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

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Abstract:

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^{-4} \text{ mol}\text{ l}^{-1}) induced an increase in the intracellular Ca^{2+} concentration as indicated by an increase in the fura-2 ratio in ganglia loaded with this Ca^{2+}-sensitive fluorescent dye. The effect of GEA 3162 was strongly reduced in the absence of extracellular Ca^{2+} suggesting an influx of Ca^{2+} from the extracellular space evoked by NO. A similar nearly complete inhibition was observed in the presence of Ca^{2+} channel blockers such as Ni^{2+} (5 \times 10^{-4} \text{ mol}\text{ l}^{-1}) or nifedipine (10^{-6} \text{ mol}\text{ l}^{-1}).

Whole-cell patch-clamp recordings confirmed the activation of voltage-dependent Ca^{2+} channels, measured as inward current carried by Ba^{2+}, by the NO donor. The peak Ba^{2+}-carried inward current increased from –100 ± 19 pA to –185 ± 34 pA in the presence of sodium nitroprusside (10^{-4} \text{ mol}\text{ l}^{-1}). The consequence was a hyperpolarization of the membrane, which was blocked by intracellular Cs^+, and thus most probably reflects the activation of Ca^{2+}-dependent K^+ channels.

Furthermore, at least two subtypes of NO-synthases, the NOS-1 (neuronal form) and the 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, does not only affect 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.

Keywords: Ca^{2+} channels, Intracellular Ca^{2+}, membrane potential, myenteric plexus, NO
Introduction

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 (30)). In vivo, NO is released from the guanidino 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. As effects of NO are often related to changes in the cytosolic Ca\(^{2+}\) concentration (see e.g. (15, 18, 37)), we measured both changes in the intracellular Ca\(^{2+}\) concentration with the Ca\(^{2+}\)-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.
Material and methods

Isolation and incubation procedure

Myenteric ganglia were isolated from the small intestine of 4 to 10 d old rats. Animals were killed by decapitation (approved by Regierungspräsidium Giessen, Giessen, Germany). After the gut was removed from the rat, the intestine was transferred to Dulbecco’s modified eagle’s medium (DMEM). The serosa was stripped away under optical control and the muscle layer was separated from the mucosa using fine forceps. Then the muscle was dissociated by incubation at 37˚C in DMEM containing 1 mg·ml⁻¹ collagenase type II (Life Technologies, Eggenstein, Germany). The ganglia, forming net-like 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⁻¹), streptomycin (10 mg·ml⁻¹), and 10 % (v/v) FCS (fetal calves serum; PAA, Cölbe, Germany). The ganglia were plated on cover slides (diameter 13 mm) coated with poly-L-lysine (molecular weight > 300 kDa; Biochrom, Berlin, Germany) in conventional four-well dishes. The cover slides 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 µl. The four-well chambers were kept in the incubator at 37 ºC with continuous carbogen (5 % CO₂ in O₂, v/v) 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⁻¹): NaCl 135, KCl 5.4, HEPES 10, CaCl₂ 1.25, MgCl₂ 1, glucose 12.2. The pH value of this solution was adjusted to
7.4 with NaOH/HCl. For the Ca\textsuperscript{2+}-free buffer, CaCl\textsubscript{2} was omitted. In order to measure Ba\textsuperscript{2+}-carried inward currents, the superfusion solution consisted of (in mmol·l\textsuperscript{-1}): BaCl\textsubscript{2} 97, HEPES 10, CaCl\textsubscript{2} 1.25, MgCl\textsubscript{2} 1, glucose 12.2.

The pipettes for the whole-cell recordings were filled with a standard solution containing (in mmol·l\textsuperscript{-1}): K\textsuperscript{+} gluconate 100, KCl 40, ethylene glycol-bis (2-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA) 0.1, tris(hydroxymethyl)-aminomethane (TRIS) 10, ATP 5, MgCl\textsubscript{2} 2. The pH was adjusted with TRIS/HCl at 7.2. When K\textsuperscript{+} currents had to be suppressed, K gluconate and KCl were replaced isomolarly by CsCl. For the measurements of Ba\textsuperscript{2+} currents, the following pipette solution was used, which has been reported to reduce run-down of voltage-dependent Ca\textsuperscript{2+} currents in myenteric neurons (5) containing (in mmol·l\textsuperscript{-1}): Cs\textsuperscript{+} gluconate 112, MgCl\textsubscript{2} 2, ATP 1, 1,2-bis-(2-aminophenoxy)ethane-N,N,N´,N´-tetraacetic acid (BAPTA) 5, HEPES 40.

