Published in final edited form as:

Gastroenterology. 2011 February ; 140(2): 608–617.e6. doi:10.1053/j.gastro.2010.09.039.

β -nicotinamide adenine dinucleotide is an enteric inhibitory neurotransmitter in human and non-human primate colons

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Abstract

Background & Aims—An important component of enteric inhibitory neurotransmission is mediated by a purine neurotransmitter, such as adenosine 5'-triphosphate (ATP), binding to P2Y1 receptors and activating small conductance K⁺ channels. In murine colon β -nicotinamide adenine dinucleotide (β -NAD) is released with ATP and mimics the pharmacology of inhibitory neurotransmission better than ATP. Here β -NAD and ATP were compared as possible inhibitory neurotransmitters in human and monkey colons.

Methods—A small-volume superfusion assay and HPLC with fluorescence detection were used to evaluate spontaneous and nerve-evoked overflow of β -NAD, ATP and metabolites. Postjunctional responses to nerve stimulation, β -NAD and ATP were compared using intracellular membrane potential and force measurements. Effects of β -NAD on smooth muscle cells (SMCs) were recorded by patch clamp. P2Y receptor transcripts and proteins were assayed by RT-PCR.

Results—In contrast to ATP, overflow of β -NAD evoked by electrical field stimulation correlated with stimulation frequency and was diminished by neurotoxins, tetrodotoxin and ω -conotoxin GVIA. Inhibitory junction potentials and responses to exogenous β -NAD, but not ATP, were blocked by P2Y receptor antagonists suramin, PPADS, MRS2179 and MRS2500. β -NAD activated non-selective cation currents in SMCs, but failed to activate outward currents.

Conclusions— β -NAD meets the criteria for a neurotransmitter better than ATP in human and monkey colons and therefore may contribute to neural regulation of colonic motility. SMCs are unlikely targets for inhibitory purine neurotransmitters because dominant responses of SMCs were activation of net inward, rather than outward, current.

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Author contributions: S.J.H. and L.D. contributed equally to this study; S.M.W., S.D.K., K.M.S., and V.N.M-Y designed research; S.J.H., L.D., L.Dw., P-L.R., S.D.K and V.N.M-Y performed research; S.M.W, S.D.K, K.M.S and V.N.M-Y wrote the paper.

Disclosures: The authors declare no conflict of interests

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Keywords

enteric nerves; P2Y receptors; gastrointestinal motility

Introduction

Enteric neural control is essential for mixing and propulsion of luminal contents in the gastrointestinal (GI) tract.¹ Motility disorders, including diabetic enteropathy, slow-transit constipation, inflammatory neuropathy, and ageing-associated motility disorders are linked, directly or indirectly, to defects in the neural control of the GI tract.^{2,3,4} However, underlying mechanisms for neurotransmitter actions and post-junctional targets for neurotransmitters are not entirely understood. Thus, accurate design and targeting of treatments for motility disorders has proven difficult.

An important component of enteric inhibitory neurotransmission is mediated by a purine neurotransmitter binding to P2Y1 purinoreceptors.^{5,6,7} Functional studies have typically suggested that adenosine 5'-triphosphate (ATP) is the inhibitory purinergic neurotransmitter in the gut.⁸ However, previous studies have largely assayed integrated responses in complex muscular tissues that are possibly mediated by mixed purine receptors. Few studies have measured release of neurotransmitters directly. ATP does not mimic the effects of the endogenous neurotransmitter⁹ in some cases, and many investigators refer to the neurotransmitter as a purine or "purine-like" substance.^{10,11,12,13,14}

β -nicotinamide adenine dinucleotide (β -NAD) is another purine substance released by nerve stimulation in vascular and visceral smooth muscles and neuro-secretory cells.^{9,15,16,17,18} We found that nerve stimulation of murine colon evoked release of β -NAD and ATP, and the pharmacological profile of β -NAD mimicked the endogenous neurotransmitter better than ATP.⁹ Here we investigated whether β -NAD serves as an inhibitory neurotransmitter in larger mammals, such as humans and non-human primates.

Materials and Methods

Tissue Preparation

Proximal colons of 94 *Cynomolgus* monkeys (*Macaca fascicularis*) of both sexes were obtained from Charles River Laboratories Preclinical Services (Reno, NV). Monkeys sedated with Ketamine (10 mg kg⁻¹) and 0.7 ml Beuthanasia-D (Schering-Plough AH, Kenilworth, NJ, USA) were exsanguinated (Charles River Laboratories Institutional Animal Care and Use Committee).

