Stimulation of voltage-dependent Ca\textsuperscript{2+} channels by NO at rat myenteric neurons

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Sitmo M, Rehn M, Diener M. Stimulation of voltage-dependent Ca\textsuperscript{2+} channels by NO at rat myenteric neurons. Am J Physiol Gastrointest Liver Physiol 293: G886–G893, 2007. First published August 16, 2007; doi:10.1152/ajpgi.00124.2007.—The aim of the present study was to characterize the action of the neurotransmitter NO on rat myenteric neurons. A NO donor such as GEA 3162 (10\textsuperscript{-4} mol/l) induced an increase in the intracellular Ca\textsuperscript{2+} concentration as indicated by an increase in the fura 2 ratio in ganglia loaded with this Ca\textsuperscript{2+}-sensitive fluorescent dye. The effect of GEA 3162 was strongly reduced in the absence of extracellular Ca\textsuperscript{2+}, suggesting an influx of Ca\textsuperscript{2+} from the extracellular space evoked by NO. A similar nearly complete inhibition was observed in the presence of Ca\textsuperscript{2+} channel blockers such as Ni\textsuperscript{2+} (5 \times 10\textsuperscript{-4} mol/l) or nifedipine (10\textsuperscript{-6} mol/l). Whole cell patch-clamp recordings confirmed the activation of voltage-dependent Ca\textsuperscript{2+} channels, measured as inward current carried by Ba\textsuperscript{2+}, by the NO donor. The peak Ba\textsuperscript{2+}-carried inward current increased from −100 ± 19 to −185 ± 34 pA in the presence of sodium nitroprusside (10\textsuperscript{-4} mol/l). The consequence was a hyperpolarization of the membrane, which was blocked by intracellular Cs\textsuperscript{+} and thus most probably reflects the activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels. Furthermore, at least two subtypes of NO synthases, NOS-1 (neuronal form) and NOS-3 (endothelial form), were found as transcripts in mRNA isolated from the rat myenteric ganglia. The expression of these NO synthases was confirmed immunohistochemically. These observations suggest that NO, released from nitrergic neurons within the enteric nervous system, not only affects target organs such as smooth muscle cells in the gut but has in addition profound effects on the enteric neurons themselves, the key players in the regulation of many gastrointestinal functions.

intracellular Ca\textsuperscript{2+}; membrane potential; nitric oxide

NITRIC OXIDE (NO) is an important neurotransmitter in the enteric nervous system, involved, e.g., in the descending relaxation of the smooth muscle layer during the peristaltic reflex (3) or regulation of mucosal blood flow (for review, see Ref. 30). In vivo, NO is released from the guanido group of L-arginine by NO synthases (NOS), from which three isoforms are known: the neuronal form, NOS-1, the inducible form, NOS-2, and the endothelial form, NOS-3 (14, 27), with NOS-1 as predominant type expressed by enteric neurons (29).

Whereas peripheral actions of NO on target cells such as the muscle layer of the gut wall or blood vessels are well known, there is only scarce information about the question whether NO might act as a neurotransmitter on the enteric neurons themselves. In guinea pig small intestine, a NO donor such as sodium nitroprusside (SNP) did not affect basal membrane potential of myenteric neurons but inhibited slow excitatory postsynaptic potentials (28). Furthermore, inhibition of NOS-1 potentiated the effect of electric field stimulation on anion secretion in guinea pig colon, suggesting a basal inhibitory effect of NO on the enteric nervous system (19). In the small intestine of the same species, NO has been reported to act as a retrograde transmitter released from interneurons affecting synaptic transmission between these interneurons and sensory neurons (38). Further evidence for potential neuronal effects of NO comes from immunohistochemical studies which show that the expression pattern of NOS isoforms in the murine myenteric plexus changes during development with an early but transient expression of NOS-2 during embryogenesis, followed by the expression of NOS-1 and NOS-3, suggesting a role for NO in the development of the enteric nervous system (1).

For other species such as rat, there is no information available about possible direct actions of NO on enteric neurons and the mechanisms involved. Therefore, in the present study, we investigated effects of NO donors on cultured rat myenteric ganglionic cells. Because effects of NO are often related to changes in the cytosolic Ca\textsuperscript{2+} concentration (see, e.g., Refs. 15, 18, 37), we measured both changes in the intracellular Ca\textsuperscript{2+} concentration with the Ca\textsuperscript{2+}-sensitive dye fura 2 as well as changes in membrane potential and membrane currents with the whole cell patch-clamp method evoked by NO donors.

