

## $\beta$ -NAD is a novel nucleotide released on stimulation of nerve terminals in human urinary bladder detrusor muscle

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**Breen, Leanne T., Lisa M. Smyth, Iliia A. Yamboliev, and Violeta N. Mutafova-Yambolieva.**  $\beta$ -NAD is a novel nucleotide released on stimulation of nerve terminals in human urinary bladder detrusor muscle. *Am J Physiol Renal Physiol* 290: F486–F495, 2006. First published September 27, 2005; doi:10.1152/ajprenal.00314.2005.—Endogenous nucleotides with extracellular functions may be involved in the complex neural control of human urinary bladder (HUB). Using HPLC techniques with fluorescence detection, we observed that in addition to ATP and its metabolites ADP, AMP and adenosine, electrical field stimulation (EFS; 4–16 Hz, 0.1 ms, 15 V, 60 s) of HUB detrusor smooth muscle coreleases novel nucleotide factors, which produce etheno-1 $N^6$ -ADP-ribose (eADPR) on etheno-derivatization at high temperature. A detailed HPLC fraction analysis determined that nicotinamide adenine dinucleotide ( $\beta$ -NAD<sup>+</sup>; 7.0  $\pm$  0.7 fmol/mg tissue) is the primary nucleotide that contributes to the formation of eADPR. The tissue superfusates collected during EFS also contained the  $\beta$ -NAD<sup>+</sup> metabolite ADPR (0.35  $\pm$  0.2 fmol/mg tissue) but not cyclic ADPR (cADPR). HUB failed to degrade nicotinamide guanine dinucleotide (NGD<sup>+</sup>), a specific substrate of ADP ribosyl cyclase, suggesting that the activity of this enzyme in the HUB is negligible. The EFS-evoked release of  $\beta$ -NAD<sup>+</sup> was frequency dependent and is reduced in the presence of tetrodotoxin (TTX; 0.3  $\mu$ M),  $\omega$ -conotoxin GVIA (50 nmol/l), and botulinum neurotoxin A (BoNT/A; 100 nmol/l), but remained unchanged in the presence of guanethidine (3  $\mu$ M),  $\omega$ -agatoxin IVA (50 nmol/l), or charbachol (1  $\mu$ M). Capsaicin (10  $\mu$ M) increased both the resting and EFS-evoked overflow of  $\beta$ -NAD<sup>+</sup>. Exogenous  $\beta$ -NAD<sup>+</sup> (1  $\mu$ M) reduced both the frequency and amplitude of spontaneous contractions. In conclusion, we detected nerve-evoked overflow of  $\beta$ -NAD<sup>+</sup> and ADPR in HUB. The  $\beta$ -NAD<sup>+</sup>/ADPR system may constitute a novel inhibitory extracellular nucleotide mechanism of neural control of the human bladder.

synaptic transmission; purinergic; neurotransmitter; nucleotide; co-transmission

NEURAL REGULATION OF URINARY bladder functions uses multiple excitatory and inhibitory pathways projecting from the central nervous system to precise targets in the bladder. There is growing appreciation that peripheral nerves release more than one transmitter, a process referred to as plurichemical neurotransmission (16) or cotransmission (8). For example, most parasympathetic constrictor neurons, which represent the major excitatory input to the bladder, release the cotransmitters acetylcholine (ACh) and adenosine 5'-triphosphate (ATP) (10, 11, 36). In addition to neurotransmitters, neuromodulatory substances modify synaptic transmission by altering the intrinsic

properties of neurons or smooth muscle cells. Therefore, cotransmission and neuromodulation ensure complex neural control of the bladder and raise diverse possibilities for synaptic integration.

Among the endogenous nucleotides ATP is a relatively well-studied substance with regard to its extracellular roles in the bladder. ATP has been determined as a parasympathetic cotransmitter in numerous species (11) as well as a primary activator of afferent sensory nerves during bladder distension (4, 14). Interestingly, while the purinergic component of neurally evoked contractions in normal human bladder is almost negligible (4, 32), the role of ATP in the human bladder enhances significantly with aging (35) and in pathological conditions including neurogenic bladder, outflow obstruction, idiopathic detrusor instability, and interstitial cystitis (2, 23, 28, 29), suggesting that endogenous nucleotides may have special roles in bladder dysfunctions.

We recently reported that activation of postganglionic nerve terminals in a number of isolated vascular and nonvascular preparations, including canine and murine bladder detrusor smooth muscle, releases  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NAD<sup>+</sup>) in a tetrodotoxin (TTX)-sensitive manner (31). Tissue superfusates collected during nerve stimulation also contained two metabolites of  $\beta$ -NAD<sup>+</sup>, namely adenosine 5'-diphospho ribose (ADPR) and cyclic ADPR (cADPR). These are intriguing observations, suggesting that the  $\beta$ -NAD<sup>+</sup>/cADPR/ADPR system may be a novel extracellular player in the urinary bladder of dog and mouse. It is not clear, however, whether this system shares similar functional significance in the human bladder. The present study was designed, therefore, to test the hypothesis that  $\beta$ -NAD<sup>+</sup> is released on activation of postganglionic nerve terminals by short-pulse electrical field stimulation (EFS) in human isolated detrusor smooth muscle. Using HPLC fraction analysis, we explored the content of  $\beta$ -NAD<sup>+</sup>, cADPR, and ADPR in tissue superfusates collected before and during EFS of detrusor smooth muscle segments. We also carried out initial characterization of the release of  $\beta$ -NAD<sup>+</sup> by testing the role of stimulation frequency as well as involvement of neuronal membrane channels, *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and smooth muscle contraction in this release. The major finding of this study is that  $\beta$ -NAD<sup>+</sup> is released on stimulation of postganglionic nerve terminals in human detrusor muscle. These results imply that the  $\beta$ -NAD<sup>+</sup>/cADPR/ADPR system may serve new extracellular functions at the neurosmooth muscle junction in the human urinary bladder.

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## MATERIALS AND METHODS

### Human Bladder Detrusor Preparations

Human bladder segments were obtained through the Cooperative Human Tissue Network, Western Division, at the National Institutes of Health. Normal human detrusor muscle segments were obtained with full informed consent from nine male and one female patients (51–82 years of age) undergoing cystectomy for cancer, who had no history of radiotherapy. Tissue was taken from a site distant to the tumor from a normal-appearing bladder area and immediately placed in RPMI medium (4°C) and transported overnight to the site of experiments. On arrival, tissues were immediately placed in oxygenated Krebs solution with the following composition (mmol/l): 118.5 NaCl, 4.2 KCl, 1.2 MgCl<sub>2</sub>, 23.8 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 11.0 dextrose, 1.8 CaCl<sub>2</sub> (pH 7.4). After the connective and fat tissues were dissected out, the detrusor smooth muscle strips were processed according to the experimental protocols described in the following sections. The use of human bladder specimens for these experiments was approved by the University of Nevada, Reno Biomedical Institutional Review Board.

### Murine Bladder Detrusor Preparations

Male C57BL6 mice (15–20 g) were euthanized with an overdose of CO<sub>2</sub>. The abdomen was opened and the urinary bladder was dissected out and bathed in oxygenated Krebs solution. The bladder was pinned in a dissecting dish, opened longitudinally, and the urothelium was gently separated from the detrusor muscle. The murine detrusor muscle was cleaned from connective tissue and blood vessels and loaded in the Brandel superfusion system for measuring ADP ribose cyclase activity as described in *Degradation of NGD in Contact with Tissue*. The use of the laboratory animals for these experiments was approved by the University of Nevada's Animal Care and Use Committee.