Human colon samples were obtained as surgical waste from 48 male and female patients (ages 32-77) undergoing colon resections for neoplasm or diverticulitis at Renown Medical Center and St. Mary's Hospital (Reno, NV), or Samsung Hospital (Seoul, South Korea). Tissues from a site distant to the disease-affected area were placed in oxygenated Krebs solution (10°C) containing (mmol/L): 118.5 NaCl; 4.2 KCl; 1.2 MgCl₂; 23.8 NaHCO₃; 1.2 KH₂PO₄; 11.0 dextrose; 1.8 CaCl₂ (pH 7.4). Use of human colon was approved by Human Subjects Research Committees at Renown and St. Mary's, and Samsung hospitals, and the Biomedical Institutional Review Board at University of Nevada, Reno. *Tunica muscularis* was processed as below after removal of mucosa and submucosa.

Purine Overflow

Muscle strips (2 × 6 mm) were prepared from monkey and human colonic *tunica muscularis*. Pure circular muscles (CM) (2 × 6 mm) of monkey colon were prepared by

peeling off longitudinal muscle (LM) with attached myenteric ganglia (MG). NADPH-diaphorase histochemistry (below) verified absence of MG in CM. Due to adhesions between smooth muscle layers in human colon, CM preparations were not viable.

Muscles (50–70 mg) were placed in 200- μ l superfusion chambers equipped with platinum electrodes.^{9, 19} Nerves were stimulated by electrical field stimulation (EFS; 480 pulses; 0.3 ms (whole muscle, WM) or 0.5 ms (CM); 4 Hz or 16 Hz; supramaximal voltage). Superfusates were collected before and during EFS and processed for derivatization to 1,N⁶-etheno-purines.^{20,21} ATP, ADP, AMP and adenosine (ADO) were converted to their 1,N⁶-etheno-derivatives eATP, eADP, eAMP, and eADO, respectively.²¹ β -NAD, ADPR and cADPR all derivatized to eADPR.¹⁵

HPLC Assay of Etheno-purines

Reverse-phase gradient HP1100 liquid chromatography equipped with fluorescence detector (Agilent Technologies, Wilmington, DE) was used as previously described.^{21,15} Amounts of nucleotides/nucleosides were calculated against standards. Results, normalized for sample volume and tissue weight, were expressed as femtomoles per milligram of tissue wet weight (fmol/mg).

HPLC Fraction Analysis

The compounds forming eADPR were determined by HPLC fraction analysis.^{9,15} Superfusates from 28 chambers were combined, concentrated and analyzed by HPLC. Samples were collected according to retention time (cADPR and ATP 7.0–7.4 min, ADPR 8.3–8.7 min, and β -NAD 10.3–10.7 min) and were etheno-derivatization and reanalyzed by HPLC for eADPR.

Electrophysiology and contractions

Muscles (10 $\times$ 10 mm) were perfused with oxygenated Krebs solution (mmol/L): NaCl 118.5; KCl 4.5; MgCl₂ 1.2; NaHCO₃ 23.8; KH₂PO₄ 1.2; dextrose 11.0; CaCl₂ 2.4, pH 7.4, at 37 $\pm$ 0.5°C, and CM cells were impaled with glass microelectrodes in the presence of nifedipine (1 μ mol/L) to reduce movement.^{9,22} Parallel platinum electrodes delivered EFS (0.5ms) from a Grass S48 stimulator (Quincy, MA, USA).

Receptor antagonists were perfused into the recording chamber. ATP and β -NAD, were applied (10 ms pulses at 10 psi) via picospritzer micropipettes (Picospritzer; General Valve, East Hanover, NJ) positioned close to electrical recording sites.

Single colonic smooth muscle cells (SMCs) were prepared from human and monkey colon as described²³. Membrane currents were recorded by patch clamp (on-cell recording or permeabilized-patch conditions with amphotericin B)²³. Currents were amplified (Axopatch 200B, Axon Instruments) and digitized (12-bit A/D converter; Digidata 1322A, Axon Instruments) on-line via pCLAMP software (V9.2.0.09, Axon Instruments). Data were sampled at 2 kHz (whole cell) or 5 kHz (single channel recordings).

Bath solution (CaPSS) for whole-cell recording contained (mmol/L): 5 KCl, 135 NaCl, 2 CaCl₂, 10 glucose, 1 MgCl₂, and 10 HEPES, adjusted to pH 7.4 with Tris. Pipette solution contained (mmol/L): 110 Cs-aspartate, 30 TEACl, 0.1 EGTA, 10 HEPES, pH7.2. For single channel recordings, bath solution contained high K⁺ (HK, mmol/L): 150 KCl, EGTA 1, and 10 HEPES, pH 7.4. The pipette solution in single channel recordings was CaPSS.