MATERIALS AND METHODS

Isolation and incubation procedure. Myenteric ganglia were isolated from the small intestine of 4- to 10-day-old rats. Animals were killed by decapitation (approved by Regierungsrätspräsidium Giessen, Giessen, Germany). After the gut was removed from the rat, the intestine was transferred to DMEM. The serosa was stripped away under optical control and the muscle layer was separated from the mucosa with fine forceps. Then the muscle was dissociated by incubation at 37°C in DMEM containing 1 mg/ml collagenase type II (Life Technologies, Eggenstein, Germany). The ganglia, forming netlike structures (24), were collected with a micropipette and placed on ice. This was followed by washing with DMEM, centrifugation for 10 min (600 rpm), and transfer into Start-V medium (Biochrom, Berlin, Germany) containing penicillin (10,000 units/ml), streptomycin (10 mg/ml), and 10% (vol/vol) FCS (PAA, Cölbe, Germany). The ganglia were plated on coverslides (diameter 13 mm) coated with poly-L-lysine (molecular weight > 300 kDa; Biochrom, Berlin, Germany) in conventional four-well dishes. The coverslides were placed in the incubator for 45 min to let the ganglionic nets settle down. Then each well was filled with Start-V medium to a final volume of 500 nl. The four-well chambers were kept in the incubator at 37°C with continuous carbon dioxide (5% CO\textsubscript{2} in O\textsubscript{2} vol/vol) supply. The slides were used in electrophysiological or imaging experiments the next day.

Solutions. The standard solution for superfusion of the myenteric ganglia during the patch-clamp or the fura 2 experiments was a HEPES-buffered Tyrode solution containing (in mmol/l) 135 NaCl,
5.4 KCl, 10 HEPES, 1.25 CaCl$_2$, 1 MgCl$_2$, 12.2 glucose. The pH value of this solution was adjusted to 7.4 with NaOH-HCl. For the Ca$^{2+}$-free buffer, CaCl$_2$ was omitted. To measure Ba$^{2+}$-carried inward currents, the superfusion solution consisted of (in mmol/l) 97 BaCl$_2$, 10 HEPES, 1.25 CaCl$_2$, 1 MgCl$_2$, 12.2 glucose.

The pipettes for the whole cell recordings were filled with a standard solution containing (in mmol/l) 100 K$^+$ gluconate, 40 KCl, 0.1 EGTA, 10 Tris, 5 ATP, 2 MgCl$_2$. The pH was adjusted with Tris-HCl to 7.2. When $K^+$ currents had to be suppressed, K gluconate and KCl were replaced isomolarly by CsCl. For the measurements of Ba$^{2+}$ currents, we used a pipette solution containing (in mmol/l) 112 Cs$^+$ gluconate, 2 MgCl$_2$, 1 ATP, 5 BAPTA, 40 HEPES, which has been reported to reduce rundown of voltage-dependent Ca$^{2+}$ currents in myenteric neurons (5).

For immunocytochemical experiments, the following solutions were used: PBS containing (in mmol/l) 130 NaCl, 8 Na$_2$HPO$_4$, and 1.2 NaH$_2$PO$_4$, and PBS with 0.05% (vol/vol) Triton X-100 (PBS-T).

Patch-clamp experiments. The myenteric ganglia grown on glass slides were transferred into the experimental chamber (volume 0.5 ml), which was superfused hydrostatically (perfusion rate ~1 ml/min). The chamber was mounted on an inverted microscope (Olympus IX-70; Olympus, Hamburg, Germany). All experiments were carried out at room temperature. The patch pipettes had resistances of 5–10 MΩ when filled with the standard pipette solution. To obtain a whole cell recording, a suction pulse was used to break the membrane patch under the tip of the pipette after seal formation. Seal resistances were 5–10 GΩ. Membrane capacitance was corrected for by cancellation of membrane capacitance transient (subtraction) using a 10 mV pulse. To distinguish between neurons and nonneuronal cells, a pulse of 50 mV amplitude (starting from a holding potential of ~80 mV) and 30 ms duration was used. In this voltage range only neurons exhibit a fast inward current in the ganglionic preparation after formation of the whole cell configuration (8). This inward current, therefore, was used as a parameter to distinguish the myenteric neurons from enteric glia (22). Current-voltage ($I$-$V$) curves were obtained by clamping the cell to a holding potential of ~80 mV and depolarization in 10 mV steps for 30 ms. After each depolarization, the cell was clamped again to the holding potential for 1 s before the following voltage step (increased by 10 mV) was applied. Inward current was measured at the point where it had reached its maximal amplitude. In other experiments, where the time constants of Ba$^{2+}$-carried inward current were measured, instead pulses of 100-ms length were applied in 20-mV steps; time constants for activation and inactivation of the currents were obtained by fitting to an exponential function.