For immunocytochemical experiments, the following solutions were used: phosphate-buffered saline (PBS; containing in mmol·l\textsuperscript{-1}: NaCl 130, Na\textsubscript{2}HPO\textsubscript{4} 8 and NaH\textsubscript{2}PO\textsubscript{4} 1.2), and phosphate-buffered saline with 0.05 % (v/v) 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 about 1 ml·min\textsuperscript{-1}). 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 the capacitance transient (subtraction) using a 10 mV pulse. To distinguish between neurons and non-neuronal 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 (IV) 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 (incremented 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 Ba²⁺-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 Ca²⁺ concentration were measured using the Ca²⁺-sensitive fluorescent dye, fura-2 as described previously (8). The myenteric ganglia were loaded for 60 min with 5·10⁻⁶ mol·l⁻¹ fura-2-acetoxymethylester (fura-2-AM) in the presence of 0.05 g·l⁻¹ 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 about 3 ml. The cells were superfused hydrostatically throughout the experiment at a flow rate at about 1 ml·min⁻¹.
Experiments were carried out at room temperature on an inverted microscope (Olympus IX-50; Olympus, Hamburg, Germany) equipped with an epifluorescence set-up 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⁻¹), a blocker of sarcoplasmic-endoplasmic reticulum Ca²⁺-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 Ca²⁺ concentration. The cells were excited alternatively at 340 nm 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.

Reverse transcriptase polymerase chain reaction (RT-PCR) experiments

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

For the PCR reaction, Eppendorf MasterMix (Eppendorf, Hamburg, Germany) was used with 2.5 mmol l⁻¹ MgCl₂. 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 bp, 812 bp, 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 ethidiumbromide.

**Immunocytochemical detection of NOS isoforms**

In order 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 %, w/v) diluted in 100 mmol l⁻¹ 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 % (v/v) FCS (PAA Laboratories, Colbe, Germany) 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 C-terminal peptide; final dilution: 1:200), or NOS-3 (Chemicon, Hofheim, Germany; rabbit polyclonal antibody against human NOS-3 amino acids 596-610; final dilution: 1:800) was performed over night at +4° C. In some double staining experiments, glial cells were stained with an antibody against glial acidic fibrillary protein (GFAP; Chemicon, Hofheim, Germany; mouse monoclonal antibody against porcine GFAP; final dilution: 1:500); neurons were labelled 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 x 5 min washing with PBS-T), followed by the incubation with the secondary antibody for 120 min (for NOS: Cy3<sup>TM</sup>-conjugated affinipure donkey anti-rabbit IgG from Jackson ImmunoResearch, West Grove, PA, USA; dissolved in PBS-T with 10 % (v/v) FCS, final dilution 1:800). Finally, the secondary antibody was removed (2 x washing with PBS-T, 1 x washing with PBS). After a further rinse with phosphate buffer, the sections were incubated for 5 min with 3·10<sup>-7</sup> mol·l<sup>-1</sup> 4′,6-diamidino-2-phenylindoldilactate (DAPI; Molecular Probes, Leiden, The Netherlands). Then the preparations were embedded in Citiflour<sup>®</sup> (Newby Castleman, Leicester, UK). For double labelling experiments, GFAP or PGP9.5 were detected with an Alexa 488-coupled secondary antibody (goat anti-mouse IgG from Molecular Probes Europe, Leiden, The Netherlands, 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 analysed qualitatively, i.e. not quantification of NOS-positive cells was performed.

