Cholinergic and GABAergic regulation of nitric oxide synthesis in the guinea pig ileum

KATALINA HEBEISS AND HEINZ KILBINGER
Department of Pharmacology, University of Mainz, D-55101 Mainz, Germany

Cholinergic and GABAergic regulation of nitric oxide synthesis in the guinea pig ileum. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G862–G866, 1999.—Nitric oxide (NO) synthesis was examined in intact longitudinal muscle-myenteric plexus preparations of the guinea pig ileum by determining the formation of [3H]citrulline during incubation with [3H]arginine. Spontaneous [3H]citrulline production after 30 min was 80–90 dpm/mg, which constituted ~1% of the tissue radioactivity. Electrical stimulation (10 Hz) led to a threefold increase in [3H]citrulline formation. Removal of calcium from the medium or addition of N-(nitro-L- arginyl)-L-arginine strongly inhibited both spontaneous and electrically induced production of [3H]citrulline. TTX reduced the electrically induced but not spontaneous [3H]citrulline formation. The electrically induced formation of [3H]citrulline was diminished by (+)-tubocurarine and mecamylamine and enhanced by scopolamine, which suggests that endogenous ACh inhibits, via muscarinic receptors, and stimulates, via nicotinic receptors, the NO synthesis in the myenteric plexus. The GABA receptor agonist muscimol and GABA also reduced the electrically evoked formation of [3H]citrulline, whereas baclofen was without effect. Bicuculline antagonized the inhibitory effect of GABA. It is concluded that nitrergic myenteric neurons are equipped with GABA receptors, which mediate inhibition of NO synthesis.

NITRIC OXIDE (NO) is a neurotransmitter of inhibitory nonadrenergic noncholinergic (NANC) nerves in the gastrointestinal tract of various species (for literature, see Ref. 17). Neuronal NO is synthesized by the isoform I of the enzyme NO synthase (6), which has been found in nerve cell bodies and nerve endings in the gastrointestinal tract of guinea pigs (7). The NO synthase-containing neurons constitute a prominent population of enteric neurons, and, in the guinea pig small intestine, ~20% of all myenteric neurons contain NO synthase immunoreactivity (4). The synthesis of neuronal NO is tightly regulated. NO is synthesized on demand from L-arginine when nerve stimulation leads to an increase in the intraneuronal calcium concentration to activate NO synthase. NO is not stored but instead simply diffuses from its site of production (29).

The activity-dependent synthesis and release of NO in the guinea pig small intestine has been studied in functional experiments in which electrical stimulation of the precontracted ileum caused smooth muscle relax-

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enized with an Ultraturrax in ice-cold 0.4 M HClO4 (1.0 ml). After centrifugation at 1,000 g for 15 min, aliquots of the supernatant (200 µl) were analyzed by HPLC according to the method described recently (1). The [3H]-labeled compounds ([3H]citrulline, [3H]ornithine, and [3H]arginine) in the tissue extract were separated on a reverse-phase column (length of 250 mm, inner diameter of 4.6 mm, prepainted with 5 µm Shandon ODS-Hypersil; Bischoff Chromatography, Leonberg, Germany) using a mobile phase 0.1 M phosphate buffer (adjusted to pH 1.8), which contained octane sulfonic acid sodium salt (300 mg/l), sodium EDTA (0.3 mM), and methanol (4.2% vol/vol). The eluate was collected in 30-s fractions into counting vials, and the radioactivity was determined by liquid scintillation spectrometry (Packard Tricarb 4430). External standardization was used to correct for counting efficiency. [3H]citrulline eluted from the column after ~8 min, and [3H]ornithine and [3H]arginine eluted after ~10 and 80 min, respectively. The identity of the eluted material as [3H]citrulline was established by regularly adding [14C]citrulline to the tissue extracts. The amount of [3H]citrulline in the sample was taken to represent the stoichiometric production of NO, and the formation of [3H]citrulline was computed as dpm per milligram tissue. Drugs were added either 30 min before addition of [3H]arginine or together with [3H]arginine. The effects of drugs on the electrically induced [3H]citrulline formation are given as percent increases above basal levels measured in parallel under resting conditions.

Data analysis. Results are expressed as means ± SE. The significance of difference between two mean values was assessed using Student’s t-test. For comparison of one control with several experimental groups, the significance of difference was estimated by one-way ANOVA followed by Dunnett’s test.