Fura 2 experiments. Relative changes in the intracellular Ca$^{2+}$ concentration were measured via the Ca$^{2+}$-sensitive fluorescent dye fura 2 as described previously (8). The myenteric ganglia were loaded for 60 min with 5 × 10$^{-6}$ mol/l fura 2-acetoxymethylene (fura 2-AM) in the presence of 0.05% (vol/vol) pluronic acid. The fura 2-AM was then washed away. The ganglia grown on glass coverslips were transferred into the experimental chamber with a volume of ~3 ml. The cells were superfused hydrostatically throughout the experiment at a flow rate of ~1 ml/min.

Experiments were carried out at room temperature on an inverted microscope (Olympus IX-50; Olympus, Hamburg, Germany) equipped with an epifluorescence setup and image-analysis system (Till Photonics, Gräfelfing, Germany). Several regions of interest, each with the size of about one cell, were selected. At the end of each experiment, cell viability was controlled by administration of cyclopiazonic acid (CPA; 5 × 10$^{-5}$ mol/l), a blocker of sarcoplasmic-endoplasmic reticulum Ca$^{2+}$-ATPases (SERCA); all cells responding to CPA were included in the statistical analysis. The wavelength at which fura 2 is maximally excited shifts depending on the cytoplasmatic Ca$^{2+}$ concentration. The cells were excited alternatively at 340 and 380 nm and the ratio of the emission signal (above 470 nm) at both excitation wavelengths was calculated. Data were sampled at 0.2 Hz.

RT-PCR experiments. For RT-PCR studies, myenteric ganglia were transferred into lysis buffer (Qiagen, Heiden, Germany) and homogenized with a mixer mill (NM301; Retsch, Haan, Germany) with a frequency of 300 Hz. Total RNA was isolated in spin columns (RNeasy kit, Qiagen). For NOS-1, the Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) was used for RNA isolation. Poly A$^+$-RNA was obtained by using an Oligotex column according to the protocol recommended by the manufacturer (Qiagen). RNA was reverse transcribed with Eppendorf cMaster RTmix (Eppendorf, Hamburg, Germany) with 20 μg oligo(dT)15 primer (Promega, Mannheim, Germany).

For the PCR reaction, Eppendorf MasterMix (Eppendorf) was used with 2.5 mmol/l MgCl$_2$. Published primers (25) were used against rat NOS-1 (http://www.ncbi.nlm.nih.gov; accession code NM_052799), NOS-2 (accession code NM_012611), and NOS-3 (accession code NM_000603), yielding expected product lengths of 599, 812, and 435 bp, respectively. Primers were obtained from MWG Biotech (Ebersberg, Germany). Cycling conditions for PCR were 10 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C followed by a final elongation for 10 min at 72°C. The reaction product was visualized after electrophoresis in an agarose gel and staining with ethidium bromide.

Immunocytochemical detection of NOS isoforms. To localize the NOS, we immunocytochemically investigated NOS signals of myenteric ganglia. The cells were fixed for 15 min at 4°C with paraformaldehyde (4% wt/vol) diluted in 100 mmol/l phosphate buffer. The paraformaldehyde was removed by washing the preparations 2 times for 5 min with PBS. To block unspecific binding sites, the ganglia were incubated with a blocking solution prepared of PBS-T with 10% (vol/vol) FCS (PAA Laboratories) at room temperature for 1 h in a moist chamber.

