Modulation by NO of acetylcholine release in the ileum of wild-type and NOS gene knockout mice

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Mang, Christian F., Sebastian Truempler, Doris Erbelding, and Heinz Kilbinger. Modulation by NO of acetylcholine release in the ileum of wild-type and NOS gene knockout mice. Am J Physiol Gastrointest Liver Physiol 283: G1132–G1138, 2002; 10.1152/ajpgi.00192.2002.—Nitric oxide (NO) inhibits the release of acetylcholine and cholinergic contractions in the small intestine of several species, but no information is available about the mouse ileum. This study examines the effects of NO on the electrically evoked release of [3H]acetylcholine and smooth muscle contraction in myenteric plexus-longitudinal muscle preparations of wild-type mice and of neuronal NO synthase (nNOS) and endothelial NOS (eNOS) knockout mice. The NOS inhibitor N\textsuperscript{G}-nitro-L-arginine (L-NNA) and the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-c]quinoxalin-1-one (ODQ) concentration dependently increased the evoked [3H]acetylcholine release and cholinergic contractions in preparations from wild-type mice and from eNOS knockout mice. Effects of L-NNA were specifically antagonized by L-arginine. In contrast, L-NNA and ODQ did not modify the release and contractions in preparations from nNOS knockout mice. The NO donor S-nitroso-N-acetyl-DL-penicillamine inhibited the electrically evoked release of [3H]acetylcholine and longitudinal muscle contractions in a quantitatively similar manner in wild-type preparations as well as in nNOS and eNOS knockout preparations. We conclude that endogenous NO released by electrical field stimulation tonically inhibits the release of acetylcholine. Furthermore, data suggest that nNOS and not eNOS is the enzymatic source of NO-mediating inhibition of cholinergic neurotransmission in mouse ileum.

neuronal nitric oxide synthase knockout mice; endothelial nitric oxide synthase knockout mice; cholinergic neurotransmission

NITRIC OXIDE (NO) is a nonadrenergic, noncholinergic neurotransmitter that causes inhibition of intestinal motility (for review, see Ref. 24). The relaxant action of NO is probably not confined to its direct action on smooth muscle, because NO decreases intestinal motility also indirectly by inhibiting the release of the functionally most prominent excitatory enteric neurotransmitters acetylcholine and substance P. For example, exogenous NO (31) and NO donors (5) inhibit the electrically evoked release of acetylcholine from guinea pig ileum. Vice versa, NO synthase (NOS) inhibitors enhance the evoked release of acetylcholine from the ileum of guinea pig (10, 20) and dog (7) as well as the electrically evoked cholinergic and tachykininergic contractions of guinea pig ileum (6, 32). This suggests that endogenous NO, released by field stimulation, decreases the evoked release of acetylcholine from enteric neurons. There are, however, tissue and species differences in the neuromodulatory effect of NO: NO or NOS inhibitors do not modify the electrically evoked acetylcholine release from the colon of guinea pig and dog (22, 29) and from stomach preparations of man (13), pig (12), and guinea pig (19).

The present investigation had two objectives: 1) to study whether a nitrergic modulation of acetylcholine release exists in the small intestine of mice. Mice have become important as subjects for neuroscience research because of the availability of genetically manipulated mice for studies of transmitter release; and 2) to investigate the type of NOS involved in the modulation of acetylcholine release. Both neuronal NOS (nNOS) and endothelial NOS (eNOS) are expressed in the gastrointestinal tract (17). NOS is primarily present in enteric neurones, whereas eNOS has been found in human and rabbit gastrointestinal smooth muscle cells (27). It has been suggested that NO synthesized from eNOS in muscle tissue constitutes the predominant component of NO formed during nerve stimulation, whereas NO derived from nNOS activity may have only a minor functional significance (27). NO produced in smooth muscle cells was thought to diffuse back and to modulate release of other neurotransmitters (e.g., vasoactive intestinal peptide) from the presynaptic nerve terminals (4). A role of NO as retrograde neurotransmitter has also been suggested in the central nervous system, where NO derived from both nNOS and eNOS can modulate the release of neurotransmitters (9).