Muscle strips (1 $\times$ 10 mm) were prepared for contractile studies and attached to Fort 10 force transducers (World Precision Instruments, Sarasota, FL) for force measurements.^{9,22}

Expression of P2Y receptors

P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptor transcripts were measured in monkey and human colons. Detailed methods are given in Supporting Information (Supplementary Materials and Table 1S).

NADPH Diaphorase Histochemistry

CMs were checked for MG using NADPH diaphorase histochemistry as described.^{24, 25} After staining, muscles were mounted in Aquamount (Lerner Laboratories, Pittsburgh, PA) and examined with a Zeiss Axiovert 200M and a Hamamatsu C5810 camera.

Data Analysis

Data presented are means $\pm$ SEM. In neurotransmitter overflow experiments means are compared by two-tailed, unpaired *t* tests (GraphPadPrism, GraphPad Software, San Diego, CA). In intracellular electrical and mechanical experiments means are compared by two-tailed paired Student's *t* tests and Mann Whitney rank sum tests. A probability of $<.05$ was considered significant. For analysis of the picospritzing data, membrane hyperpolarization area following picospritzing was plotted as a function of $\text{mV}\cdot\text{ms}^{-1}$ until the membrane repolarized to control level. For force measurements, relaxation responses (10 min) were calculated as percents of the maximal inhibition following application of β -NAD.

Drugs

ATP, ADP, AMP, adenosine, β -NAD, nifedipine, PPADS, suramin, apamin, L-NNA, atropine, ω -conotoxin GVIA, and amphotericine B were purchased from Sigma-Aldrich (St. Louis, MO). ADPR and cADPR came from Biolog (Germany). MRS2179 and MRS2500 came from Tocris Bioscience (Ellisville, MO). Nifedipine, dissolved in ethanol at 10mmol/L, was added to the perfusion to make 1 $\mu\text{mol/L}$. Other drugs were dissolved in de-ionized H_2O and diluted in perfusion solutions.

Results

Neural release of purines

Stimulation of intrinsic nerves caused accumulation of ATP and β -NAD and metabolites, ADP, AMP, ADO, ADPR and cADPR in tissue superfusates (Figs. 1-2, Tables 2S and 3S in Supplementary Materials). ADP is a product of ATP. AMP and ADO are products of ATP and β -NAD, and cADPR and ADP-ribose are products of β -NAD.^{26,27,28} Therefore, ATP and β -NAD detected in superfusates are remnants of purines released less metabolic products. Fig. 1 shows overflow of purines from human colonic muscles. EFS evoked β -NAD release at 4 Hz (Fig. 1A, C) and release increased at 16 Hz (Fig. 1C, $P=.0227$). In contrast, ATP did not increase significantly with stimulation frequency (Fig. 1B, $P=.3325$). β -NAD was released from nerves, as it was reduced by 0.5 $\mu\text{mol/L}$ TTX ($P=.0028$), a blocker of neural Na^+ channels, and 50nmol/L ω -conotoxin GVIA ($P=.0469$), an inhibitor of neuronal voltage-dependent, N-type Ca^{2+} channels (Fig. 1C). ATP release was not significantly reduced by TTX ($P=.2360$) or ω -conotoxin GVIA ($P=.2994$), Fig. 1B, suggesting that most ATP released during EFS may occur by TTX-insensitive mechanisms. EFS-evoked overflow of ADP, AMP, and ADO was not significantly reduced by TTX and ω -conotoxin GVIA ($P>.05$), but total purine release followed β -NAD overflow (Table 2S, Supplementary Material).

In human colon β -NAD, ADPR and cADPR contributed 82%, 17.4% and 0.6% of the eADPR peak, respectively (Fig. 1D). Therefore, β -NAD was the dominant purine nucleotide

in β -NAD+ADPR+cADPR peaks. β -NAD release exceeded ATP by about 15 fold ($P=0.0072$) (Fig. 1E).