Incubation with the primary antibodies against NOS-1 (Becton Dickinson, Heidelberg, Germany; rabbit polyclonal antibody against human NOS-1 amino acids 1095–1289; final dilution: 1:800), NOS-2 (Chemicon, Hofheim, Germany; rabbit polyclonal antibody against NOS-2 murine COOH-terminal peptide; final dilution: 1:200), or NOS-3 (Chemicon; rabbit polyclonal antibody against human NOS-3 amino acids 596–610; final dilution: 1:800) was performed overnight at +4°C. In some double-staining experiments, glial cells were stained with an antibody against glial fibrillary acidic protein (GFAP; Chemicon; mouse monoclonal antibody against porcine GFAP; final dilution: 1:500); neurons were labeled with an antibody against protein gene product 9.5 (PGP9.5; Dianova, Hamburg, Germany; mouse polyclonal antibody against human PGP 9.5, final dilution: 1:200–500). In control experiments, the primary antibody was omitted to check for antibody specificity.

Then the primary antibody was removed (3 × 5 min washing with PBS-T), followed by the incubation with the secondary antibody for 120 min [for NOS: Cy3-conjugated AffiniPure donkey anti-rabbit IgG from Jackson ImmunoResearch, West Grove, PA; dissolved in PBS-T with 10% (vol/vol) FCS, final dilution 1:800]. Finally, the secondary antibody was removed (2 × washing with PBS-T, 1 × washing with PBS). After a further rinse with phosphate buffer, the sections were incubated for 5 min with 3 × 10$^{-7}$ mol/l 4',6-diamidino-2-phenylindol-diacetate (DAPI; Molecular Probes, Leiden, The Netherlands). Then the preparations were embedded in Citifluor (Newby Castleman, Leicester, UK). For double-labeling experiments, GFP or PGP9.5 were detected with an Alexa 488-coupled secondary antibody (goat anti-mouse IgG from Molecular Probes Europe, final dilution 1:500). The ganglia were examined on a fluorescence microscope (Nikon 80i). Digital images were taken with a B/W camera (DS-2M B/Wc) using the NIS Elements 2.30 software (all from Nikon, Düsseldorf, Germany) to finally adjust brightness, color, and contrast. Images were only analyzed qualitatively, i.e., no quantification of NOS-positive cells was performed.

Drugs. GEA 3162 [1,2,3,4-oxiazatolium-5-amino-3-(3,4-dichlorophenyl) chloride; Axxora, Grünberg, Germany], SNP (Calbiochem, G887
Ganglia were loaded with the Ca\(^{2+}\) NO-induced increase in the cytosolic Ca\(^{2+}\) by SNP (10\(^{-4}\) mol/l; Table 1). In contrast, in the presence of nifedipine (10\(^{-6}\) mol/l; D), at the end of each experiment, the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) blocker cyclopiazonic acid (CPA, 5 \times 10^{-5}\) mol/l; open bar) was added to test cell viability. Typical tracings; for statistics, see Table 1.

**RESULTS**

**Effect of a NO donor on the fura 2 ratio.** Because neuronal excitation is in general linked with an increase in the intracellular Ca\(^{2+}\) concentration, in the first series of experiments the ganglia were loaded with the Ca\(^{2+}\)-sensitive dye fura 2 and changes in the fura 2 ratio signal as indicator for an increase in the cytosolic Ca\(^{2+}\) concentration were measured. The ganglia were exposed to a drug known to liberate NO, GEA 3162 (10\(^{-4}\) mol/l), a lipophilic NO donor (26). The agonist evoked an increase in the fura 2 ratio signal from 0.43 \pm 0.013 to 0.51 \pm 0.018 (P < 0.05, n = 66) indicating an increase in the cytosolic Ca\(^{2+}\) concentration (Fig. 1A, Table 1). At the end of the experiment, a further Ca\(^{2+}\) increase was evoked by addition of CPA (5 \times 10^{-5}\) mol/l), a blocker of SERCA (for references to this inhibitor, see, e.g., Refs. 23), inducing a release of stored Ca\(^{2+}\). The action of GEA 3162 was mimicked by SNP (10\(^{-4}\) mol/l), another NO donor, which caused an increase in the fura 2 ratio from 0.56 \pm 0.047 to 0.60 \pm 0.051 (P < 0.05, n = 19; not statistically different from the increase in the fura 2 ratio evoked by GEA 3162).