### Overflow Experiments

Detrusor muscle tissue segments (~50–60 mg wet wt) were placed in small-volume (200  $\mu$ l) water-jacket Brandel superfusion chambers (Biomedical Research and Development Laboratories, Gaithersburg, MD) for neurotransmitter overflow experiments as described previously (6, 7, 26, 31). 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 at 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 s with a train of supra-threshold pulses of 0.1 ms at 4, 8, or 16 Hz. 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. Time controls parallel to drug treatment protocols were carried out with each group of experiments. Samples were analyzed for nucleotide/nucleoside contents by HPLC techniques with fluorescence detection as described previously (5–7, 31).

### Experimental Protocols

**Protocol 1: treatment with TTX,  $\omega$ -CTx GVIA, and  $\omega$ -AgaTx IVA.** After the conditioning stimulation, the tissues were superfused for 30 min with either TTX (0.3  $\mu$ mol/l),  $\omega$ -CTx GVIA (10–50 nmol/l), or  $\omega$ -AgaTx IVA (20 and 50 nmol/l) to block neuronal fast Na<sup>+</sup> channels, Cav2.2 (N-type), and Cav2.1 (P/Q-type) voltage-operated Ca<sup>2+</sup> channels (VOCC), respectively. Three superfusate samples for each treatment were collected (S1: in the absence of toxin, S2: in the presence of toxin before EFS, and S3: in the presence of toxin during EFS) and processed as usual (see *Sample Preparation and HPLC Analysis of Etheno-Nucleotides and Etheno-Nucleosides*).

**Protocol 2: treatment with BoNT/A.** Tissue segments were incubated for 5.5 h at 37°C in a sealed vial containing either 250  $\mu$ l of RPMI-1640 solution (control group) or 250  $\mu$ l of RPMI-1640 and 100 nM BoNT/A (BoNT/A group). At the end of the incubation period, tissue segments were washed three times (5 min each) in Krebs solution without BoNT/A. The tissues were then loaded in the Brandel system, equilibrated as usual, and superfusate samples were collected before and during EFS.

**Protocol 3: treatment with guanethidine.** The tissue segments were incubated for 2 h (37°C) in Krebs solution containing 3–10  $\mu$ mol/l guanethidine to inhibit action potential-induced activation of sympathetic nerve terminals. Superfusate samples were collected before and during EFS.

**Protocol 4: treatment with capsaicin.** To test whether activation of capsaicin-sensitive receptors (and hence capsaicin-sensitive sensory neurons) mediate  $\beta$ -NAD<sup>+</sup> release, tissue segments were superfused with capsaicin (10  $\mu$ mol/l) for either 5 or 30 min after the conditioning stimulation. Superfusate samples were collected in the absence and in the presence of capsaicin before and during EFS.

**Protocol 5: treatment with carbachol.** To test the possibility that contraction of the smooth muscle is the primary cause of  $\beta$ -NAD<sup>+</sup> overflow, tissues were perfused with carbachol (CCh; 1  $\mu$ mol/l) for 5 min. Superfusate samples were collected in the absence and presence of CCh.

### Sample Preparation and HPLC Assay of Etheno-Nucleotides and Etheno-Nucleosides

A method modified from Levitt et al. (22), which originally describes a procedure for detection of 1,N<sup>6</sup>-etheno-derivatives of ATP, ADP, AMP, and ADO, was employed. Briefly, 100  $\mu$ l of a citrate phosphate buffer (pH 4.0) were added to 200  $\mu$ l of the superfusate sample in a 1.5 ml Eppendorf tube. Chloroacetaldehyde was synthesized according to a method modified from Secrist et al. (30) and Levitt et al. (22) and described previously (5). Ten microliters of 2-chloroacetaldehyde were added to the samples in a fume hood; the samples were heated for 40 min at 80°C in a dry bath incubator (Fisher Scientific) to produce 1,N<sup>6</sup>-etheno-nucleotides and 1,N<sup>6</sup>-etheno-nucleosides.

The liquid chromatographic system used throughout this study was an HP1100 LC module system (Agilent Technologies, Wilmington, DE) as described previously (5, 31). The mobile phase comprised of 0.1 mol/l KH<sub>2</sub>PO<sub>4</sub> (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 and run time was 20 min. Column temperature was ambient while the autosampler temperature was 4°C. The fluorescence 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 (5). The nonderivatized compounds were detected at an excitation wavelength of 270 nm and emission wavelength of 410 nm as described previously (31).

### Sample Concentration and HPLC Fraction Analysis

To identify the compound that is released during EFS in human detrusor muscle, superfusate samples from 8–10 chambers containing ~40–50 mg tissue per chamber were placed together in two 2-ml Eppendorf tubes containing the prestimulation samples and the samples were collected during EFS, respectively. The two resulting samples were further concentrated by Speed Vacuum (Savant SVC100, Thermo Electron, Westmont, IL) to 1 ml volume. Seven hundred and fifty microliters of each concentrated sample were injected into the HPLC system and 400- $\mu$ l 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  $\beta$ -NAD (10.3–10.7 min, "10.5-min fraction") were collected in borosilicate culture tubes

containing 180  $\mu$ l of citric buffer. The exact retention times for the three nucleotides were determined by injecting  $\beta$ -NAD<sup>+</sup>, cADPR, and ADPR standards (40 nmol/injection) in the same sequence before the concentrated superfusate samples. The HPLC fractions were further subjected to etheno-derivatization with 20  $\mu$ l 2-chloroacetaldehyde as described in *Sample Preparation and HPLC Assay of Etheno-Nucleotides and Etheno-Nucleosides*. The derivatized samples were injected into the HPLC and analyzed for 1,N<sup>6</sup>-etheno-ADPR content.

#### *Degradation of NGD in Contact with Tissue*

Tissue segments from human or murine bladder detrusor smooth muscle were loaded in a Brandel superfusion system as described in *Overflow Experiments*. Following a 45-min equilibration period, the tissues were superfused with nicotinamide guanine dinucleotide (NGD<sup>+</sup>; 0.2 mmol/l). A 200- $\mu$ l 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 by freezing with liquid N<sub>2</sub>. The samples were then processed for nucleotide detection by a reverse-phase HPLC technique in conjunction with fluorescence detection as described above. The ADP ribosyl cyclase activity was determined by measuring the amount of substrate NGD that had decreased and the amount of the product cGDPR that had increased in the S2 compared with S1.

#### *Western Immunoblot Analysis of SNAP-25*

After pharmacological experimentation, human detrusor muscle segments from control and BoNT/A-treated groups were weighed and frozen by immersion in liquid nitrogen. Frozen strips were pulverized and total protein was extracted by glass-glass homogenization with a buffer composed of 20 mM Tris, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 100  $\mu$ M AEBSF, and 1  $\mu$ M leupeptin. Insoluble material was pelleted by centrifugation at 15,000 g for 1 h at 4°C. Total protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad kit, Hercules, CA) using BSA for standards. Tissue homogenates were reduced with Laemmli reagent and equal amounts of total protein (15  $\mu$ g) were resolved by SDS-PAGE (15% acrylamide) and transferred onto nitrocellulose membranes for 1.5 h at 24 V and 4°C (Genie blotter, Idea Scientific, Minneapolis, MN). Membranes were blocked for 1 h with LI-COR blocking buffer (LI-COR, Lincoln, NE) and probed for 18 h at 4°C with a SNAP-25 primary mouse monoclonal antibody (Synaptic Systems, Göttingen, Germany), diluted 2,000-fold in LI-COR buffer. After removal of excess primary antibody, membranes were incubated for 45 min at room temperature with a secondary mouse antibody coupled to IR800 infrared fluorescence marker (emission wavelength 800 nm, Rockland Immunochemicals, PA), diluted 100,000-fold in LI-COR buffer. Fluorescent images were obtained with an infrared Odyssey scanner (LI-COR).

