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 nerves, and, in the guinea pig small intestine, ~20% of all myenteric nerves 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 relaxation (22, 27). Evidence to support NO as an inhibitory neurotransmitter was provided from the use of NO synthase inhibitors, which abolished or reduced the relaxations. Another approach to study NO synthase activity uses the citrulline assay, i.e., the conversion of [3H]arginine to [3H]citrulline. This reaction involves the oxidation of a guanidino nitrogen of L-arginine to NO together with the stoichiometric production of L-citrulline (for review, see Ref. 3). With the use of this method, NO synthase activity has been studied in the past in a variety of gastrointestinal preparations of the guinea pig, such as stomach (12), taenia coli (9), and isolated ganglia from the myenteric plexus (8). Surprisingly, no comparable studies have been performed on intact nerve-muscle preparations of the guinea pig small intestine, although this tissue has been widely used as a model system for the analysis of enteric inhibitory and excitatory neurotransmission.

The present investigation has two objectives. First, we aimed to investigate systematically NO synthesis in an intact myenteric plexus-longitudinal muscle preparation under a variety of different conditions using the citrulline assay. Second, we aimed to study whether [3H]citrulline formation is modulated by neurotransmitters of the enteric nervous system, such as ACh and GABA. GABA is colocalized with NO synthase in guinea pig myenteric plexus (28), but the functional significance of such colocalization is not known.

MATERIALS AND METHODS

Guinea pigs of either sex weighing 200–400 g were stunned by a blow to the head and bled. A 30-cm section of ileum was taken, with the distal end being 10 cm from the ileocecal junction. Longitudinal muscle-myenteric plexus strips (20–40 mg wet wt) were prepared as described previously (23). The strips were suspended isometrically under a tension of 5 mN in a 2-mL organ bath and superfused (2 mL/min) with a physiological salt solution (composition in mmol/l: 149.3 NaCl, 2.7 KCl, 1.8 CaCl2, 1.05 MgCl2, 145.4 Cl−, 11.9 HCO3−, 0.42 H3PO4, and 5.6-glucose) of 37°C that was bubbled with a mixture of 95% O2 and 5% CO2. L-Arginine (10 µM) was added to the solution, since it was shown that NO synthase activity in the intestine increases with the L-arginine concentration, reaching a maximum at ~10 µM (10). After an equilibration period of 45 min, superfusion was stopped and the tissue was incubated for 30 min (in a few experiments for 10 or 45 min) with [3H]arginine (0.5 µCi/ml). During the incubation with [3H]arginine, the tissue was stimulated by electrical field stimulation using Grass S6 stimulators. Square wave pulses of 1 ms duration and 10 or 30 Hz were applied intermittently (10-s stimulation periods at intervals of 30 s) by two platinum electrodes that were positioned parallel to the strips (distance of 0.6 cm). The applied current was 300–320 mA, depending on the individual stimulator, and was controlled by monitoring the potential drop across a 20 Ω resistor. At the end of the incubation, the strips were blotted, weighed, and homog-
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, prepacked with 5 µm Shandon ODS-Hypersil; Bischoff Chromatography, Leonberg, Germany) using as 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 [¹¹C]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.

Results. 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-[¹⁴C]ornithine (40–60 Ci/mmol) were from NEN (Dreieich, Germany). (±)-Bicuculline methiodide was from Research Biochemicals International (Natick, MA). L-Arginine hydrochloride, (±)-bafodex, N⁵-nitro-L-arginine, mecamylamine hydrochloride, muscimol, oxotremorine sesquifumurate, scopolamine 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 threefold 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 N⁵-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-
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</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76 ± 6</td>
<td>5</td>
</tr>
<tr>
<td>Oxotremorine, 1.0 µM</td>
<td>79 ± 10</td>
<td>5</td>
</tr>
<tr>
<td>Scopolamine, 0.1 µM</td>
<td>70 ± 7</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>88 ± 6</td>
<td>9</td>
</tr>
<tr>
<td>(+)-Tubocurarine, 10 µM</td>
<td>70 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>(+)-Tubocurarine, 100 µM</td>
<td>86 ± 23</td>
<td>4</td>
</tr>
<tr>
<td>Mecamylamine, 10 µM</td>
<td>97 ± 13</td>
<td>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.

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.

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.

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 GABA into [3H]citrulline. The GABAergic inhibition of [3H]citrulline formation was observed with GABA (10 and 100 µM) and the GABAA agonist muscimol (10 µM), whereas the GABAB agonist baclofen (10 µM) was without effect. Neither drug had an effect on spontaneous [3H]citrulline production (Table 2).

The GABAA 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).
[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-phenylpiperazinium 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. Oxtremorine 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 oxtremorine 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 costored 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

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>7.7 ± 0.4</td>
<td>9</td>
</tr>
<tr>
<td>Baclofen, 10 µM</td>
<td>0</td>
<td>69 ± 8.6</td>
<td>3</td>
</tr>
<tr>
<td>Muscimol, 10 µM</td>
<td>0</td>
<td>8.3 ± 12.2</td>
<td>8</td>
</tr>
<tr>
<td>GABA, 10 µM</td>
<td>0</td>
<td>8.5 ± 14.8</td>
<td>5</td>
</tr>
<tr>
<td>GABA, 100 µM</td>
<td>0</td>
<td>8.5 ± 14.8</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>63 ± 2.4</td>
<td>5</td>
</tr>
<tr>
<td>GABA, 100 µM</td>
<td>10</td>
<td>82 ± 13.7</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.
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<sub>A</sub> receptors, reduces motility through inhibition of the electrically evoked ACh release (14). In contrast, GABA, via GABA<sub>A</sub> receptors, may stimulate motility, since GABA<sub>A</sub> receptor blockade reduces peristalsis (21). This excitatory effect can be attributed to the stimulation of cholinergic neurons by GABA<sub>A</sub> receptors located on the neuronal cell somata (14). A similar biphasic GABAergic modulation probably exists for the release of NO from myenteric neurons. On the one hand, GABA causes NANC relaxation in the ileum of dogs (2) and guinea pigs (15) via activation of GABA<sub>A</sub> receptors, which presumably are located on the cell somata of nitricergic neurons. On the other hand, the present study shows that GABA inhibits NO formation through GABA<sub>A</sub> receptors, which may be localized prejuncturally on nitricergic neurons. The inhibition of NO synthesis and release may, in turn, lead to an increase of intestinal motility.

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