In the present study, we used wild-type mice and mice deficient in either nNOS or eNOS gene expression to clarify the role of either NOS isoform in modulating cholinergic neurotransmission in the ileum. The effects of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine (L-NNA), of the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP),...
and of the inhibitor of the soluble guanylyl cyclase 1H-1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (2) on release of acetylcholine and smooth muscle contractions were studied in myenteric plexus-longitudinal muscle (MPLM) preparations. Release of acetylcholine was measured in the absence of a cholinesterase inhibitor as overflow of [3H]radioactivity from preparations labeled with [3H]choline.

MATERIALS AND METHODS

Adult male homozygote nNOS knockout mice (B6,129S-Nos1) were obtained from Jackson Laboratory. Breeding pairs of homozygote eNOS knockout (C57BL/6J-Nos3) and of wild-type mice (B6129SF2) were also obtained from Jackson Laboratory and bred at the animal facilities of the University of Mainz. The wild-type strain served as control for both types of knockout mice. The deficiency of NOS expression was verified by PCR amplification of specific DNA fragments using as templates mouse-tail DNA (DNase tissue kit, Quiagen). The knockout mice had normal size and showed no obvious physical or neurological deficit. The stomachs of the nNOS knockout mice were enlarged as described previously (8).

Male mice weighing 30–40 g were used in all experiments. The mice were killed by a blow to the head and bled. The proximal ileum was removed and placed in a physiological salt solution (composition in mM: 137 NaCl, 2.7 KCl, 1.8 CaCl2, 1.05 MgCl2, 11.9 NaHCO3, 0.42 NaH2PO4, 5.6 D-glucose, 0.001 choline chloride). The ileum was drawn over a horizontal glass rod, and the longitudinal muscle with the myenteric plexus attached was separated from the underlying circular muscle with a damp wisp of cotton as described for the guinea pig ileum (21). MPLM preparations (length: 1.5–2.5 cm) were suspended isometrically under a tension of 4.9 mN between two platinum electrodes in a 2-ml organ bath, superfused (2 ml/min) with medium at 37°C and bubbled with a mixture of 95% O2–5% CO2.

After a 30-min equilibration, superfusion was stopped, and muscle strips were incubated with [3H]choline (2.5 μCi/ml) for 30 min during which the tissue was stimulated electrically with square-wave pulses of 1 Hz and 1-ms duration (voltage drop 10 V/cm). The myenteric plexus preparations were then again superfused with the physiological salt solution that contained an additional 10 μM hemicholinium-3. After a washout period of 70 min, the superfusate was collected in 3-min fractions and the tritium content of the samples was measured by liquid-scintillation spectrometry. Strips were stimulated twice 51 min apart (S1, S2) with a frequency of 1 Hz for 3 min. The stimulation-evoked outflow of [3H]-radioactivity was calculated from the difference between the total outflow during and after stimulation, and the basal outflow was calculated by interpolation from two samples before stimulation and two samples 15–18 min after the onset of stimulation. At the end of the experiment, the tissue was immersed overnight in 3 ml of 0.4 M HClO4, and the radioactivity contained in the extract was determined. The outflow of tritium was calculated as percent tritium content in the tissue at the beginning of the respective collection period. The tone of the longitudinal muscle was recorded simultaneously to the [3H]-outflow. All responses of the smooth muscle were measured as peak contractile and relaxant responses.

Drugs were added to the superfusion solution 33 min before S1 and remained in the medium up to the end of the experiments. To characterize the stimulation-evoked [3H]-outflow in the superfusate, the preparations were superfused with a medium containing an additional 10 μM neostigmine plus 0.3 μM scopolamine from the last 27 min of the washout period onward. [3H]choline and [3H]acetylcholine were separated by reverse-phase HPLC as described previously (30). Superfusate (200 μl) was injected onto the HPLC column, and radioactivity was measured in the effluent collected in 1-min fractions.

Data analysis. Results are given as means ± SE. Statistical significance of differences was estimated by the unpaired Student’s t-test, and significance was assumed at the 5% level. If one control group was compared with more than one group of treatments, a one-way ANOVA was carried out followed by Dunnett’s test. Concentration-response data for L-NNA were evaluated by sigmoid curve fitting and log EC50 values with their 95% confidence limits calculated from individual values by nonlinear regression analysis by GraphPad Prism (San Diego, CA).