#### *Force Development*

Detrusor smooth muscle strips (5-mm long) were mounted in 10-ml organ baths by means of loop-like sutures, and force displacements were further monitored with Fort 10 isometric force transducers in a Myobath 4 system (World Precision Instruments, Sarasota, FL). A resting force of 1 g was applied to each muscle segment. This was found to stretch tissue segments to near the optimum length for tension development. In all experiments, tissues were initially equilibrated for 1 h followed with at least three 3-min exposures to KCl (30 mmol/l) every 15 min to establish viability and equilibrate the tissue.

CCh (1  $\mu$ mol/l) was applied to the preparations and the contractile response of the smooth muscle strip was monitored for 3–5 min. In

some experiments, exogenous  $\beta$ -NAD<sup>+</sup> (1  $\mu$ mol/l) was applied to the bath and spontaneous contractile activity (frequency and amplitude) of the tissue segment monitored.

#### *Drugs*

TTX, guanethidine,  $\omega$ -conotoxin GVIA, agatoxin IVA, capsaicin, cADPR, ATP, ADP, AMP, ADO,  $\beta$ -NAD, and NGD were all purchased from Sigma (St. Louis, MO). ADPR was purchased from Calbiochem (San Diego, CA) and cADPR was purchased from Biolog (San Diego, CA). Botulinum toxin A was purchased from List Biological Laboratories (Campbell, CA) and RPMI-1640 from Invitrogen (GIBCO, Carlsbad, CA). Anti-SNAP 25 mouse monoclonal antibody (Synaptic Systems, Cat. no. 111011). All drugs were initially dissolved in redistilled water with the exception of capsaicin, which was initially dissolved in DMSO, and further diluted in Krebs solution (final concentration 0.1% DMSO).

#### *Statistics*

Data are presented as means  $\pm$  SE. Means were compared by ANOVA (one-way ANOVA; GraphPadPrism v. 3, GraphPad Software). A probability value of <0.05 was considered significant.

## RESULTS

### *EFS Evokes Overflow of Nucleotides and Adenosine*

Figure 1 shows typical results from superfusate samples collected before (PS, prestimulation) and during EFS (ST) of human isolated detrusor muscle preparations subjected to EFS at 4 and 16 Hz, 0.1-ms pulse width, for 60 s. In samples subjected to etheno-derivatization and analyzed with a fluorescence detector, peaks that correspond to 1,N<sup>6</sup>-etheno-ATP (eATP, elution time  $\sim$ 9.9 min), 1,N<sup>6</sup>-etheno-ADP (eADP, elution time  $\sim$ 10.8 min), 1,N<sup>6</sup>-etheno-ADPR (elution time  $\sim$ 11.2 min), 1,N<sup>6</sup>-etheno-AMP (eAMP, elution time  $\sim$ 12.6 min), and 1,N<sup>6</sup>-etheno-ADO (eADO, elution time  $\sim$ 16.6 min) were observed. The overflow of the five purines increased with the stimulation frequencies. At 4 Hz, the eATP peak exceeded the eADPR peak, whereas at 16 Hz a reverse relationship was observed. Thus the ratio eATP:eADPR was  $1.5 \pm 0.2$  at 4 Hz and  $0.07 \pm 0.02$  at 16 Hz, suggesting that the composition of the neurotransmitter cocktail released from nerve terminals depends on the level of neural activity. All peaks were significantly reduced in the presence of TTX (0.3  $\mu$ mol/l). Figure 2 shows the overflow of ATP, ADP, AMP, and ADO in the human bladder detrusor at 4, 8, and 16 Hz. While eATP, eADP, eAMP, and eADO correspond to ATP, ADP, AMP, and ADO, respectively, eADPR could be formed from either  $\beta$ -NAD<sup>+</sup>, cADPR, or ADPR during etheno-derivatization of the superfusate samples at pH 4.0 and 80°C (31). Therefore, an HPLC fraction analysis was carried out to identify which one of the three nucleotides contributed the most to the peak of eADPR.

#### *HPLC Fraction Analysis*

As we reported previously (31), authentic cADPR, ADPR, and  $\beta$ -NAD<sup>+</sup> elute at different retention times (e.g., 7.2, 8.5, and 10.5 min, respectively; Fig. 3A). When etheno-derivatized, however, the three nucleotides form eADPR, which elutes at 11.2 min (Fig. 3A). The three nucleotides have different fluorescence efficiency as illustrated by the size of the eADPR peaks produced from equal amounts of ADPR, cADPR, and  $\beta$ -NAD<sup>+</sup> (Fig. 3B). If a fraction col-