β -NAD released in intact muscles might originate from enteric ganglia rather than motor nerve terminals. Thus, we measured purine release from pure CMs with LM and MG removed. Fig. 2 shows overflow of β -NAD and ATP and metabolites from monkey colonic WM (containing MG) and pure CM, with nerve processes but no cell bodies (Fig. 2A). ATP release was not increased between 4 Hz and 16 Hz (Fig. 2B, WM, $P=0.7831$; CM, $P=0.6706$), and was not significantly reduced by TTX (WM, $P=0.2487$; CM, $P=0.4649$) or ω -conotoxin GVIA (WM, $P=0.1688$; CM, $P=0.2371$). In contrast β -NAD release increased with stimulus frequency (Fig. 2C, WM, $P=0.0398$; CM, $P=0.0300$). Release of β -NAD from CM was 2- to 3-fold lower than from WM at 16 Hz ($P=0.0289$), but no difference was observed at 4 Hz ($P=0.6117$). Thus, at lower stimulation frequencies, β -NAD may be released exclusively from nerve endings, but at 16 Hz, β -NAD may also be released in ganglia. β -NAD release (16 Hz) was inhibited by TTX (WM $P=0.0374$, CM $P=0.0024$) and ω -conotoxin GVIA (WM, $P=0.0092$, CM, $P=0.0340$), indicating that β -NAD was released from enteric motor nerves.

In monkey WM, β -NAD, ADPR and cADPR contributed ~64%, 33% and 3% of the eADPR peak, respectively (Fig. 2D). In CM, β -NAD, ADPR and cADPR contributed 86%, 12.5% and 1.5% of the eADPR peak, respectively. Therefore, β -NAD is the dominant nucleotide in β -NAD+ADPR+cADPR peaks. β -NAD exceeded the mass of ATP by 25-fold in WM ($P=0.0094$) and 15-fold in CM ($P=0.0043$, Fig. 2E).

Purinergetic component of inhibitory junction potentials (IJPs)

Functional responses of colonic muscles were tested by measuring membrane potentials and contractions (Supplementary materials; Figs. 1S, 2S). CM cells of monkey proximal colon and human colon had resting membrane potentials averaging -51.2 ± 1.1 mV ($n=39$) and -43.0 ± 0.6 ($n=41$), respectively. The effects of antagonists, including suramin (100 μ mol/L), PPADS (30 μ mol/L), MRS2179 (10 μ mol/L), MRS2500 (1 μ mol/L) and apamin (0.3 μ mol/L) were tested on the purinergetic component of IJPs (Fig. 3). Apamin reduced IJPs in monkey colon (by $70 \pm 3.9\%$; $n=19$, $P<0.00001$), suramin (by $51 \pm 1.7\%$; $P<0.00005$, $n=10$), PPADS (by $61 \pm 4.8\%$; $P<0.00001$, $n=15$), and MRS2179, selective P2Y1 antagonist,^{29,30} (by $85 \pm 6.4\%$; $P<0.00001$, $n=14$), Fig. 3A. Apamin tended to reduce IJPs of human colon (by $16 \pm 4.7\%$; $n=19$; but did not reach significance; $P>0.05$), Fig. 3B. Human IJPs were reduced by suramin (by $60 \pm 6.0\%$; $P<0.0001$, $n=5$), PPADS (by $81 \pm 1.6\%$; $P<0.0005$, $n=5$), MRS2179 (by $83 \pm 2.1\%$; $P<0.00001$, $n=15$), and MRS2500 (by $95 \pm 1.3\%$; $P<0.0000000001$, $n=10$), Fig. 3B. Therefore, non-nitroergic inhibitory responses in human colon were mediated via P2Y1 receptors, as previously reported¹⁰ and these responses have similar pharmacology in monkey colon.

Antagonists of purinergetic inhibitory responses also inhibited responses to exogenous transmitters. With atropine (1 μ mol/L) and L-NNA (100 μ mol/L) present, picospritzing of ATP onto monkey colonic muscles caused hyperpolarization averaging 12 ± 0.9 mV ($n=21$). Apamin (0.3 μ mol/L) inhibited this response by $97 \pm 2.4\%$ ($n=5$, $P<0.05$, Fig. 4A, left). Responses to exogenous ATP were not inhibited significantly by suramin (100 μ mol/L, reduced by $5 \pm 5.2\%$ of control, $P>0.05$, $n=5$), PPADS (30 μ mol/L, by $12 \pm 9.6\%$, $P>0.05$, $n=6$), or MRS2179 (10 μ mol/L, by $23 \pm 12\%$, $n=5$, $P>0.05$) (Fig. 4B-D, left). Picospritzing ATP onto human muscles under the same conditions caused hyperpolarization averaging 10.9 ± 0.7 mV ($n=11$). Apamin did not reduce responses to ATP significantly, and ATP responses were not reduced by MRS2179 (10 μ mol/L, by $9 \pm 2.1\%$, $n=5$, $P>0.05$, Fig. 4E, left) or MRS2500 (1 μ mol/L, by $12.3 \pm 7.3\%$, $n=3$, $P>0.05$). Thus, IJPs and hyperpolarizations to ATP displayed different pharmacological profiles, suggesting responses to the endogenous neurotransmitter and exogenous ATP are mediated by different receptors.

