2: An unidentified peak with elution time ~11.2 min is observed in superfusate samples collected during EFS (0.1 ms, 15 V, 16 Hz) of several vascular and non-vascular preparations. Original chromatograms from HPLC-FLD analysis of samples collated from vascular smooth muscle (A) including canine mesenteric vein (CMV), guinea-pig mesenteric artery (GPMA), guinea-pig mesenteric vein (GPMV), rat tail artery (RTA), and mouse tail artery (MTA), and non-vascular smooth muscles (B) including canine (CUB), rabbit (RbUB), guinea-pig (GPUB), murine (MUB) urinary bladder, and rat vas deferens (RVD). Aliquots were derivatized with 2-chloroacetaldehyde (80°C, pH 4, 40 min) to 1,N⁶-etheno (e)-nucleotides (i.e., eATP, eADP, eAMP) and 1,N⁶-etheno-(e)ADO. At all frequencies of stimulation an additional peak with unknown identity (marked “?”) with elution time of ~11.2 min is observed. Scale applies to all chromatograms.
Fig. 3: ADPR reacts with 2-chloroacetaldehyde and, at 80°C, pH 4.0 for 40 min, is derivatized to 1,N\textsuperscript{6}-etheno-ADPR (eADPR). (A) Original chromatogram of authentic non-derivatized ADPR (40 nmol). (B) Original chromatogram of derivatized ADPR (1 pmol). Scale applies to (A) and (B). (C) MALDI-MS analysis of non-derivatized ADPR. (D) MALDI-MS analysis of etheno-derivatized ADPR.

Fig. 4: (A) Original chromatograms of authentic and etheno-derivatized cADPR. Authentic cADPR (40 nmol) elutes at 7.2 min when detected by the HPLC-FLD method (upper panel). cADPR reacts with 2-chloroacetaldehyde and, at 80°C, pH 4.0 for 40 min, is derivatized to 1,N\textsuperscript{6}-etheno-ADPR (eADPR) (bottom panel). The chromatogram shows eADPR generated from 1 pmol cADPR. (B) Original chromatograms of authentic and etheno-derivatized \(\beta\)-NAD. Authentic \(\beta\)-NAD (40 nmol) elutes at 10.5 min (upper panel). \(\beta\)-NAD reacts with 2-chloroacetaldehyde and, at 80°C, pH 4.0 for 40 min, is derivatized to 1,N\textsuperscript{6}-etheno-ADPR (eADPR) (bottom panel). The chromatogram shows eADPR generated from 1 pmol \(\beta\)-NAD.

Fig. 5: Structural formulae of \(\beta\)-NAD, cADPR and ADPR. All three nucleotides give 1,N\textsuperscript{6}-etheno-ADPR (eADPR) after reaction with 2-chloroacetaldehyde at 80°C (pH 4.0) for 40 min.

Fig. 6: (A) Canine mesenteric artery. Original chromatograms of the 7.2-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.3 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (B) Canine mesenteric artery. Original chromatograms of the 8.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper
panel) and during EFS (ST, 16 Hz, 0.3 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (C) Canine mesenteric artery. Original chromatograms of the 10.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.3 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. eADPR is detected in all fractions; the amount of eADPR is greater in the ST samples. (D) Canine urinary bladder. Original chromatograms of the 7.2-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (E) Canine urinary bladder. Original chromatograms of the 8.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (F) Canine urinary bladder. Original chromatograms of the 10.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. In all fractions different amounts of eADPR are detected: the amount of eADPR is greater in the ST samples.

Fig. 7: The EFS (16 Hz, 0.1 ms, 15V)-evoked release of both β-NAD (A) and NE (B) in canine isolated mesenteric artery is sensitive to chemical denervation with TTX (1 µmol/L), guanethidine (10 µmol/L), 6-OHDA (300 mg/L), and ω-conotoxin GVIA (5 nmol/L). Data represent mean±SEM from 4-6 experiments. Asterisks denote significant differences from controls, P<0.05.
Fig. 2

200 LU

CMV

GPMA

GPMV

RTA

MTA

CUB

RbUB

GPUB

MUB

RVD

Fig. 2
Fig. 3
Fig. 4
Fig. 5

\[
\begin{align*}
\text{NAD}^+ & \xrightarrow{\text{ClCH}_2\text{CHO}} \text{eADPR} \\
\text{eADP-R} & \xrightarrow{\text{Citric buffer, pH ~ 4}} \text{eADPR} \\
\text{ADPR} & \xrightarrow{80 \degree C, 40 \text{ min}} \text{eADPR}
\end{align*}
\]
Fig. 6

A. 7.2-min fraction (cADPR)

B. 8.5-min fraction (ADPR)

C. 10.5-min fraction (β-NAD)

D. 7.2-min fraction (cADPR)

E. 8.5-min fraction (ADPR)

F. 10.5-min fraction (β-NAD)
Fig. 7
A. 7.2-min fraction (cADPR)

B. 8.5-min fraction (ADPR)

C. 10.5-min fraction (β-NAD)

SUPPLEMENTAL MATERIALS
Fig. 1: Murine urinary bladder. (A) Original chromatograms of the 7.2-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (B) Original chromatograms of the 8.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. (C) Original chromatograms of the 10.5-min fraction of tissue superfusate samples (12 chambers combined) collected before EFS (PS, upper panel) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, lower panel) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. In all fractions different amounts of eADPR are detected: the amount of eADPR is greater in the ST samples.
Fig. 2: Canine mesenteric artery. HPLC-FLD analysis of fractions of tissue superfusates collected during EFS (16 Hz, 0.3 ms, 60 s) and etheno-derivatized with 2-chloroacetaldehyde, at 80°C, pH 4.0 for 40 min. (A) Original chromatograms of the 7.2-min fraction containing ATP. (B) Original chromatograms of the 8-min fraction containing ADP. (C) Original chromatograms of the 9-min fraction containing AMP. (D) Original chromatograms of the 15-min fraction containing ADO.
Fig. 3: Canine mesenteric artery. Original chromatograms of the 7.2-min fraction (A), 8.5-min fraction (B) and 10.5-min fraction (C) of tissue superfusates collected during EFS (16 Hz, 0.3 ms, 120 s), treated with 0.2 U NPP for 10 min at 26°C, and then etheno-derivatized with 2-chloroacetaldehyde (80°C, pH 4.0, for 40 min). 1,N\textsuperscript{6}-etheno-AMP is the major product in both the 8.5-min and 10.5-min fractions, whereas in the 7.2-min fraction 1,N\textsuperscript{6}-etheno-ADPR is the primary product.
SUPPL. Fig. 4

A. 
(-) tissue 
(+/-) tissue 

B. 
(-) tissue 
(+/-) tissue 

C. 
(-) tissue 
(+/-) tissue 

$eta$-NAD 
NGD 
cADPR 
LU
Fig. 4: Canine mesenteric artery. Original chromatograms from (A) β-NAD (0.2 mmol/L), (B) NGD (0.2 mmol/L), and (C) cADPR (0.2 mmol/L), 25 nmol/injection, in the absence [(-) tissue] and presence of tissue [(+) tissue]. In all cases the substrate concentration was reduced during the 2-min contact with the artery strips. In the case of NGD as substrate an increase of the product cGDPR is also observed (B).
Release of nicotinamide adenine dinucleotide (β-NAD) upon stimulation of postganglionic nerve terminals in blood vessels and urinary bladder
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