**Drugs**

GEA 3162 (1,2,3,4-oxatriazolium-5-amino-3-(3,4-dichlorophenyl) chloride; Axxora, Grünberg, Germany), sodium nitroprusside (SNP; Calbiochem, Bad Soden, Germany), and verapamil were dissolved in aqueous stock solution. ω-Conotoxin GVIA and ω-agatoxin IVA (both from Alomone, Jerusalem, Israel) were dissolved in Tyrode solution containing 1 g·l<sup>-1</sup> bovine serum albumin (BSA). Nifedipine was dissolved in ethanol (final content of ethanol 0.02 %, v/v). Ni<sup>2+</sup>
was administered as chloride salt. Fura-2-acetoxy methyl ester (fura-2-AM), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Tocris, Bristol, UK), and pluronic® acid (both from Molecular Probes Europe, Leiden, The Netherlands) were dissolved as stock in dimethylsulphoxide (DMSO). If not indicated differently, chemicals were obtained from Sigma (Taufkirchen, Germany).

Statistics

Results are given as mean ± standard error of the mean (SEM) with the number (n) of investigated cells. For the comparison of two groups, either a Student's t-test or a Mann Whitney U-test was applied. An F-test decided which test method had to be used. Both paired and unpaired two-tailed Student's t-tests were applied as appropriate. When the mean values of more than two groups had to be compared, an one-way anova test was performed followed by analysis of linear contrasts with the test of Scheffé.
Results

Effect of a NO donor on the fura-2 ratio

As 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\(\cdot\)l\(^{-1}\)), a lipophilic NO donor (26). The agonist evoked an increase in the fura-2 ratio signal from 0.43 ± 0.013 to 0.51 ± 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\(\cdot\)10\(^{-5}\) mol\(\cdot\)l\(^{-1}\)), a blocker of sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA; for references to this inhibitor, see e.g. (23)), inducing a release of stored Ca\(^{2+}\). The action of GEA 3162 was mimicked by sodium nitroprusside (10\(^{-4}\) mol\(\cdot\)l\(^{-1}\)), another NO donor, which caused an increase in the fura-2 ratio from 0.56 ± 0.047 to 0.60 ± 0.051 (p < 0.05, n = 19; not statistically different from the increase in the fura-2 ratio evoked by GEA 3162).

In order 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\(^{2+}\)
inflow (with the Ca\textsuperscript{2+} extrusion mechanisms such as plasma membrane Ca\textsuperscript{2+}-ATPases or Na\textsuperscript{+}-Ca\textsuperscript{2+}-exchangers still running) might cause a gradual reduction of the Ca\textsuperscript{2+}-stores.

A similar, nearly complete inhibition of the GEA 3162 response was observed in the presence of Ni\textsuperscript{2+} (10\textsuperscript{-3} mol\textsuperscript{-1}; Fig. 1C, Table 1), a blocker of voltage-dependent Ca\textsuperscript{2+} channels (for reference for this and all other Ca\textsuperscript{2+} channel blockers used, see (4)). In order to differentiate the subtype of the voltage-dependent Ca\textsuperscript{2+} channel involved, more specific inhibitors were tested. A strong inhibition was observed with the L-type channel blocker, nifedipine (10\textsuperscript{-6} mol\textsuperscript{-1}; Fig. 1D, table 1), an action, which was mimicked by another L-type blocker, verapamil (10\textsuperscript{-5} mol\textsuperscript{-1}; table 1). In contrast, an N-type blocker, ω-conotoxin GVIA (10\textsuperscript{-6} mol\textsuperscript{-1}), only partially inhibited the response to the NO donor (Table 1). ω-Agatoxin IVA (10\textsuperscript{-7} mol\textsuperscript{-1}), a blocker of P- and Q-type Ca\textsuperscript{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\textsuperscript{2+} currents**