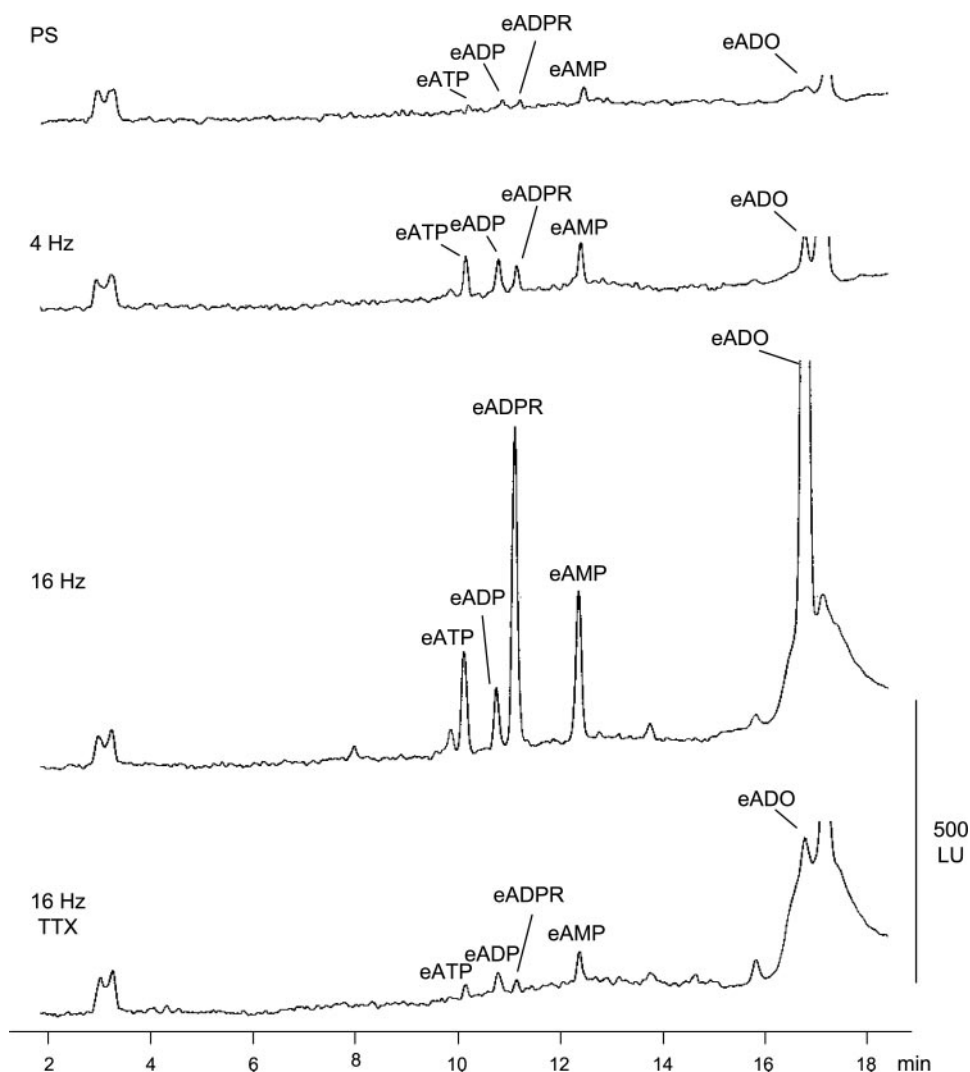


Fig. 1. Electrical field stimulation (EFS; 0.1 ms, 15 V, 60 s) evokes frequency-dependent overflow of adenine nucleotides in human isolated bladder detrusor smooth muscle. Original chromatograms are shown from HPLC-FLD analysis of purines in tissue superfusate samples collected before (pre-stimulation, PS) and during EFS (ST) at 4 and 16 Hz. Aliquots were derivatized with 2-chloroacetaldehyde (80°C, pH 4, 40 min) to 1,*N*<sup>6</sup>-etheno (e)-nucleotides (i.e., eATP, eADP, eAMP) and 1,*N*<sup>6</sup>-etheno-(e)ADO. At all frequencies of stimulation, 1,*N*<sup>6</sup>-etheno-(e)ADPR with elution time of ~11.2 min is also observed. The EFS (16 Hz)-evoked release of purines is significantly reduced in the presence of TTX (0.3  $\mu$ mol/l for 30 min). Scales apply to all chromatograms.

lected at a retention time specific for either  $\beta$ -NAD<sup>+</sup>, cADPR or ADPR produces 1,*N*<sup>6</sup>-etheno-ADPR (and hence a peak at ~11.2 min) after etheno-derivatization with 2-chloroacetaldehyde at 80°C (pH 4.0), then this fraction contains the corresponding nucleotide. As described in MATERIALS AND METHODS, superfusate samples collected before (PS) or during EFS (ST) of 8–10 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<sup>+</sup> (10.5-min fraction) were then collected, etheno-derivatized, and reinjected in the HPLC system. Figure 4 shows representative chromatograms from an experiment with human detrusor muscle. In the 7.2-min fraction of both the PS and ST samples, no peak at ~11.2 min was observed (Fig. 4A), suggesting that cADPR was absent both at rest and during EFS. In the 8.5-min fraction (presumably containing ADPR), a small peak at 11.2 min was observed in the PS sample (Fig. 4B, top) and a peak with greater area was observed in the samples collected during EFS (ST, Fig. 4B, bottom). Thus ADPR was present in small amounts in the PS sample; additional ADPR was formed or released during EFS. Finally, in the 10.5-min fraction (and hence the frac-

tion presumably containing  $\beta$ -NAD<sup>+</sup>), no peak was observed in the PS sample (Fig. 4C, top). However, there was a well-defined peak in the ST sample (Fig. 4C, bottom). The results from the HPLC fraction identification experiments were normalized to tissue weight and injection volume and compared with standard curves of 1,*N*<sup>6</sup>-etheno-ADPR produced from equal concentrations of  $\beta$ -NAD<sup>+</sup>, cADPR, and ADPR standards (Fig. 5). It is likely that the absolute amounts of  $\beta$ -NAD<sup>+</sup> and ADPR are underestimated in the HPLC fractions due to loss of nucleotides during the multiple steps of the assay. However, it is clear that  $\beta$ -NAD<sup>+</sup> is the primary nucleotide that is released on nerve stimulation of HUB especially at higher frequency of stimulation.  $\beta$ -NAD<sup>+</sup> is accompanied with small amounts of its metabolite ADPR. These findings are in accordance with the findings in canine mesenteric artery and vein as well as canine and murine bladder (31). No cADPR was detected in these samples. Thus  $\beta$ -NAD<sup>+</sup> represents 90–95% of the eADPR generated during etheno-derivatization of the samples collected during EFS of HUB, whereas ADPR is 5–10% and cADPR 0%.

To provide positive controls, samples containing standards of cADPR, ADPR, and  $\beta$ -NAD<sup>+</sup> were injected in the HPLC system; then, the 7.2-min fraction was collected from

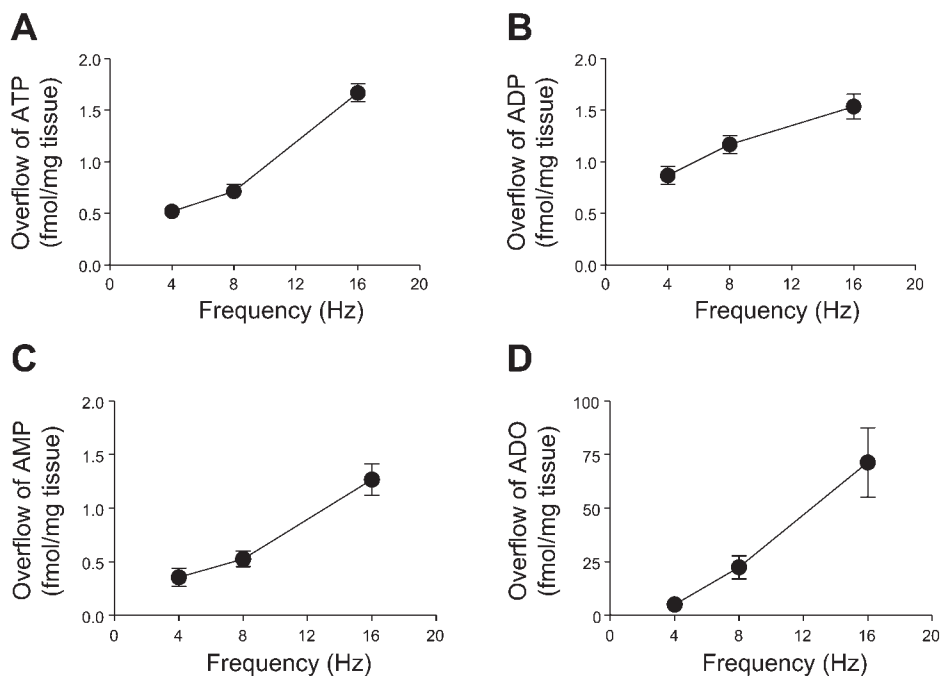


Fig. 2. Human bladder detrusor smooth muscle. EFS (0.1 ms, 15 V, 60 s) evokes frequency-dependent overflow of ATP (A), ADP (B), AMP (C), and ADO (D).

the cADPR standard, the 8.5-min fraction was collected from the ADPR standard, and the 10.5-min fraction was collected from the  $\beta$ -NAD<sup>+</sup> standard. These fractions were then etheno-derivatized and analyzed with the HPLC-FLD.

In all three samples, a single peak of 1,N<sup>6</sup>-etheno-ADPR at 11.2 min was observed (data not shown). In other set of experiments, fractions from human bladder superfusate samples with retention times corresponding to authentic