Picospritzing β -NAD caused hyperpolarization of monkey colon averaging 7.9 ± 0.8 mV ($n=23$). Responses to β -NAD were reduced by apamin ($0.3 \mu\text{mol/L}$) by $98 \pm 2.4\%$ ($n=6$, $P < .05$), suramin ($100 \mu\text{mol/L}$) by $95 \pm 3.8\%$ ($n=5$, $P < .05$), PPADS ($30 \mu\text{mol/L}$) by $88 \pm 3.2\%$ ($n=6$, $P < .05$) and MRS2179 ($10 \mu\text{mol/L}$) by $98 \pm 1.3\%$ ($n=6$, $P < .005$), Fig. 4A-D, right. β -NAD hyperpolarized human colon by 6.8 ± 0.5 mV ($n=13$). β -NAD solutions are acidic, but picospritzing of acidified Krebs solution (pH 3.6) had no effect on resting membrane potential. Apamin did not affect the hyperpolarization responses to β -NAD ($n=2$). However, β -NAD responses were reduced by MRS2179 ($10 \mu\text{mol/L}$) by $68 \pm 6.5\%$ ($n=5$, $P < .05$, Fig. 4E, right) and by MRS2500 ($1 \mu\text{mol/L}$) by $97 \pm 1.7\%$ ($n=3$, $P < .05$, Fig. 4F, right). Thus, β -NAD mimicked the pharmacology of the purine inhibitory neurotransmitter.

Monkey and human colonic muscles expressed P2Y receptor transcripts, including P2Y1 receptors (Fig. 5).

Effects of β -NAD and ATP on SMC conductance

β -NAD induced hyperpolarization in human and monkey colonic muscles, which might be accomplished by activation of K^+ channels or inhibition of a tonic inward current in colonic SMCs. The effects of β -NAD on isolated SMC were tested with cell-attached patch clamp recording. Monkey colonic SMC were held at -80 mV and depolarized by ramping potential to $+80$ mV. Control single channel openings at -80 mV were negligible, but β -NAD (1 mmol/L) increased channel openings (-53 ± 17 pA, $n=5$, $P < .05$, Fig. 6A). These effects returned to control level upon washout and were repeatable. Currents activated by β -NAD reversed at 0 mV (Fig. 6B), suggesting that non-selective cation channels, but not Cl^- channels ($E_{\text{Cl}} \approx -30$ mV) or K^+ channels ($E_K \approx -80$ mV) were responsible. ATP (1 mmol/L) on cell-attached patches also activated non-selective cation channels at -80 mV ($n=2$, Fig. 6C).

β -NAD was also tested on human colonic SMC using permeabilized patch, whole-cell recording. Contamination from K^+ and Cl^- currents were eliminated with Cs-TEA pipette solution with E_{Cl} set to -40 mV. Under these conditions, β -NAD (5 mmol/L) activated inward current, averaging -424 ± 0.4 pA (-80 mV; $n=7$, $P < .01$ control net inward current was -37 ± 4 pA) (Fig. 6D). The currents activated reversed at about 0 mV (Fig. 6E). Therefore, β -NAD-activated currents were not due to K^+ and Cl^- conductances, but appeared to be due to activation of non-selective cation channels. Activation of non-selective cation channels would generate net inward current and depolarize SMC. ATP also activated non-selective cation current at -80 mV (-99 ± 47 pA, $n=4$, Fig. 6F).

Discussion

This study demonstrates several new features of enteric inhibitory motor neurotransmission in human and non-human primate colonic muscles. Stimulation of intrinsic neurons caused release of ATP and β -NAD, and β -NAD was the dominant purine released. β -NAD was previously suggested as an enteric inhibitory neurotransmitter in murine colonic muscles,⁹ but it has never been considered as a candidate for neurotransmission in human GI smooth muscles. The characteristics of β -NAD release suggest it is more likely to be a neurotransmitter than ATP. For example, release of β -NAD, but not ATP, was frequency-dependent and blocked by neurotoxins. Localized β -NAD application mimicked purinergic responses to enteric inhibitory neurotransmission; antagonists that successfully blocked neural responses also blocked responses to exogenous β -NAD. This was not true for ATP, because MRS2179 and MRS2500 effectively blocked purinergic IJPs but failed to block responses to ATP. These data suggest that β -NAD could be the primary purinergic enteric inhibitory neurotransmitter in human and non-human primate colonic muscles. Purinergic neurotransmission may be the dominant form of enteric inhibitory control in human colon as

brief stimuli failed to elicit nitrergic responses (Supplementary Data), as commonly elicited in laboratory animals, and block of NO synthesis failed to reveal tonic nitrergic inhibition, as commonly observed in animal models.³¹