In order to be able to measure effects of NO donors on Ca\textsuperscript{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\textsuperscript{2+} solution, as most voltage-dependent Ca\textsuperscript{2+} channels are more permeable for Ba\textsuperscript{2+} than for Ca\textsuperscript{2+} itself (31). At the start of each experiment, during superfusion with the standard 135 mmol\textsuperscript{-1} NaCl solution, neurons were identified by their fast, Na\textsuperscript{+}-carried inward current during voltage-clamp conditions, before the superfusion was changed towards the 97 mmol\textsuperscript{-1} BaCl\textsubscript{2} solution in order to measure the Ba\textsuperscript{2+}-carried inward current. When subsequently the NO donor was administered, the Ba\textsuperscript{2+} inward current was
stimulated, a response, which could be completely suppressed by addition of nifedipine \((10^{-6}\) mol\(\text{l}^{-1}\); Fig. 2A). Measurement of IV-curves revealed that the inward current (measured at its maximum, i.e. during a depolarizing pulse to +20 mV) increased from \(-100 \pm 19\) pA under control conditions to \(-185 \pm 34\) 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 to a previous study at rat myenteric neurons (5), where it developed at \(-20\) mV with 2.4 mmol\(\text{l}^{-1}\) Ca\(^{2+}\) as charge carrier or 0 mV with 20 mmol\(\text{l}^{-1}\) Ba\(^{2+}\) as charge carrier, a phenomenon, which might be caused by the higher concentration of divalent cations used in our experiments.

A stimulation of Ba\(^{2+}\)-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 Ba\(^{2+}\) currents were fitted in the absence and in the 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 Ba\(^{2+}\) currents was suppressed by ODQ, an inhibitor of the soluble guanylate cyclase (10). In the presence of ODQ \((10^{-5}\) mol\(\text{l}^{-1}\); applied with the superfusion), the peak Ba\(^{2+}\)-carried inward current amounted to \(-123 \pm 23\) 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: \(-121 \pm 22\) pA, \(n = 7\)).

*Effect of a NO donor on membrane potential*

In order to investigate changes in membrane potential evoked by NO, membrane potential was measured in the current-clamp mode using a standard pipette solution (see Methods). Superfusion with the NO donor evoked a hyperpolarization of the membrane (Fig. 4). In average, membrane
potential changed from $-29.3 \pm 3.5$ mV under control conditions to $-39.0 \pm 5.7$ 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 A$_2$, induce an increase in the cytosolic Ca$^{2+}$ concentration followed by a similar hyperpolarization at rat myenteric neurons as observed for the NO donor; a response, which is mediated by the activation of Ca$^{2+}$-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 recording. After blockade of K$^+$ channels with Cs$^+$, basal membrane potential only amounted to $-10.0 \pm 2.0$ mV ($n = 6$). Sodium nitroprusside, applied under these conditions, caused only a marginal, insignificant change of membrane potential to $-11.0 \pm 1.8$ mV ($n = 6$), suggesting that an activation of probably Ca$^{2+}$-dependent K$^+$ channels is responsible for the hyperpolarization evoked by NO.

Detection of NOS isoforms in rat myenteric ganglia

Finally we asked for the ability of the myenteric ganglia to produce NO. First, mRNA was isolated from the ganglia and a RT-PCR was performed for the 3 different isoforms of NO synthase. The mRNA for the neuronal form of the enzyme, the 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, the 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), the NOS-1, as shown already by others (see e.g. (17, 32)). However, also the NOS-3 could be detected in the ganglia.
immunohistochemically (Fig. 7). In contrast, the inducible form of the enzyme, the NOS-2, was not observed (or below our limit of detection), at least not under basal conditions (data not shown).

Double-labelling 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 colabelled with NOS-1 or NOS-3, indicating that NOS expression is restricted to a subset of ganglionic cell.
Discussion

NO donors evoke an increase in the cytosolic Ca\textsuperscript{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 nitric oxide. Effects of NO on the cytosolic Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} signalling, probably through an interaction with the enzyme cADP-ribosyl cyclase responsible for the production of cADP ribose (37). Similarly, in murine skeletal muscle fibres, NO exerts an inhibitory action on Ca\textsuperscript{2+} release via ryanodine receptors (18). In contrast, in the rat myenteric ganglia, the increase in the cytosolic Ca\textsuperscript{2+} concentration was completely dependent on the presence of extracellular Ca\textsuperscript{2+} (Fig. 1B) and was inhibited by typical Ca\textsuperscript{2+} channel blockers, especially L-type Ca\textsuperscript{2+} blockers (Fig. 1C-D, Table 1), clearly indicating that in these cells NO stimulates an influx of Ca\textsuperscript{2+} from the extracellular space.