Drugs. L-[2,3-3H]arginine HCl (38.5 Ci/mmol), L-[ureido-14C]citrulline (57.8 Ci/mmol), L-[1-14C]ornithine (40–60 Ci/mmol) were from NEN (Dreieich, Germany). (-)-Bicuculline methiodide was from Research Biochemicals International (Natick, MA). L-Arginine hydrochloride, (±)-baclofen, Nω-nitro-arginine, mecamylamine hydrochloride, muscimol, oxotremorine sesquifumarate, scopalamine hydrobromide, and TTX were from Sigma (Munich, Germany). GABA and (+)-tubocurarine chloride pentahydrate were from Fluka (Buchs, Switzerland). Drugs were dissolved in distilled water.

RESULTS

Formation of [3H]citrulline. Incubation of the longitudinal muscle-myenteric plexus preparation with [3H]arginine led to a spontaneous formation of [3H]citrulline. [3H]ornithine was not detected. Spontaneous [3H]citrulline production after 30 min was 89 ± 4 dpm/mg (n = 13), which accounted for 1.1 ± 0.05% of the total [3H]radioactivity in the tissue. Intermittent field stimulation at 10 Hz for 30 min led to a twofold increase in the [3H]citrulline content (276 ± 21 dpm/mg; n = 13), which accounted for 3.0 ± 0.26% of the tritium content of the tissue. Increasing the stimulation period to 45 min did not further significantly enhance the [3H]citrulline content (Fig. 1). Moreover, there was no further increase of [3H]citrulline in response to stimulation at 30 Hz (30 min). Therefore, in all subsequent experiments, the strips were stimulated at 10 Hz for 30 min.

In the presence of the NO synthase inhibitor Nω-nitro-L-arginine (100 µM), both the spontaneous and the electrically evoked formations of [3H]citrulline were strongly inhibited (Fig. 2). Likewise, omission of calcium from the incubation medium significantly reduced the [3H]citrulline production under resting and stimulation conditions. Neuronal NO synthase is a calcium- and calmodulin-dependent enzyme that is maximally active at already 0.5 µM calcium (6). To prevent endogenous calcium from activating NO synthase, the chelator EGTA (2 mM) was added to the medium in these experiments. TTX (1 µM) did not affect [3H]citrulline production under resting conditions but significantly inhibited the electrically evoked increase in [3H]citrulline production by ~32%.

Effects of cholinergic agonists and antagonists. [3H]citrulline formation under resting conditions was not significantly changed by the muscarinic agonist oxotremorine or the antagonist scopolamine (Table 1). However, scopolamine significantly enhanced [3H]citrulline production during electrical stimulation (Fig. 3, top), whereas oxotremorine did not modify the stimulation-evoked [3H]citrulline formation.

Spontaneous [3H]citrulline formation was also not significantly affected by the nicotinic receptor antago-
nists (+)-tubocurarine and mecamylamine (Table 1), but both antagonists strongly inhibited the stimulation-evoked [3H]citrulline production (Fig. 3, bottom).

GABAergic inhibition of [3H]citrulline formation. GABA (10 and 100 µM) inhibited the electrically induced formation of [3H]citrulline by 43 and 78%, respectively (Fig. 4). Similarly, the GABA<sub>A</sub> agonist muscimol (10 µM) reduced the stimulation-evoked production of [3H]citrulline, whereas the GABA<sub>A</sub> agonist baclofen (10 µM) was without effect. Neither drug had an effect on spontaneous [3H]citrulline production (Table 2).

The GABA<sub>A</sub> antagonist bicuculline (10 µM) applied 30 min before incubation with [3H]arginine did not significantly modify spontaneous or evoked [3H]citrulline formation compared with the respective control values in the absence of drugs. However, bicuculline significantly antagonized the inhibitory effect of 100 µM GABA (Fig. 4B).

DISCUSSION

Our study shows that NO synthase can easily be measured in the myenteric plexus-longitudinal muscle preparation of the guinea pig ileum by the conversion of

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Table 1. Effects of cholinergic agonists and antagonists on [3H]citrulline formation under resting conditions

<table>
<thead>
<tr>
<th>Drug</th>
<th>[3H]Citrulline Formation, dpm/mg n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76 ± 4 (5)</td>
</tr>
<tr>
<td>Oxotremorine, 1.0 µM</td>
<td>79 ± 10 (5)</td>
</tr>
<tr>
<td>Scopolamine, 0.1 µM</td>
<td>70 ± 7 (5)</td>
</tr>
<tr>
<td>Control</td>
<td>88 ± 6 (9)</td>
</tr>
<tr>
<td>(+)-Tubocurarine, 10 µM</td>
<td>70 ± 8 (4)</td>
</tr>
<tr>
<td>(+)-Tubocurarine, 100 µM</td>
<td>86 ± 23 (4)</td>
</tr>
<tr>
<td>Mecamylamine, 10 µM</td>
<td>97 ± 13 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of preparations. Longitudinal muscle-myenteric plexus preparations were incubated for 30 min with [3H]arginine. Oxotremorine was added together with [3H]arginine, and antagonists were added 30 min before incubation with [3H]arginine.