ATP and β -NAD were released from human and monkey colonic muscles spontaneously and during nerve stimulation. At low frequency stimulation, β -NAD appeared to be released from motor nerve terminals, but ganglionic release may also occur at higher frequencies. ATP release was equivalent in muscles with and without ganglia, and ATP release was not significantly reduced by neurotoxins. EFS-evoked overflow of the ATP metabolite, ADP, however, was significantly reduced by ω -conotoxin GVIA in WM, suggesting that a portion of ATP might have originated in ganglia but was degraded to ADP. β -NAD release exceeded ATP by 15-25 fold, suggesting that higher concentrations of β -NAD might be achieved near post-junctional receptors. Taken together, these data suggest that β -NAD may serve as an inhibitory neurotransmitter.

Tissue superfusates contained not only β -NAD and ATP but also their metabolites ADP, AMP, ADPR, cADPR, and adenosine. Thus, mechanisms for terminating actions of purines are available in colon muscles. As in murine colon⁹ β -NAD is the primary purine in β -NAD + ADPR + cADPR peaks in human and monkey colons. Unlike murine colon, where ADPR comprised only ~6% of the β -NAD + ADPR + cADPR mixture, ADPR contributes 12%-33% in the human and monkey colons. Future studies should determine whether ADPR originates only from degradation of β -NAD, or whether ADPR is also released from nerves. If the latter is true, ADPR may also elicit responses in post-junctional cells and be a co-transmitter.

Comparisons of postjunctional responses to neurotransmitters released during EFS with responses to exogenous, putative transmitters, ATP and β -NAD, shed new light on the nature of enteric inhibitory neurotransmission in the human colon. In agreement with previous studies^{7,9,10,12,32} electrical and mechanical responses of human and monkey colons to EFS demonstrated P2Y receptor- and NO-mediated components (Supplementary Data). In monkey and human muscles the primary component of IJPs, a non-nitrergic, fast hyperpolarization, was significantly reduced by the nonselective P2Y receptor antagonists PPADS and suramin and by the P2Y1 inhibitors MRS2179 and MRS2500.^{29, 33} IJPs in the human colon were less sensitive to 0.3 μ mol/L apamin than monkey responses, and this finding is consistent with previous reports demonstrating relative insensitivity of human colonic^{10,32} and intestinal³⁴ muscles to apamin. Therefore, our data are in agreement with previous studies^{6,10} showing that IJPs in human colon are mediated by P2Y1 receptors through a pathway that involves, in part, activation of apamin-sensitive channels. The major K^+ conductance activated during purinergic IJPs in the human is unknown. Of major importance in this study is the finding that β -NAD also elicited hyperpolarization via P2Y1 receptors, but ATP responses were not blocked by MRS2179 and MRS2500. These data, coupled with findings on neurotransmitter release, suggest that β -NAD is a better candidate for the purine inhibitory neurotransmitter than ATP. There are, however, additional explanations (as below) why the P2Y1 receptor antagonists were effective in blocking neurogenic responses but incapable of blocking responses to exogenous ATP. Thus, we cannot completely rule out a role for ATP as a neurotransmitter.

IJPs may be mediated by different post-junctional receptors, possibly expressed by different cell types, than hyperpolarization responses to exogenous substances. Utilization of specific receptors may result from compartmentalization of P2Y1 receptors in restricted receptive fields close to sites of neurotransmitter release and broader expression of purinergic receptors in cells not closely associated with active zones of transmitter release. In fact *tunica muscularis* expresses multiple P2Y receptors, including P2Y1, P2Y2, P2Y4, P2Y6,

and P2Y₁₁, but specific junctional and/or extrajunctional distribution could not be delineated because the antibodies we tested performed poorly in immunohistochemistry. β -NAD might be a more exclusive agonist for P2Y₁ receptors than ATP, and therefore responses to exogenous β -NAD were readily blocked by MRS2179 and MRS2500. In contrast, responses to exogenous ATP appear to be mediated by receptors other than P2Y₁ receptors, and therefore insensitive to the antagonists. ATP, if released from nerves, might generate responses via a specialized, junctional population of P2Y₁ receptors, and our data do not rule out this possibility.