Whole-cell patch-clamp experiments confirmed the stimulation of a Ca\textsuperscript{2+} conductance (measured as Ba\textsuperscript{2+}-carried inward current) by NO (Fig. 2A). NO did not change the voltage-dependence of the Ca\textsuperscript{2+} currents (Fig. 2B) and also not the time constants for activation and inactivation (Fig. 3), suggesting either an increase in the density of active Ca\textsuperscript{2+} channels in the membrane or an increase in open probability after exposure to NO.

The mechanism, by which NO affects voltage-dependent Ca\textsuperscript{2+} channels, is unknown. Many actions of NO are mediated by an increase in the intracellular cGMP concentration after
stimulation of the soluble guanylate cyclase with the consecutive stimulation of protein phosphorylation via protein kinase G (33). Indeed, at myenteric neurons from the guinea pig after administration of NO donors a stimulated production of cGMP has been demonstrated (36), suggesting the presence of soluble guanylate cyclase as target enzyme for NO in these cells. On the other hand, NO is well known to induce the S-nitrosylation of proteins at cysteine thiols (for review see (2, 9)) thereby modulating protein functions. A well-studied example for an action of NO on an ion channel mediated by a S-nitrosylation is the activation of a Ca^{2+}-activated K^{+} channel by NO donors in guinea-pig intestinal smooth muscle (12) or rat brain (13). Blockade of the action of the NO donor by ODQ, an inhibitor of the soluble guanylate cyclase, suggests a predominant mediation of the NO response by cGMP, although further experiments are necessary to finally clarify the mechanism of action of NO at rat myenteric neurons.

What is the functional consequence of these NO actions on enteric neurons? NO donors have been shown to inhibit the release of acetylcholine (measured as ^3H-choline release) in guinea pig ileum (34). Similar observations have been made in other segments of the gastrointestinal tract (reviewed by (30)). Vice versa, inhibition of NO synthesis by NOS-1 blockade enhanced the effect of electric field stimulation on anion secretion in guinea pig colon (19), and in guinea pig small intestinal myenteric neurons, NO inhibited slow excitatory postsynaptic potentials (28). Consequently, NO seems to exert in generally an inhibitory action on enteric neurons; an effect, which might be explained by the present observation that the stimulation of Ca^{2+} influx via voltage-dependent Ca^{2+} channels causes 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.
Nitrergic 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. (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 does not only affect 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 upregulation of NOS-2 (see e.g. (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 up-regulation of NOS-2 in the enteric glia; a response, which has been shown to be responsible for the massive change in the action of acetylcholine derivatives on intestinal anion transport (6). These observations
suggest that the inhibitory action of NO on the enteric nervous system might also be of pathophysiological significance.

Acknowledgement

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References:


17. Porras, M., Martín, M.T., Torres, R., and Vergara, P. Cyclical upregulation of iNOS and long-term downregulated nNOS are the bases for relapse and quiescent phases in a rat model of IBD. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290**: G423-G430, 2006.