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Fig. 3. Effects of oxotremorine (Oxo, 1 µM), scopolamine (Sc, 0.1 µM), (+)-tubocurarine (Tc, 10 and 100 µM), and mecamylamine (Me, 10 µM) on electrically evoked [3H]citrulline formation. Longitudinal muscle-myenteric plexus preparations were incubated with [3H]arginine and stimulated electrically (10 Hz) for 30 min. Oxotremorine was added at the onset of incubation with [3H]arginine; antagonists were present from 30 min before incubation with [3H]arginine onward. Ctr, control experiments. Results are means ± SE (n = 5 and 4–9 for top and bottom, respectively). Significance of difference from corresponding control value: *P < 0.05, **P < 0.01.

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Fig. 4. Effects of GABAergic agonists on electrically evoked [3H]citrulline formation. Preparations were incubated with [3H]arginine and stimulated electrically (10 Hz) for 30 min. A: effects of baclofen (Bac, 10 µM, n = 3), GABA (10 µM, n = 4), and muscimol (Mus, 10 µM, n = 8). B: effects of 100 µM GABA in the absence (open bar, n = 5) and presence (hatched bar, n = 7) of 10 µM bicuculline. Agonists were present from the onset of incubation and bicuculline from 30 min before the onset of incubation with [3H]arginine. Control experiments (Ctr) were also conducted in the absence (open bar, n = 9) and presence (hatched bar, n = 5) of bicuculline. Significance of difference from corresponding control value: **P < 0.01. Significance of difference from the effect of GABA in the presence of bicuculline: ++P < 0.01.
Table 2. Effects of GABAergic agonists in the absence and presence of bicuculline on [3H]citrulline formation under resting conditions

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Bicuculline, µM</th>
<th>[3H]Citrulline Formation, dpm/mg</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>77 ± 4</td>
<td>9</td>
</tr>
<tr>
<td>Baclofen, 10 µM</td>
<td>0</td>
<td>69 ± 8</td>
<td>3</td>
</tr>
<tr>
<td>Muscimol, 10 µM</td>
<td>0</td>
<td>83 ± 12</td>
<td>8</td>
</tr>
<tr>
<td>GABA, 10 µM</td>
<td>0</td>
<td>85 ± 14</td>
<td>5</td>
</tr>
<tr>
<td>GABA, 100 µM</td>
<td>0</td>
<td>85 ± 14</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>63 ± 2</td>
<td>5</td>
</tr>
<tr>
<td>GABA, 100 µM</td>
<td>10</td>
<td>82 ± 13</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of preparations. Longitudinal muscle-myenteric plexus preparations were incubated for 30 min with [3H]arginine. GABA agonists were added at the onset of incubation with [3H]arginine, and bicuculline was added 30 min before incubation with [3H]arginine.

[3H]arginine to [3H]citrulline. This method has recently been used to measure NO synthase activity in the rat myenteric plexus-longitudinal muscle preparation (1). The [3H]citrulline formation under resting conditions and during field stimulation was nearly prevented by the NO synthase inhibitor N^o^-nitro-L-arginine, which indicates that this [3H]citrulline originates specifically from the NO synthase reaction. Moreover, spontaneous and electrically induced formation of [3H]citrulline was largely inhibited by removal of extracellular calcium, suggesting that the NO synthase was of the constitutive form, as neuronal NO synthase is known to be.

The electrically induced formation of [3H]citrulline was significantly reduced but not abolished by TTX, which implies that a component of the [3H]citrulline formation is not due to the sodium-dependent neuronal action potential propagation. A similar result has been reported in studies on canine enteric neurons, in which the NO release evoked by nicotinic receptor stimulation was only partly TTX sensitive (24). We suppose that strong electrical currents of 300 mA depolarize the entire neuron well beyond its threshold even in the presence of TTX. This leads to increased intracellular calcium concentrations and hence increased NO synthesis in all parts of the neuron. NO synthase is present in the whole nerve cell of guinea pig myenteric neurons (19), and electrical stimulation releases NO not only from the nerve terminal but also from the axon and soma (26). Such direct effects of electrical field stimulation at high current strength on nerves of the autonomic nervous system are well known. Illes et al. (11), for instance, showed that field stimulation of sufficient strength caused a TTX-resistant but calcium-dependent release of norepinephrine from the mouse vas deferens. A complete inhibition of the electrically induced NO synthesis and release was reported for other isolated gastrointestinal preparations, but in these studies both current strength and pulse duration were submaximal compared with the present experiments (in Ref. 1, rat myenteric plexus-longitudinal muscle strips were 240 mA and 0.4 ms; in Ref. 5, rat gastric fundus strips were 120 mA and 1 ms, respectively). The spontaneous [3H]citrulline production was not modified by TTX. Thus there is probably a spontaneous, stimulus-independent activity of NO synthase similar to that in the rat small intestine (1).