To find out the source of the Ca\(^{2+}\) responsible for the NO-induced increase in the cytosolic Ca\(^{2+}\) concentration, influx of Ca\(^{2+}\) from the extracellular space was prevented by superfusion of the cells with Ca\(^{2+}\)-free buffer. In Ca\(^{2+}\)-free buffer, the action of the NO donor was completely blocked (Fig. 1B, Table 1). Cell viability can again be recognized at the response to the SERCA blocker, which still induced an increase in the cytosolic Ca\(^{2+}\) concentration in the absence of extracellular Ca\(^{2+}\). The obvious reduction of the CPA response in Ca\(^{2+}\)-free buffer (or after Ca\(^{2+}\) channel blockade) may be explained by the assumption that an inhibition of Ca\(^{2+}\) influx (with Ca\(^{2+}\)-extrusion mechanisms such as plasma membrane Ca\(^{2+}\)-ATPases or Na\(^{+}\)-Ca\(^{2+}\) exchangers still running) might cause a gradual reduction of the Ca\(^{2+}\) stores.

A similar, nearly complete inhibition of the GEA 3162 response was observed in the presence of Ni\(^{2+}\) (10\(^{-3}\) mol/l; Fig. 1C, Table 1), a blocker of voltage-dependent Ca\(^{2+}\) channels (for reference for this and all other Ca\(^{2+}\) channel blockers used, see Ref. 4). To differentiate the subtype of the voltage-dependent Ca\(^{2+}\) channel involved, more specific inhibitors were tested. A strong inhibition was observed with the L-type channel blocker nifedipine (10\(^{-6}\) mol/l; Fig. 1D, Table 1), an action that was mimicked by another L-type blocker, verapamil (10\(^{-5}\) mol/l; Table 1). In contrast, an N-type blocker, \(\omega\)-conotoxin GVIA (10\(^{-6}\) mol/l), only partially inhibited the response to the NO donor (Table 1). \(\omega\)-Agatoxin IVA (10\(^{-7}\) mol/l), a blocker of P- and Q-type Ca\(^{2+}\) channels, did not reduce the action of GEA 3162 on the fura 2 ratio. In contrast, in the presence of this inhibitor, a paradoxical stimulation of the action of the NO donor was observed (Table 1).

**Effect of a NO donor on Ca\(^{2+}\) currents.** To be able to measure effects of NO donors on Ca\(^{2+}\) conductances more directly, whole cell patch-clamp recordings were performed using SNP as NO donor. In these experiments, the myenteric neurons were superfused with a Ba\(^{2+}\) solution, because most voltage-dependent Ca\(^{2+}\) channels are more permeable for Ba\(^{2+}\) than for Ca\(^{2+}\) itself (31). At the start of each experiment, during superfusion with the standard 135 mmol/l NaCl solution, neurons were identified by their fast, Na\(^{+}\)-carried inward current during voltage-clamp conditions, before the superfusion was changed toward the 97 mmol/l BaCl\(_2\) solution to measure the Ba\(^{2+}\)-carried inward current. When subsequently the NO donor was administered, the Ba\(^{2+}\) inward current was stimulated, a response that could be completely suppressed by

**Fig. 1.** Effect of the nitric oxide donor GEA 3162 (10\(^{-4}\) mol/l; shaded bar) on the fura 2 ratio under control conditions (A), in the absence of extracellular Ca\(^{2+}\) (B), in the presence of Ni\(^{2+}\) (10\(^{-3}\) mol/l; C), or in the presence of nifedipine (10\(^{-6}\) mol/l; D). At the end of each experiment, the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) blocker cyclopiazonic acid (CPA, 5 \times 10^{-5}\) mol/l; open bar) was added to test cell viability. Typical tracings; for statistics, see Table 1.
addition of nifedipine (10⁻⁶ mol/l; Fig. 2A). Measurement of I-V curves revealed that the inward current (measured at its maximum, i.e., during a depolarizing pulse to +20 mV) increased from −100 ± 19 pA under control conditions to −185 ± 34 pA in the presence of SNP (Fig. 2B; P < 0.05, n = 7). The maximum inward current was observed at a more positive clamp potential compared with a previous study at rat myenteric neurons (5), where it developed at −20 mV with 2.4 mmol/l Ca²⁺ as charge carrier or 0 mV with 20 mmol/l Ba²⁺ as charge carrier, a phenomenon that might be caused by the higher concentration of divalent cations used in our experiments.

A stimulation of Ba²⁺-carried inward current by the NO donor was observed in all neurons, where a successful whole cell recording was possible. When time constants for activation and inactivation of Ba²⁺ currents were fitted in the absence and presence of SNP, there was no obvious difference in both after administration of the NO donor, indicating that SNP did not modify the process of voltage-dependent activation or inactivation (Fig. 3).