β -NAD- and ATP-induced hyperpolarizations of human and monkey colonic muscles were modest in comparison to IJPs. Multiple factors may be involved in this difference, including degradation of purines, insufficient penetration of β -NAD and ATP to appropriate receptors, or simultaneous activation of opposing conductances. Picospritzing drugs close to recording sites, instead of superfusing drugs, was meant to simulate rapid direct application and avoid some of the factors that might dampen responses. β -NAD and ATP in picospritz pipettes were likely comparable to concentrations of neurotransmitters achieved near nerve terminals following exocytosis, since synaptic vesicles have been estimated to contain 5-1000 mmol/L neurotransmitters.^{35,36} Close examination of electrical responses to β -NAD in monkey colonic muscles (Fig. 4) after P2Y antagonists revealed transient depolarizations in response to β -NAD. Equivalent observations were made in contractile responses of human muscles to β -NAD where transient contractions often occurred before relaxation (Fig. 2S). We considered the possibility that β -NAD might activate opposing conductances in different cells, and tested the effects of this compound on isolated SMC. The endogenous purine neurotransmitter and exogenous β -NAD caused hyperpolarization. In monkey cells, hyperpolarization resulted, in part, from activation of apamin-sensitive (SK) channels. Thus, if the effects of β -NAD were mediated by SMC, one would expect activation of SK channels in these cells. In fact, the dominant effect of both ATP and β -NAD was activation of non-selective cation channels in SMC. These observations support the idea that purinergic neurotransmission is targeted toward specific cells in GI muscles, and SMC are unlikely to mediate purinergic IJPs.

There are two additional cells in close proximity to nerve terminals and coupled to SMCs via gap junctions in GI muscles: i) interstitial cells of Cajal (ICC)³⁷, and ii) fibroblast-like cells (FLC).³⁸ It has not been possible to identify either ICC or FLC in dispersions of human or monkey GI muscles. Previous studies on mice, however, suggest that ICC are unnecessary for purinergic responses³⁹, but FLC express SK3 channels abundantly.^{40, 41,42,43} Thus, FLC, not SMC, may mediate purinergic IJPs in colonic muscles. Our data demonstrate that when β -NAD and ATP are spritzed or bath applied to colonic muscles, activation of opposing conductances tends to antagonize hyperpolarization responses.

The present study demonstrates the complexity of the enteric purinergic signaling and suggests that the β -NAD system can be a new target for treating motility disorders of the large intestine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A program project grant from the NIDDK: P01 DK41315 supported this study. We are grateful to Charles River Laboratories, Preclinical Services for monkey colonic muscles and Renown, St. Mary's and Samsung hospitals for human specimens.

Grant Support: This work was supported by National Institutes of Health Program Project Grant P01 DK41315 (to S.M.W., S.D.K., K.M.S., and V.N.M.-Y).

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Abbreviations

ADP	adenosine 5'-diphosphate
ADO	adenosine
ADPR	adenosine 5'-diphospho-ribose
ATP	adenosine 5'-triphosphate
cADPR	cyclic adenosine 5'-diphospho-ribose
CM	circular muscle
EFS	electrical field stimulation
GI	gastrointestinal
HEPES	<i>N</i> -2-hydroxyethyl piperazine- <i>N'</i> -ethanesulphonic acid
HPLC	high-pressure liquid chromatography
IJP	inhibitory junction potential
LM	longitudinal muscle
L-NNA	NG-nitro-L-arginine
MG	myenteric ganglia
MRS2179	2'-deoxy-N6-methyladenosine 3',5'-bisphosphate
MRS2500	(1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]- 2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt
β-NAD	β-nicotinamide adenine dinucleotide
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate
Tris	tris hydroxymethyl aminomethane

Figure 1. Overflow of ATP and β -NAD in human colonic muscle

(A) Chromatograms of tissue superfusates collected during EFS (4 and 16 Hz) and with neural blockers at 16 Hz. EFS-evoked overflow of β -NAD, but not ATP, increased with stimulation frequency and decreased with TTX (0.5 μ mol/L) or ω -conotoxin GVIA (50 nmol/L); LU, luminescence units. Note that each chromatogram shows data from a different experimental tissue. (B and C) Averaged data are means $\pm$ SEM; ($^{\circ}$) denote significant differences from 4 Hz controls ($P < .05$); (*) denote significant differences from 16 Hz controls ($*P < .05$, $**P < .01$). Experiment number shown in parentheses. (D and E) HPLC fraction analysis demonstrated that β -NAD is the primary purine nucleotide in β -NAD

+ADPR+cADPR peak. (*E*) Fraction analysis showed that the amount of β -NAD exceeded ATP; (*) denote significant differences from ATP (** $P<.01$).