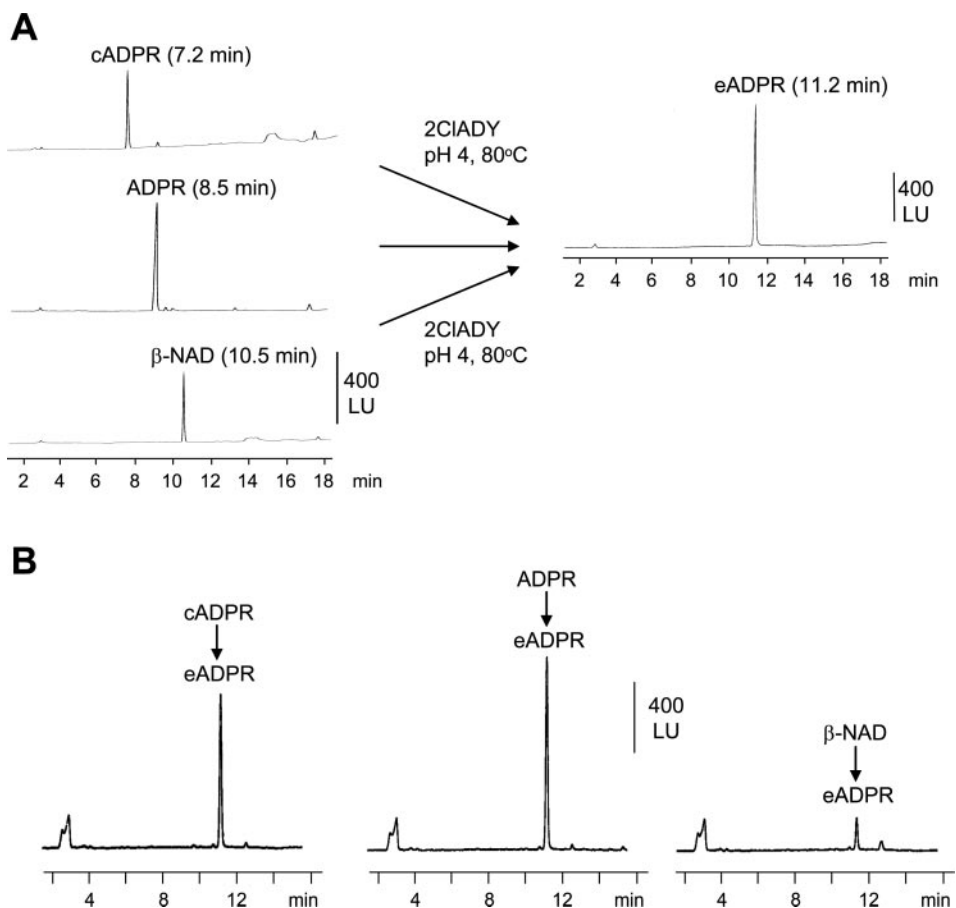


Fig. 3. A: original chromatograms of authentic nonderivatized cADPR, ADPR, and  $\beta$ -NAD<sup>+</sup> (40 nmol). The 3 nucleotides elute at different retention times. When subjected to etheno-derivatization at 80°C, however, the 3 nucleotides produce eADPR, which elutes at ~11.2 min. B: original chromatograms of eADPR produced from equal amounts (1 pmol) of cADPR, ADPR, and  $\beta$ -NAD<sup>+</sup>. Scales apply to each group of chromatograms.

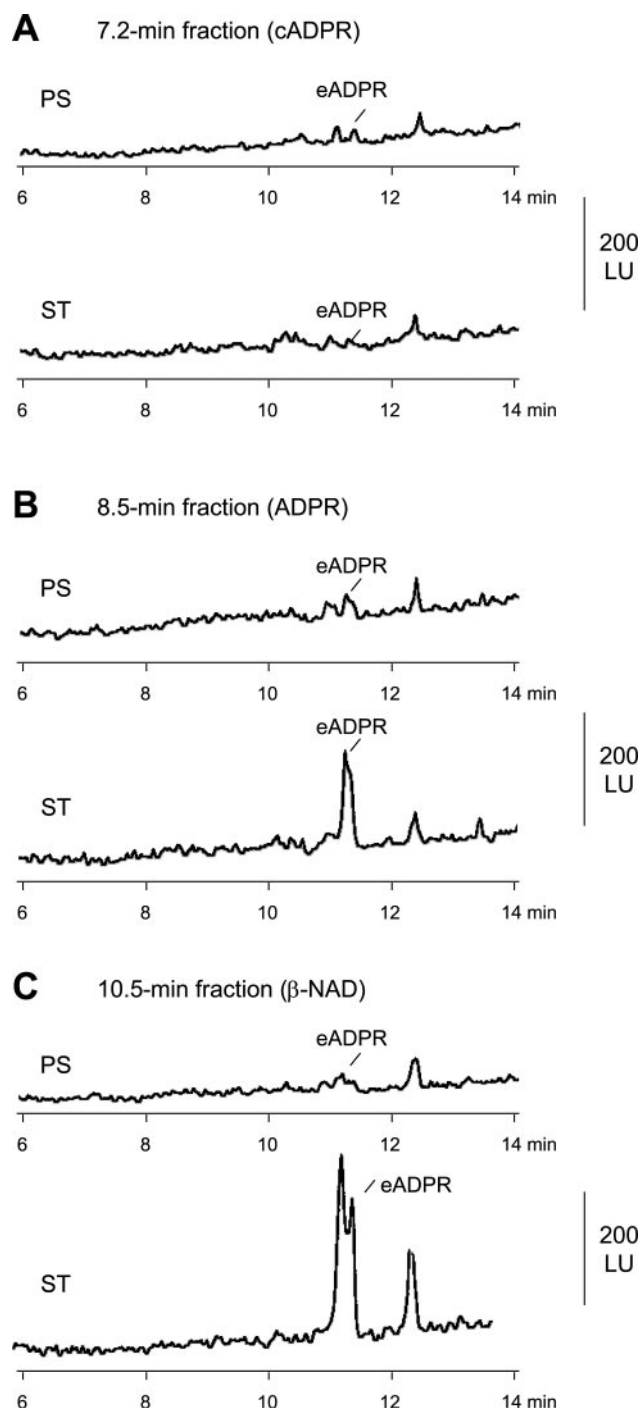


Fig. 4. Human isolated bladder detrusor smooth muscle. Original chromatograms of tissue superfusate samples (10 chambers combined) collected before EFS (PS, top) and during EFS (ST, 16 Hz, 0.1 ms, 60 s, bottom) and etheno-derivatized with 2-chloroacetaldehyde at 80°C, pH 4.0 for 40 min. A: 7.2-min (cADPR-containing) fraction. B: 8.5-min (ADPR-containing) fraction. C: 10.5-min ( $\beta$ -NAD<sup>+</sup>) fraction. Highest amounts of eADPR are observed in the 10.5-min ( $\beta$ -NAD<sup>+</sup>-containing) fraction during stimulation.

ATP, ADP, AMP, and ADO were also collected, etheno-derivatized, and reinjected in the HPLC. As expected, peaks corresponding to eATP, eADP, eAMP, and eADO were detected in the correlated samples, supporting the validity of our experimental approach.

#### NGD Degradation in Human and Murine Detrusor Muscle

Previous studies have shown that ADP-ribosyl cyclase converts both  $\beta$ -NAD<sup>+</sup> into cADPR and NGD<sup>+</sup> into cGDPR (18). However, unlike cADPR, cGDPR is not hydrolyzed by tissue cADPR hydrolase. Therefore, we used the conversion rate of NGD<sup>+</sup> into cGDPR to evaluate the ADP-ribosyl cyclase activity avoiding the influences of cADPR hydrolysis. Figure 6 shows chromatograms from NGD<sup>+</sup> (0.2 mM), a substrate for ADP-ribosyl cyclase, before (no tissue) and after 2-min contact with murine detrusor muscle (+ tissue) (Fig. 6A). A decrease in the substrate NGD and an increase in the product cGDPR were seen after NGD contact with the murine tissue. In contrast, no degradation of the NGD substrate (and hence no formation of cGDPR) was seen in the human bladder detrusor (Fig. 6B). This suggests that in the human bladder the ADP-ribosyl cyclase activity, if any, is negligible, whereas in the mouse bladder the formation of cADPR appears to have a greater role.