Drugs. Drugs used were [Methyl-3H]choline (NEN, Dreieich, Germany); L-arginine hydrochloride, d-arginine hydrochloride, hemicholinium-3 bromide, l-NNA, (–)-scopolamine hydrochloride, neostigmine methyl sulfate, SNAP, tetrodotoxin (all from Sigma, St Louis, MO); and ODQ, (Tocris, Bristol, UK). Drugs were dissolved in water or in DMSO (SNAP, ODQ). DMSO at 116 mM (maximum concentration) neither affected basal nor electrically evoked outflow of tritium nor basal tone nor evoked contractions of the smooth muscle strips.

RESULTS

Wild-type mice. Electrical field stimulation of the MPLM preparations at 1 Hz produced a clear increase in the outflow [3H]-radioactivity (Fig. 1). In control experiments, the [3H]-outflow evoked by S2 was 82 ± 3% (n = 13) of that caused by S1. Tetrodotoxin (300 nM; n = 2) or omission of calcium (n = 2) from the super-

<table>
<thead>
<tr>
<th>Drug Added</th>
<th>Before S1</th>
<th>S1, %</th>
<th>S2/S1</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>DMSO 116 mM</td>
<td>2.30 ± 0.27</td>
<td>0.82 ± 0.03</td>
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<td>2.88 ± 0.46</td>
<td>0.94 ± 0.05</td>
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<tr>
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<tr>
<td>nNOS−/−</td>
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<td>0.92 ± 0.01</td>
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<tr>
<td>eNOS−/−</td>
<td>DMSO 116 mM</td>
<td>1.98 ± 0.22</td>
<td>0.83 ± 0.04</td>
<td>5</td>
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</tbody>
</table>

Table 1. Effects of drugs added before S1 in control experiments on electrically-evoked outflow of [3H]acetylcholine in wild-type and NOS knockout mice.
fusion medium prevented the increase in $^3$H-outflow by field stimulation (not shown). The $^3$H-radioactivity in the superfusate was further analyzed after separation of $[^3$H]acetylcholine and $[^3$H]choline in the medium that contained an additional 100 nM neostigmine and 0.3 μM scopolamine. Electrical field stimulation elicited an increase in outflow of tritium that consisted of 95.6% ($n = 6$) of $[^3$H]acetylcholine; the outflow of $[^3$H]choline remained unchanged. Thus the electrically evoked outflow of tritium as determined under the present experimental conditions can be regarded as a reliable indicator for the Ca$^{2+}$-dependent release of acetylcholine due to the propagation of action potentials along cholinergic neurones.

Smooth muscle contractions were recorded simultaneously. Electrical stimulation caused a transient initial relaxation of the longitudinal muscle followed by a contraction (Fig. 1). In the presence of scopolamine (0.1 μM) the relaxant response was enhanced from 0.4 ± 0.1 to 1.1 ± 0.1 mN ($n = 5$), whereas the contraction was abolished.

L-NNA increased both the electrically evoked outflow of $[^3$H]acetylcholine and the longitudinal muscle contraction (Fig. 1). Basal tone of the longitudinal muscle remained unchanged by L-NNA, but the field stimulation-induced initial relaxation was abolished. Concentration-response curves for the facilitatory effects of L-NNA are shown in Fig. 2. The $-\log EC_{50}$ values for the evoked release (5.18 ± 0.43) and contractions (4.52 ± 0.44) were not significantly different. To confirm the specificity of the effect of L-NNA, interaction experiments were performed with L- and D-arginine (1 mM). In control experiments, L- and D-arginine did not affect basal tone of the longitudinal muscle but abolished the stimulation-induced initial relaxation.