The action of SNP on Ba²⁺ currents was suppressed by ODQ, an inhibitor of the soluble guanylate cyclase (10). In the presence of ODQ (10⁻⁵ mol/l; applied with the superfusion), the peak Ba²⁺-carried inward current amounted to −123 ± 23 pA (n = 7). When SNP was administered in the presence of this inhibitor, the NO donor did not stimulate the inward current any more (peak inward current after administration of SNP: −121 ± 22 pA, n = 7).

**Effect of a NO donor on membrane potential.** To investigate changes in membrane potential evoked by NO, membrane potential was measured in the current-clamp mode using a standard pipette solution (see MATERIALS AND METHODS). Superfusion with the NO donor evoked a hyperpolarization of the membrane (Fig. 4). On average, membrane potential changed from −29.3 ± 3.5 mV under control conditions to −39.0 ± 5.7 mV in the presence of SNP (P < 0.05, n = 8).

Other substances such as the short-chain fatty acid butyrate or the eicosanoid thromboxane A₂ induce an increase in the cytosolic Ca²⁺ concentration followed by a similar hyperpolarization at rat myenteric neurons as observed for the NO donor, a response that is mediated by the activation of Ca²⁺-dependent K⁺ channels (7, 20). Therefore, the effect of SNP was tested after blockade of K⁺ channels by intracellular Cs⁺ applied with the patch-clamp pipette during the whole cell (Fig. 2A: stimulation of Ba²⁺-carried inward current (Iₙa) by sodium nitroprusside (SNP, 10⁻⁴ mol/l) at a depolarizing pulse from −80 mV to +20 mV for 30 ms as indicated by the inset, and inhibition by nifedipine (10⁻⁶ mol/l), administered in the presence of SNP. Current-voltage relationship of inward currents (B) under control conditions (□), in the presence of SNP (10⁻³ mol/l; ●), and in the combined presence of SNP and nifedipine (10⁻⁶ mol/l; ▲). Values are means ± SE, n = 7. Vclamp, clamped membrane potential.
recording. After blockade of K\textsuperscript{+} channels with Cs\textsuperscript{+}, basal membrane potential only amounted to 10.0 ± 2.0 mV (n = 6). SNP applied under these conditions caused only a marginal, insignificant change of membrane potential to 11.0 ± 1.8 mV (n = 6), suggesting that an activation of probably Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels is responsible for the hyperpolarization evoked by NO.

Detection of NOS isoforms in rat myenteric ganglia. Finally, we investigated the ability of the myenteric ganglia to produce NO. First, mRNA was isolated from the ganglia and a RT-PCR was performed for the three different isoforms of NOS. The mRNA for the neuronal form of the enzyme, NOS-1, was clearly present, when the agarose gels were stained with ethidium bromide (Fig. 5A). In addition, there was a very weak but highly reproducible band for the endothelial form of the enzyme, NOS-3 (Fig. 5B). For the inducible form (NOS-2), no mRNA was found in the preparation (data not shown).

These findings were confirmed by immunohistochemical staining. The myenteric ganglia express the neuronal form of the NO-producing enzyme (Fig. 6), NOS-1, as shown already by others (see, e.g., Refs. 17, 32). However, also NOS-3 could be detected in the ganglia immunohistochemically (Fig. 7). In contrast, the inducible form of the enzyme, NOS-2, was not observed (or below our limit of detection), at least not under basal conditions (data not shown).

Double-labeling experiments against the neuronal marker, PGP9.5, and the glial marker, GFAP, revealed that NOS-1 and NOS-3 were expressed both by myenteric neurons as well as by glia (Fig. 8). Not all PGP9.5- or GFAP-positive cells could be coabeled with NOS-1 or NOS-3, indicating that NO expression is restricted to a subset of ganglionic cells.
DISCUSSION