#### Release of $\beta$ -NAD<sup>+</sup> Is Sensitive to Tetrodotoxin But Insensitive to Guanethidine

The amount of eADPR in samples collected during EFS at 16 Hz (0.1-ms pulse width, 15 V, for 60 s) in human detrusor muscle tissue, and hence the EFS-evoked release of  $\beta$ -NAD<sup>+</sup>, was significantly reduced in the presence of the blocker of fast Na<sup>+</sup> channels TTX (0.3  $\mu$ mol/l; Fig. 7). However, the inhibitor of action potential-induced release of neurotransmitters from the sympathetic nerve terminals guanethidine did not significantly affect the evoked release of  $\beta$ -NAD<sup>+</sup>. These results taken together suggest that  $\beta$ -NAD<sup>+</sup> is released from postganglionic nerve terminals, which are not sensitive to guanethidine and hence are not part of the sympathetic nervous system.

#### Release of $\beta$ -NAD<sup>+</sup> Is Sensitive to $\omega$ -Conotoxin GVIA, But Insensitive to $\omega$ -Agatoxin IVA

In human bladder detrusor muscle inhibition of Cav2.2 (N-type) VOCC with  $\omega$ -conotoxin GVIA (50 nmol/l) reduced the EFS (16 Hz, 0.1 ms, 60 s)-evoked release of  $\beta$ -NAD<sup>+</sup> from  $15.6 \pm 3$  fmol/mg tissue in the controls ( $n = 13$ ) to  $3.6 \pm 1.2$

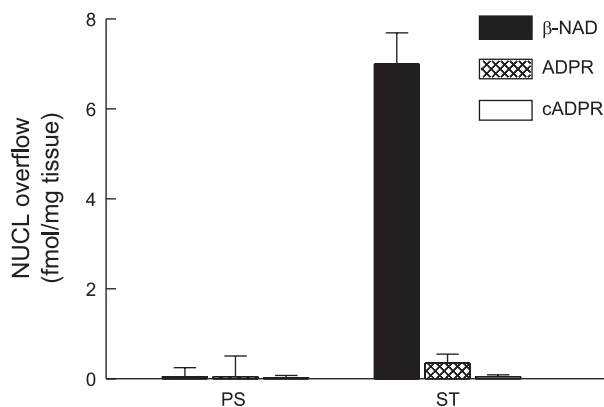


Fig. 5. Human isolated bladder detrusor smooth muscle. Overflow of cADPR, ADPR, and  $\beta$ -NAD<sup>+</sup> based on the HPLC fraction analysis of tissue superfusate samples (10 chambers combined) collected before EFS (PS) and during EFS at 16 Hz, 0.1 ms, 60 s (ST).  $\beta$ -NAD<sup>+</sup> is the primary nucleotide that is released on EFS.

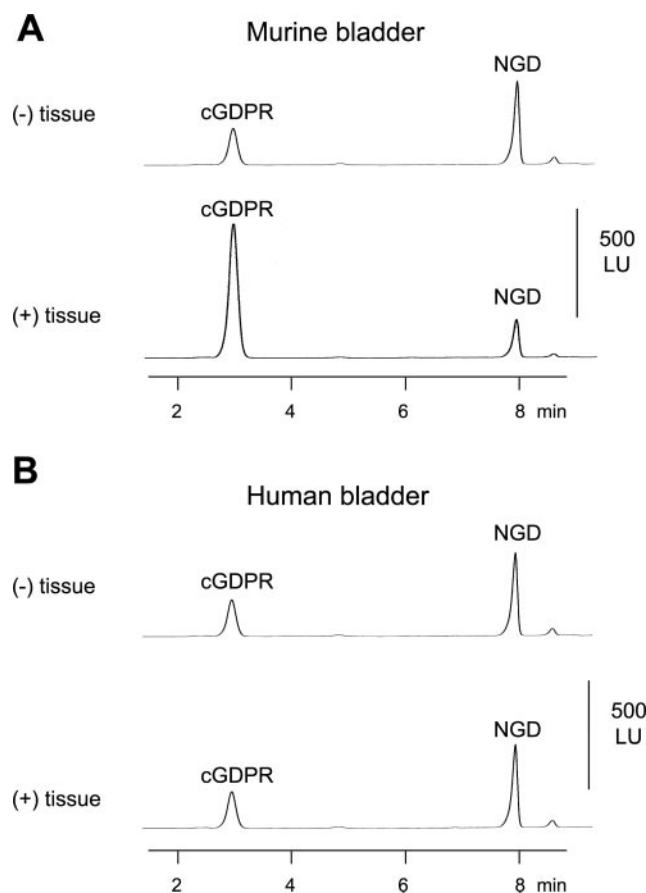


Fig. 6. Original chromatograms of NGD in the absence (– tissue) and presence (+ tissue) of murine (A) or human (B) isolated bladder detrusor smooth muscle. A decrease of the substrate NGD (0.2 mmol/l) and an increase of the product cGDPR were seen in the murine isolated bladder detrusor muscle (A), whereas no degradation was seen in the bladder isolated from human.

fmol/mg tissue ( $n = 6$ ,  $P < 0.05$ ), whereas in the presence of the blocker of Cav2.1 (P/Q-type) VOCC agatoxin IVA (50 nmol/l) the EFS-evoked overflow was  $15.3 \pm 4.5$  fmol/mg tissue ( $n = 6$ ,  $P > 0.05$  vs. controls).

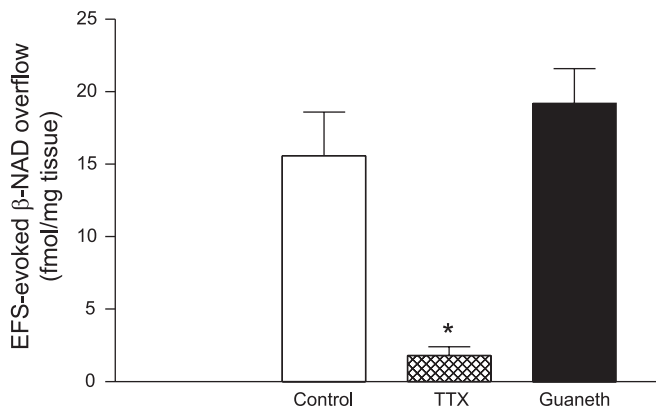


Fig. 7. EFS (16 Hz, 0.1 ms, 15 V, 60 s)-evoked release of  $\beta$ -NAD<sup>+</sup> in human isolated bladder detrusor muscle is sensitive to chemical denervation with TTX (0.3  $\mu$ mol/l), but insensitive to guanethidine (3–10  $\mu$ mol/l). Data represent means  $\pm$  SE from 6–12 experiments. \*Significant differences from controls,  $P < 0.05$ .

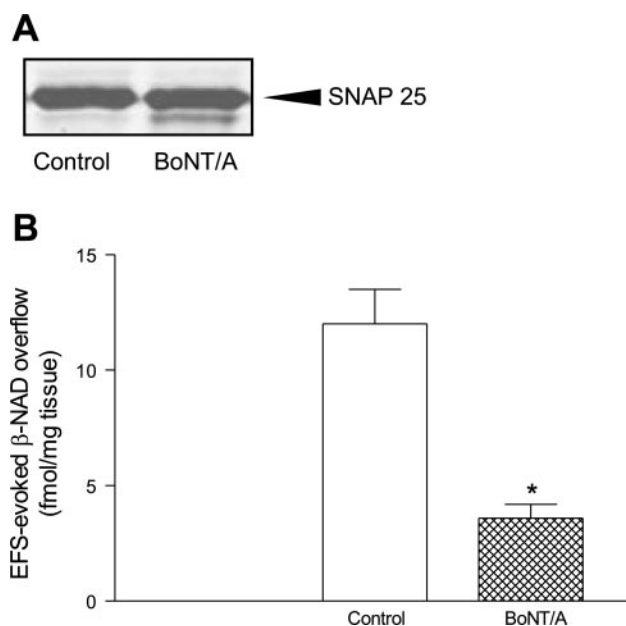


Fig. 8. Human isolated bladder detrusor smooth muscle. A: content of SNAP-25 was analyzed by immunoblotting in tissue homogenates from tissues incubated with either RPMI-1640 (control) or BoNT/A (100 nmol/l). BoNT/A causes cleavage of SNAP-25; thus the 25-kDa immunoreactive band is reduced in area or density after BoNT/A, and an additional band at 24 kDa is apparent after 100 nM BoNTA. B: EFS (16 Hz, 0.1 ms, 15 V, for 60 s)-evoked release of  $\beta$ -NAD<sup>+</sup> is diminished after treatment with BoNT/A. Data represent means  $\pm$  SE from 4 experiments. \*Significant difference from controls,  $P < 0.05$ .