Figure 2. ATP and β-NAD released in monkey whole and circular muscle

(A) NADPH-diaphorase staining of LM and CM of monkey colon. Left panel shows NADPH-diaphorase⁺ neurons (arrows) in myenteric plexus LM preparation. Inset is magnification showing neurons within MG (*). Right panel shows that CM is free of ganglia. Nerve fibers parallel to CM remain (arrow heads). Inset is magnification showing nerve fibers. Scale bars are 500μm in both panels and 50μm in insets. (B and C) ATP and β-NAD released from monkey WM and CM (4 and 16 Hz) and with TTX (0.5 μmol/L) and ω-conotoxin-GVIA (50 nmol/L). Averaged data (fmol/mg tissue) are means±SEM; (°) denote significant differences from 4 Hz controls ($P<.05$); (*) denote significant differences from 16 Hz controls ($*P<.05$, $**P<.01$). (D and E) HPLC fraction analysis demonstrates that β-NAD is the primary nucleotide in β-NAD+ADPR+cADPR peak in WM and CM. β-NAD was greater in WM than CM. (°) denote significant difference from WM ($P<.05$). β-NAD exceeded ATP in WM and CM ($**P<.01$).

Figure 3. Purinergic component of IJPs

IJPs generated by EFS (0.5 ms; 1 pulse; solid circles) in monkey (A) and human colonic muscles (B) in the presence of atropine (1 $\mu\text{mol/L}$), L-NNA (100 $\mu\text{mol/L}$) and nifedipine (1 $\mu\text{mol/L}$). Purinergic component of IJP in monkey colon was reduced by apamin, suramin, PPADS, and MRS2179. In human colon apamin only partially reduced IJPs, but surmain, PPADS, MRS2179 and MRS2500 significantly reduced IJPs.

Figure 4. Membrane responses to exogenous purines

Picospritzed ATP (10mmol/L, *left panels*) and β -NAD (50mmol/L, *right panels*) hyperpolarized monkey (*A-D*) and human muscles (*E, F*). Responses to β -NAD, but not to ATP, were inhibited by purine receptor antagonists (as labeled). In monkey muscles, apamin inhibited both ATP and β -NAD induced hyperpolarization (*A, left and right panels*). In some muscles β -NAD caused transient depolarization after MRS2179 (*D, right*). In human muscles MRS2179 and MRS2500 did not affect ATP response (*E, F left*), but significantly reduced β -NAD response (*E, F right*). Scale bars in D&F apply to traces in *A-D* and *E-F*, respectively.

Figure 5. Expression of P2Y receptors in monkey and human tissues

(A) Expression of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptor transcripts in the *tunica muscularis* of the monkey fundus, antrum, jejunum and proximal colon. (B) Expression of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptor transcripts in human brain, fundus, gastric body (G. Body), antrum, ascending colon (A. Colon), distal colon (D. Colon) and sigmoid colon (S. Colon). Cytoglobin was used as a house keeping gene and M represents base pair marker.

Figure 6. β -NAD and ATP activate inward currents in SMCs of monkey (A-C) and human (D-F) (A) In cell-attached patches (pipette CaPSS; bath HK), channel openings were negligible. β -NAD (1mmol/L) increased openings at -80 mV. (B) Stepping from -80 to +80 mV showed β -NAD-activated currents reversed at 0 mV in monkey SMC. *a* (control) and *b* (β -NAD) show expanded traces from panel A during ramp depolarization (-80 mV to +80 mV). (C) ATP (1mmol/L) increased channel activity at -80 mV in monkey SMC. (D) Perforated whole-cell conditions (pipette Cs-TEA, bath CaPSS), β -NAD (5mmol/L) activated inward current at -80 mV. β -NAD activated-currents reversed upon washout. (E) *a*, *b*, and *c* show expanded traces from panel D during ramp depolarization. β -NAD activated-currents reversed at 0 mV, demonstrating non-selective cation conductance was activated by β -NAD. Dotted lines in B and E denote 0 mV and 0 pA. (F) ATP (1 mmol/L) activated inward currents at -80 mV.