The production of [3H]citrulline was strongly impaired by (+)-tubocurarine and mecamylamine. This finding was not unexpected, since previous studies showed that the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinum iodide stimulated [3H]citrulline formation in myenteric ganglia from guinea pig small intestine (8) and increased neuronal NO synthase mRNA expression in rat gastric myenteric ganglia (20). Nicotinic receptors have recently been detected on the soma of NO synthase-immunoreactive neurons in the myenteric plexus of guinea pig small intestine (13). These findings together with those in this study suggest that nitrergic neurons are equipped with nicotinic receptors whose stimulation by endogenous ACh enhances NO synthesis.

Scopolamine increased the formation of [3H]citrulline, which indicates that NO synthesis is inhibited by endogenous ACh via muscarinic receptors. Oxotremorine caused only a slight but statistically not significant reduction in [3H]citrulline production. It is possible that the inhibitory muscarinic receptors were already maximally activated by endogenous ACh released during field stimulation so that oxotremorine could not exert the expected inhibition. A similar muscarinic receptor-mediated inhibition of [3H]citrulline synthesis has been reported in studies on rat myenteric plexus preparation (1). Thus ACh may enhance (via nicotinic receptors) and diminish (via muscarinic receptors) NO synthesis and release in the small intestine.

The most interesting finding of the present study is the inhibition of NO synthesis through GABA. The effect of GABA was mimicked by muscimol, but not by baclofen, and was antagonized by bicuculline, which indicates that the inhibition was mediated by GABA_A receptors. Another neurotransmitter whose release from guinea pig myenteric neurons is inhibited via GABA_A receptors is GABA itself (25). A prejunctional location of these GABA_A autoreceptors is suggested by the finding that the GABA_A agonist muscimol inhibited the release of [3H]GABA evoked by high potassium in the presence of TTX, i.e., when axonal conduction was blocked (25). Although our study gives no information on the location of the GABA_A receptors inhibiting NO synthesis, it seems likely that they are located on nerve terminals of nitrergic neurons. In experiments on the rat colon, GABA_A receptor immunoreactivity was found on cell somata of NO synthase-positive neurons but not on nerve fibers (16). There might thus be a species difference, since the above-mentioned [3H]GABA release experiments suggest the occurrence of prejunctional GABA_A receptors on guinea pig myenteric neurons (25). GABA is cotored with NO in a subpopulation (~20%) of nitrergic neurons, which are inhibitory motoneurons supplying the longitudinal and circular muscles of the guinea pig small intestine (28). If the GABA_A receptors are located at these neurons, they may be regarded as autoreceptors, which inhibit not only the synthesis and...
subsequent release of NO but also the release of the cotransmitter GABA.

The actions of exogenous GABA on gastrointestinal motility are complex and comprise stimulation and inhibition of the release of both ACh and NO. GABA, via presynaptic GABA_A receptors, reduces motility through inhibition of the electrically evoked ACh release (14). In contrast, GABA, via GABA_B receptors, may stimulate motility, since GABA_B receptor blockade reduces peristalsis (21). This excitatory effect can be attributed to the stimulation of cholinergic neurons by GABA_A receptors located on the neuronal cell soma (14). A similar biphasic GABAergic modulation probably exists for the release of NO from myenteric neurons. On the one hand, GABA causes NANC relaxations in the ileum of dogs (2) and guinea pigs (15) via activation of GABA_B receptors, which presumably are localized on the cell soma of nitricergic neurons. On the other hand, the present study shows that GABA inhibits NO formation through GABA_A receptors, which may be localized prejunctionally on nitricergic neurons. The inhibition of NO synthesis and release may, in turn, lead to an increase of intestinal motility.

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Address for reprint requests and other correspondence: H. Kilbinger, Dept. of Pharmacology, Univ. of Mainz, D-55101 Mainz, Germany (E-mail: KILBINGE@mail.uni-mainz.de).

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