#### Inhibition of SNAP-25 by Botulinum Toxin A (BoNT/A) Reduces the EFS-Evoked Release of $\beta$ -NAD

Incubation of human bladder detrusor muscle with BoNT/A (100 nmol/l for 5.5 h) caused significant reduction of the EFS (16 Hz, 0.1 ms, 60 s)-evoked release of  $\beta$ -NAD<sup>+</sup> ( $n = 6$ ,  $P < 0.05$ ; Fig. 8B). Immunoblot analysis of control detrusor strips revealed a single immunoreactive band with molecular size of  $\sim 25$  kDa, presumably representing intact SNAP-25 (Fig. 8A). Incubation of muscle strips with BoNT/A produced a secondary immunoreactive band with apparent molecular size of  $\sim 24$  kDa, which was less dense and was found in none of the control tissue protein extracts. Because BoNT/A has previously been shown to catalyze cleavage of SNAP-25 (25), the 24-kDa band is likely to represent degradation product(s) of SNAP-25. The reducing effect of BoNT/A on the EFS-evoked release of  $\beta$ -NAD<sup>+</sup> suggests that the release of  $\beta$ -NAD<sup>+</sup> is most likely occurring through exocytosis.

#### Capsaicin Increases the Release of $\beta$ -NAD<sup>+</sup>

Superfusion with capsaicin (10  $\mu$ mol/l) for 5 min elicited overflow of  $\beta$ -NAD<sup>+</sup> in the PS samples ( $1.44 \pm 0.23$  fmol/mg tissue,  $n = 4$ ), collected from human bladder detrusor muscle tissue. The EFS (16 Hz)-evoked release of  $\beta$ -NAD<sup>+</sup> was also increased from  $12.0 \pm 3$  in the DMSO-treated controls to  $72.0 \pm 6$  fmol/mg tissue in the presence of capsaicin ( $n = 4$ ,  $P < 0.05$ ). Thirty-minute incubation of the tissues with capsaicin also increased both the basal and EFS-evoked overflow of  $\beta$ -NAD<sup>+</sup> (data not shown).

### Contraction of the Smooth Muscle Does Not Cause Release of $\beta$ -NAD<sup>+</sup>

CCh (1  $\mu$ mol/l) caused a robust contraction of human bladder strips at a magnitude of  $32 \pm 0.7$  mN ( $n = 5$ ). However, in the superfusate samples collected in the presence of CCh, no eADPR (and hence  $\beta$ -NAD<sup>+</sup>) was observed, suggesting that neither the activation of muscarinic receptors nor contraction of the smooth muscle per se caused the release of  $\beta$ -NAD<sup>+</sup>.

### Exogenous $\beta$ -NAD<sup>+</sup> Affects the Contractility of Human Detrusor Muscle

In tissue segments exhibiting spontaneous activity, the frequency and the amplitude of the spontaneous contraction were reduced in the presence of 1  $\mu$ mol/l  $\beta$ -NAD<sup>+</sup> (Fig. 9). Thus the frequency was reduced from  $3.0 \pm 0.03$  contractions per min (cpm) in the absence of  $\beta$ -NAD<sup>+</sup> to  $2.1 \pm 0.01$  cpm in the presence of 1  $\mu$ mol/l  $\beta$ -NAD<sup>+</sup> ( $n = 3$ ). Likewise, the amplitude of these spontaneous contractions was diminished from  $1 \pm 0.2$  to  $0.5 \pm 0.05$  mN in the presence of exogenous  $\beta$ -NAD<sup>+</sup> (1  $\mu$ mol/l).

### DISCUSSION

The major finding in the present study is that  $\beta$ -NAD<sup>+</sup> is released on stimulation of postganglionic nerve terminals in the human bladder detrusor smooth muscle in a TTX- and  $\omega$ -Ctx GVIA-sensitive manner and thus introduces  $\beta$ -NAD<sup>+</sup> as a putative novel extracellular player in the neural control of the human bladder.

As discussed earlier, the neural control of smooth muscle in general is characterized by increasing complexity; that is, multiple neurotransmitter and neuromodulator substances contribute to the realization and fine tuning of peripheral neurotransmission. Cotransmission and neuromodulation might have particularly important implications for the functions of the human bladder as disbalances in neural control mechanisms are thought to play an important role in human pathology, including detrusor overactivity or instability (1–3). It is well established that the urinary bladder features complex innervation comprising of parasympathetic excitatory innervation, sympathetic innervation (inhibitory in the dome, and excitatory in the neck), and sensory innervation (1, 12, 15).

Among the endogenous nucleotides, ATP attracts much attention as a molecule with important extracellular roles in addition to the well-known intracellular functions. In the context of bladder function, ATP is recognized to be the fast neurotransmitter coreleased with ACh from the parasympa-

thetic nerve terminals and a primary activator of the sensory nerve terminals. In the human urinary bladder, the large majority of the studies on ATP as a parasympathetic cotransmitter are based on the analysis of the purinergic component of the nerve-evoked contractions (e.g., Refs. 28, 32) and hardly ever on direct measurements of the nerve-evoked release of ATP (e.g., 19). To our knowledge, no studies report simultaneous measurements of ATP and its metabolites in the human bladder, although the degradation of neurotransmitter substances (and hence termination of neurotransmitter action) is an important issue in the context of neurotransmission and cotransmission. In the present study, we show for the first time that short-pulse EFS of the human isolated bladder detrusor muscle evokes overflow of not only ATP, but also of ATP metabolites ADP, AMP, and ADO. This is in accordance with previous studies of ours and others in a variety of vascular and nonvascular smooth muscle preparations, including canine and guinea pig mesenteric arteries and veins, rat tail artery, and guinea pig vas deferens (6, 7, 26, 27, 31, 34).

The present study expands on these observations and shows that in addition to ATP, ADP, AMP, and ADO, the tissue superfusates collected during stimulation of postganglionic nerve terminals in human isolated bladder detrusor muscle also contain  $\beta$ -NAD<sup>+</sup> along with its metabolite ADPR. These data, added to prior findings, show that  $\beta$ -NAD<sup>+</sup> is released on nerve stimulation from urinary bladders of mouse, rat, guinea pig, rabbit, dog, monkey, and human (31; our unpublished observations; the present study). Therefore, these results may be considered broadly significant, which suggest novel mechanisms of neural control of the bladder in a variety of mammalian species, including humans.