NO donors evoke an increase in the cytosolic Ca\(^{2+}\) concentration of rat myenteric neurons (Fig. 1A). Despite the well-known heterogeneity of enteric neurons concerning morphology, transmitter expression, or electrophysiological properties (35), the response was consistently observed in the cultured myenteric ganglia, suggesting that most of these neurons respond to NO. Effects of NO on the cytosolic Ca\(^{2+}\) concentration are well known from other tissues but seem to be strongly cell type specific. For example, in rat hepatocytes, NO evokes a release of Ca\(^{2+}\) from intracellular stores via inositol-1,4,5-trisphosphate receptors (15). In other cells, however, such as coronary smooth muscle cells, NO donors inhibit Ca\(^{2+}\) signaling, probably through an interaction with the enzyme cADP-ribosyl cyclase responsible for the production of cADP ribose (37). Similarly, in murine skeletal muscle fibers, NO exerts an inhibitory action on Ca\(^{2+}\) release via ryanodine receptors (18). In contrast, in the rat myenteric ganglia, the increase in the cytosolic Ca\(^{2+}\) concentration was completely dependent on the presence of extracellular Ca\(^{2+}\) (Fig. 1B) and was inhibited by typical Ca\(^{2+}\) channel blockers, especially L-type Ca\(^{2+}\) blockers (Fig. 1, C–D; Table 1), clearly indicating that in these cells NO stimulates an influx of Ca\(^{2+}\) from the extracellular space.

Whole cell patch-clamp experiments confirmed the stimulation of a Ca\(^{2+}\) conductance (measured as Ba\(^{2+}\)-carried inward current) by NO (Fig. 2A). NO did not change the voltage-gated Ca\(^{2+}\) channels.

Fig. 6. Immunocytochemical staining against NOS-1 (left), nuclear staining with DAPI (middle), and overlay of both signals (right). Bottom: the primary antibody against NOS-1 was omitted to evaluate background fluorescence. Typical photograph of at least 3 experiments with similar results. Bars: 20 μm.

Fig. 7. Immunocytochemical staining against NOS-3 (left), nuclear staining with DAPI (middle), and overlay of both signals (right). Bottom: the primary antibody against NOS-3 was omitted to evaluate background fluorescence. Typical photograph of at least 3 experiments with similar results.
the respective cell type marker as well as the respective NOS form.

... either an increase in the density of active Ca\(^{2+}\) channels, predominantly of the L type. The consequence is the opening of Ca\(^{2+}\)-dependent K\(^+\) channels, and thereby hyperpolarizes the membrane (Fig. 4). This shifts the membrane potential away from the threshold for the opening of voltage-dependent Na\(^+\) channels and will therefore reduce excitability of enteric neurons.

Nitirgic neurons project predominantly in an anal direction, e.g., to innervate the circular muscle, where they contribute to the descending relaxation during the peristaltic reflex, or the mucosa, where they affect epithelial ion transport (see, e.g., Ref. 16). In addition, descending interneurons seem to release NO as a retrograde transmitter to modify synaptic transmission between them and sensory neurons (38). The changes in the cytoplasmic Ca\(^{2+}\) concentration as well as in the basal membrane potential observed in the present study may be involved in these descending reflex pathways.

Taken together, these observations demonstrate that NO, which can be produced in rat myenteric ganglia by NOS-1 and NOS-3 (Figs. 5–8), which both are activated by intracellular Ca\(^{2+}\) (14), causes a stimulation of voltage-dependent Ca\(^{2+}\) channels, predominantly of the L type. The consequence is the opening of Ca\(^{2+}\)-dependent K\(^+\) channels and a hyperpolarization of the membrane. Thus NO released from enteric neurons not only affects effector cells within the gastrointestinal tract, such as smooth muscles, but modifies in addition functions of the enteric neurons themselves, which act as the key players for the control of most autonomous gut functions (35). Interestingly, inflammatory bowel diseases are known to be related to changes in NO synthase activity and expression patterns. For example, experimentally induced colitis is related to an up-regulation of NOS-2 (see, e.g., Refs. 32), whereas the ability of the enteric neurons to produce NO via NOS-1 is reduced (11, 17). NOS-2 knockout mice and NOS-3 knockout mice develop a more severe colitis when exposed to trinitrobenzenesulfonic acid. In another model for colitis, i.e., mice treated with dextran sodium sulfate, there is a prominent upregulation of NOS-2, whereas the ability of the enteric neurons to produce NO via NOS-1 is reduced (11, 17). NOS-2 knockout mice and NOS-3 knockout mice develop a more severe colitis when exposed to trinitrobenzenesulfonic acid. In another model for colitis, i.e., mice treated with dextran sodium sulfate, there is a prominent upregulation of NOS-2, whereas the ability of the enteric neurons to produce NO via NOS-1 is reduced (11, 17). NOS-2 knockout mice and NOS-3 knockout mice develop a more severe colitis when exposed to trinitrobenzenesulfonic acid. 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