![Fig. 1](image1.png)

**Fig. 1.** Effects of N⁶-nitro-L-arginine (L-NNA) on outflow of $^3$H-radioactivity and longitudinal muscle contraction. Preparations were stimulated twice at 1 Hz (3 min) (S1, S2). L-NNA (300 μM) was present as indicated by the horizontal lines. $A$: outflow of $^3$H as percentage of the tritium present in the tissue at the start of each fraction. The x-axis starts at the end of the washout period. Values are means ± SE of $n = 7$ (control) and $n = 5$ (L-NNA) experiments. $B$: original recording of the effect of 300 μM L-NNA on the electrically evoked contractile and relaxant responses. Note that the chart speed was reduced from the beginning of the perfusion with L-NNA until 2 min before S2. L-NNA did not affect basal tone of the longitudinal muscle but abolished the stimulation-induced initial relaxation.

![Fig. 2](image2.png)

**Fig. 2.** Increase by L-NNA of the electrically evoked outflow of $[^3$H]acetylcholine (A) and muscle contractions (B). Myenteric plexus-longitudinal muscle preparations were stimulated twice (S1, S2) at 1 Hz (3 min), and L-NNA was added 33 min before S2 ($n = 4–9$). Additionally, in interaction experiments, L-arginine ($n = 5$) and D-arginine ($n = 4$) were present from 40 min before S1 onward. Effects of drugs on the evoked outflow and contractions were calculated from the S2/S1 ratios and expressed as a percentage of the corresponding average ratio in control experiments without L-NNA. Values are means ± SE. Significant differences from control values: $^*P < 0.05; ^{**}P < 0.01$. 

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not change either the evoked outflow of $[^3]$H during S1 or the ratio S2/S1 (Table 1). Fig. 2 shows that the facilitatory effects of L-NNA were stereospecifically prevented by L-arginine but not by D-arginine. Thus these experiments suggest that endogenous NO released by electrical field stimulation inhibits the evoked release of acetylcholine.

To test whether the inhibitory action of endogenous NO is mediated via activation of soluble guanylyl cyclase, the effects of ODQ were studied. Figure 3 shows that 1 μM ODQ significantly enhanced both the evoked release of $[^3]$H acetylcholine and longitudinal muscle contraction. A lower concentration (0.1 μM) had no effect. Basal outflow of $[^3]$H-radioactivity was not changed by 0.1 or 1 μM ODQ.

Finally, the effects of the NO donor SNAP were studied. Concentrations of 100 and 300 μM SNAP significantly decreased the evoked release of $[^3]$H acetylcholine (Fig. 4). Similarly, the electrically evoked longitudinal muscle contractions were significantly inhibited to 76 ± 4% (100 μM; n = 6; P < 0.05) and to 70 ± 4% (300 μM; n = 4; P < 0.01). Basal outflow of $[^3]$H was not changed, whereas basal tone of the longitudinal muscle was slightly reduced by 0.7–1 mN by both 100 and 300 μM SNAP. Specificity of the effects of SNAP was tested in interaction experiments with ODQ. A low concentration of ODQ (0.1 μM), which by itself did not affect $[^3]$H acetylcholine release (see Fig. 3), prevented the inhibitory effects of 100 μM SNAP on the evoked release (Fig. 4) and contractions (n = 4; not shown).

Knockout mice. The initial relaxation in response to electrical stimulation was missing in MPLM preparations of nNOS-deficient mice but not in preparations of eNOS knockout mice. The outflow of tritium evoked by 1 Hz at S1 and the ratios of S2/S1 were similar regardless of the mouse strain (Table 1).

L-NNA (300 μM) and ODQ (1 μM) had no effect on either the electrically evoked $[^3]$H acetylcholine outflow or muscle contraction in preparations from nNOS knockout mice. However, both compounds increased outflow in eNOS knockout preparations to a similar degree as in preparations from wild-type mice (Fig. 5).
The main findings of the present study are that 1) NO inhibits acetylcholine release and cholinergic contraction in the ileum of wild-type mice and 2) the source of the endogenous NO is the nNOS.