Stimulation of peripheral nerve terminals causes release of ATP and  $\beta$ -NAD<sup>+</sup>, two endogenous nucleotides largely known for their important intracellular functions. The two nucleotides are subjected to rapid degradation by multiple enzyme systems at the synaptic cleft, which explains the presence of ADP, AMP, ADPR, and ADO in the tissue superfusates. ATP is degraded by ecto-nucleoside-triphosphate diphosphohydrolases (E-NTPDases) to ADP and AMP, which in turn is degraded to ADO by 5'-nucleotidase (37).  $\beta$ -NAD<sup>+</sup>, on the other hand, is degraded by NAD glycohydrolase to ADPR (20), which in turn is degraded to AMP by ecto-nucleotide pyrophosphatase (E-NPP) (13) and ADO by 5'-nucleotidase. Therefore, ADO could be formed by the degradation of both  $\beta$ -NAD<sup>+</sup> and ATP. An interesting consequence of this is that our data for release of  $\beta$ -NAD<sup>+</sup> along with ATP in numerous systems show that ATP can no longer be considered the only

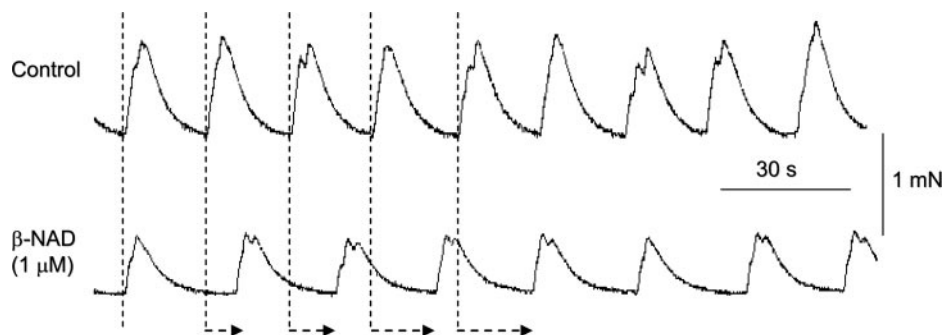


Fig. 9. Original traces showing that exogenous  $\beta$ -NAD<sup>+</sup> (1  $\mu$ mol/l) inhibits both the frequency and amplitude of muscle contractions in a human detrusor muscle segment exhibiting spontaneous contractile activity.



source of AMP and ADO in the tissue superfusates collected during EFS, as previously suggested (e.g., 33).

In addition to the pathway served by NAD glycohydrolases, soluble and membrane-bound ADP-ribosyl cyclases produce cADPR from  $\beta$ -NAD<sup>+</sup> (17). cADPR, in turn, is degraded to ADPR by cADPR hydrolase (19, 21). We previously showed that superfusate samples collected during stimulation of postganglionic nerve terminals of canine and murine isolated bladder detrusor muscles contain small amounts of cADPR along with  $\beta$ -NAD<sup>+</sup> and ADPR (31), suggesting that ADP ribosyl cyclase activity is present in these systems. Interestingly, the human bladder tissue superfusates appear to contain  $\beta$ -NAD<sup>+</sup> and ADPR, but not cADPR. Therefore, a question arises as to whether human bladder lacks ADP ribosyl activity. Indeed, no significant degradation of NGD<sup>+</sup> and formation of cGDPR, respectively, occurred when NGD<sup>+</sup> was in contact with human detrusor muscle, suggesting that the ADP ribosyl cyclase activity in the human bladder detrusor muscle, if any, is negligible. In contrast, murine bladder detrusor muscle significantly degraded NGD<sup>+</sup>, suggesting that the mouse bladder detrusor expresses ADP ribosyl cyclase activity and hence can form cADPR from  $\beta$ -NAD<sup>+</sup>. These findings are in agreement with the results from the HPLC fraction analysis of the superfusate samples collected during EFS of both human (present study) and murine (31) bladder detrusor smooth muscle. Although HUB appears to lack significant ADP-ribosyl cyclase activity, mechanisms for the degradation (and hence removal) of  $\beta$ -NAD<sup>+</sup> are still present as suggested by the presence of ADPR, AMP, and ADO in the tissue superfusates. This issue is becoming particularly important in the context of our hypothesis that  $\beta$ -NAD<sup>+</sup> might be a putative neurotransmitter at the neuromuscular junction in the bladder.

Closely related to the issue of  $\beta$ -NAD<sup>+</sup> being a putative neurotransmitter is the issue of possible source(s) of  $\beta$ -NAD<sup>+</sup> release. CCh (at a concentration causing a robust contraction) failed to induce release of  $\beta$ -NAD<sup>+</sup>, suggesting that contraction of the smooth muscle per se is not responsible for the release of this novel extracellular nucleotide. However, the release of  $\beta$ -NAD<sup>+</sup> was sensitive to a number of inhibitors of the neural activity. The EFS-evoked release of  $\beta$ -NAD<sup>+</sup> was significantly reduced by 1) inhibition of fast Na<sup>+</sup> channels, which are attributable to neurons and not smooth muscle cells, by TTX; 2) inhibition of neuronal Cav2.2 (N-type) voltage-operated Ca<sup>2+</sup> channels by  $\omega$ -conotoxin GVIA; and 3) BoNT/A-induced disruption of SNAP-25, a member of soluble SNARE proteins with a critical role in the exocytotic machinery. These findings taken together indicate possible neuronal origin of the novel nucleotide. The release of  $\beta$ -NAD<sup>+</sup> appears to be triggered primarily by the influx of extracellular Ca<sup>2+</sup> through N-type VOCC as shown previously for canine mesenteric blood vessels and murine urinary bladder (31). Ca<sup>2+</sup> influx through P/Q type VOCC seems to be of lesser importance for the human detrusor muscle.

An intriguing difference between human bladder and canine mesenteric blood vessels is presented by the effects of guanethidine, an inhibitor of neurotransmitter release from sympathetic nerve terminals (24). The release of  $\beta$ -NAD<sup>+</sup> was significantly reduced by guanethidine in the canine mesenteric vessels (31) and murine bladder detrusor muscle (Breen LT, Smyth LM, Yamboliev IA, and Mutafova-Yambolieva VN, unpublished observations), suggesting that sympathetic nervous system

may be the primary source of  $\beta$ -NAD<sup>+</sup> in blood vessels and one of the multiple sources in murine bladder. However, guanethidine had no effect on the EFS-evoked release of  $\beta$ -NAD<sup>+</sup> in the human bladder detrusor, suggesting that neurons different from guanethidine-sensitive sympathetic nerve terminals contribute the most to the source of  $\beta$ -NAD<sup>+</sup> release. In the present study, we show that stimulation of C-type sensory nerves with capsaicin evoked release of  $\beta$ -NAD<sup>+</sup>, suggesting sensory nerves as a potential source. The role of parasympathetic nerve terminals in this release as well as the precise source(s) of  $\beta$ -NAD<sup>+</sup> release remain to be elucidated. It is anticipated that multiple sources are involved in the release of  $\beta$ -NAD<sup>+</sup> in the human bladder.

The present study clearly shows that the EFS-evoked release of  $\beta$ -NAD<sup>+</sup> in the human bladder detrusor muscle is 1) evoked by short-pulse EFS in a frequency-dependent manner, 2) significantly reduced by blockers of neuronal membrane channels (i.e., fast Na<sup>+</sup> channels and Cav2.2 VOCC), 3) mediated by intact SNARE protein complexes, 4) not mediated by guanethidine-sensitive mechanisms, 5) increased by capsaicin, and 6) is not evoked by contraction of the smooth muscle mediated by muscarinic acetylcholine receptors. Moreover,  $\beta$ -NAD<sup>+</sup> seems to have a direct inhibitory effect on the smooth muscle cells, as demonstrated by its effect on the spontaneous mechanical activity in isolated bladder strips. We conclude therefore that  $\beta$ -NAD<sup>+</sup> represents a novel inhibitory factor that is released on stimulation of postganglionic nerve terminals in the human bladder and meets some of the criteria for a neurotransmitter and/or neuromodulator substance (9). Understanding the specific extracellular functions of  $\beta$ -NAD<sup>+</sup> and its metabolites in neurotransmission/neuromodulation may therefore be an important new direction of research into the neural control of bladder function and dysfunction.

#### GRANTS

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