NOS inhibitor L-NNA increased the electrically evoked release of acetylcholine and the cholinergic contractions of the longitudinal muscle of wild-type mice. This suggests that endogenous NO, which is released concurrently by field stimulation, tonically inhibits the release of acetylcholine. In line with this suggestion is the finding that the NO donor SNAP decreased the electrically evoked release and contractions. The effects of NO are probably mediated via activation of soluble guanylyl cyclase, because a low concentration of the selective inhibitor of the NO-sensitive guanylyl cyclase, ODQ, antagonized the inhibitions caused by SNAP. A higher concentration of ODQ alone, similar to L-NNA, facilitated the evoked release and contractions. This finding supports the above-mentioned suggestion that endogenous NO exerts a tonic inhibitory effect on acetylcholine release, which is disinhibited by L-NNA or ODQ. It is, however, unclear from the present study, whether inhibition of acetylcholine release is the sole cause of the decrease of smooth muscle contraction or whether a concomitant direct relaxant effect of NO contributes to the suppressed contraction on field stimulation.

In preparations from nNOS knockout mice, electrical field stimulation did not produce a relaxation of the longitudinal muscle and L-NNA and ODQ did not modify the evoked acetylcholine release and cholinergic contractions. On the other hand, the facilitatory effects of L-NNA and ODQ on release and contractions were quantitatively similar in MPLM preparations of eNOS.

**DISCUSSION**

Immunohistochemical studies have shown that the longitudinal muscle of the mouse ileum is densely innervated by cholinergic neurons and, in addition, by NOS-containing neurones (25, 33). In the present experiments, electrical field stimulation of the MPLM preparation of wild-type mice caused a release of acetylcholine determined biochemically as outflow of [3H]acetylcholine and as scopolamine-sensitive contraction. In addition, field stimulation caused a release of NO, which elicited a transient, L-NNA-sensitive relaxation of smooth muscle. Release of [3H]acetylcholine and nerve-mediated cholinergic contractions and nitrergic relaxations of the mouse ileum have also previously been reported (3, 23, 33).

![Fig. 5](http://ajpgi.physiology.org/) Effects of 300 μM L-NNA (solid bars; n = 4 and 7) and 1 μM ODQ (hatched bars; both n = 4) on the electrically evoked outflow of [3H]acetylcholine from preparations of neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) knockout mice. Open bars, control experiments with nNOS (n = 4 and 5) and eNOS (n = 6 and 8) preparations. Values are means ± SE. In nNOS knockout mice, L-NNA and ODQ caused no significant change in release. **Significant (P < 0.01) increases of release in eNOS knockout mice.

![Fig. 6](http://ajpgi.physiology.org/) Inhibition by 300 μM SNAP (hatched bars) of electrically evoked [3H]acetylcholine outflow in preparations of nNOS and eNOS (both n = 4) knockout mice. Open bars, control experiments (n = 5 and 6). Values are means ± SE. Significance of inhibition: **P < 0.01.
knockout mice to those in wild-type mice. eNOS has been reported to be present in human and rabbit gastrointestinal smooth muscle cells, from where it was thought to act as a retrograde mediator modulating neurotransmitter release (27). On the other hand, nNOS is involved in nitric oxide release and contraction in wild-type mice. One would therefore expect that the lack of endogenous NO production in nNOS knockout mice would have enhanced acetylcholine release and contraction during electrical stimulation by S1. This was, however, not seen. Similar discrepancies between acute experiments using NO inhibitors and experiments with NO knockout mice are well known and have been explained by compensatory changes (e.g., in release of other neurotransmitters) throughout embryonic development (17).

SNAP inhibited the release of acetylcholine in tissues from wild-type mice as well as in preparations from both nNOS and eNOS knockout mice. This shows that the sensitivity of the cholinergic nerve to the inhibitory effect of NO was not altered in nNOS-deficient mice. SNAP did not affect basal release of acetylcholine. This is different in the guinea pig ileum, where NO donors and exogenous NO cause a large increase in basal acetylcholine release (5) and an atropine- and tetrodotoxin-sensitive contraction of the longitudinal muscle (1).

An inhibition by NO of cholinergic neurotransmission has been detected in a variety of gastrointestinal tissues (see introduction). In the guinea pig myenteric plexus, NOS immunoreactive axons were found to make synapses with other myenteric neurons (14), which also indicates that NO has presynaptic neuromodulatory effects. The present investigation adds the mouse ileum and supports the suggestion (6) that the NO-mediated gastrointestinal relaxations in these species are, in part, due to the inhibition of the release of excitatory neurotransmitters.

Parts of this study have been presented as preliminary reports (see Refs. 15 and 16).

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REFERENCES