Fig. 1: Effect of the NO donor GEA 3162 (10^{-4} \text{ mol}\cdot\text{l}^{-1}; \text{grey 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} \text{ mol}\cdot\text{l}^{-1}; \text{C}), or in the presence of nifedipine (10^{-6} \text{ mol}\cdot\text{l}^{-1}; \text{D}). At the end of each experiment, the SERCA blocker cyclopiazonic acid (CPA, 5 \cdot 10^{-5} \text{ mol}\cdot\text{l}^{-1}; \text{white bar}) was added to test cell viability. Typical tracings; for statistics, see Table 1.
Fig. 2A: Stimulation of Ba\textsuperscript{2+}-carried inward current by sodium nitroprusside (SNP, 10^{-4} mol\text{L}^{-1}) 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\text{L}^{-1}), administered in the presence of SNP. Current-voltage relationship of inward currents (B) under control conditions (open squares), in the presence of SNP (10^{-3} mol\text{L}^{-1}; closed circles), and in the combined presence of SNP and nifedipine (10^{-6} mol\text{L}^{-1}; closed triangles). Values are means ± SEM, n = 7.
Fig. 3: Time constants (τ) for activation (left) and inactivation (right) of Ba\textsuperscript{2+}-carried inward current before (open squares) and after administration of sodium nitroprusside (10\textsuperscript{-4} mol\textsuperscript{-1}; closed circles) at a depolarizing pulse from –80 mV to 0 mV, +20 mV, +40, +60, or + 80 mV for 100 ms. Values are means ± SEM, n = 6. No significant differences between both groups were observed at any voltage.
Fig. 4: Hyperpolarization evoked by sodium nitroprusside (SNP, $10^{-4}$ mol l$^{-1}$; grey bar). In order to test viability, carbachol (CCh, $5 \times 10^{-5}$ mol l$^{-1}$; black bars) was administered, which stimulates nicotinic receptors and thereby causes a depolarization of the cell via opening of non-selective cation channels (21, 22). Typical tracing from 8 experiments with similar results; for statistics, see text.
Fig. 5: cDNA prepared by RT-PCR using NOS-1 (A) and NOS-3 (B) specific primers. The expected size of the RT-PCR product was 599 bp for NOS-1 and 435 bp for NOS-3. The faint (but highly reproducible) NOS-3 signal is marked by an arrow. The DNA ladder at the left contains cDNA from 100 to 1000 bp in 100 bp intervals. Representative for at least 3 experiments with similar results.
Fig. 6: Immunocytochemical staining against NOS-1 (left column), nuclear staining with DAPI (middle column), and overlay of both signals (right column). In the lower row, 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 column), nuclear staining with DAPI (middle column), and overlay of both signals (right column). In the lower row, the primary antibody against NOS-3 was omitted to evaluate background fluorescence. Typical photograph of at least 3 experiments with similar results.
Fig. 8: Immunocytochemical doublestaining against the glial marker, GFAP (row 1 and 2; green) or the neuronal marker, PGP9.5 (row 3 and 4; green), and NOS-1 (row 1 and 3; red) or NOS-3 (row 2 and 4; red). Arrows mark cells positive for both the respective cell type marker as well as the respective NOS form.
### Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Fura-2 ratio before GEA 3162</th>
<th>Fura-2 ratio after GEA 3162</th>
<th>Δ Fura-2 ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.43 ± 0.013</td>
<td>0.51 ± 0.018*</td>
<td>0.077 ± 0.013a</td>
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<tr>
<td>Ca²⁺-free</td>
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<td>0.24 ± 0.007</td>
<td>0.009 ± 0.001b</td>
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<tr>
<td>Ni²⁺</td>
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<td>0.18 ± 0.013</td>
<td>0.010 ± 0.004b</td>
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<tr>
<td>Nifedipine</td>
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<td>0.40 ± 0.018</td>
<td>0.016 ± 0.001b</td>
<td>52</td>
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<tr>
<td>Verapamil</td>
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<td>0.48 ± 0.014</td>
<td>0.019 ± 0.002b</td>
<td>38</td>
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<tr>
<td>ω-Conotoxin GVIA</td>
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<td>0.57 ± 0.015</td>
<td>0.014 ± 0.003b</td>
<td>132</td>
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<tr>
<td>ω-Agatoxin IVA</td>
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<td>0.64 ± 0.035*</td>
<td>0.209 ± 0.023c</td>
<td>53</td>
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</tbody>
</table>

Fura-2 ratio before and after administration of GEA 3162 (10⁻⁴ mol·l⁻¹), and difference between both (Δ fura-2 ratio) in the absence of any inhibitors, in the absence of extracellular Ca²⁺, and in the presence of Ni²⁺ (10⁻³ mol·l⁻¹), nifedipine (10⁻⁶ mol·l⁻¹), verapamil (10⁻⁵ mol·l⁻¹), ω-conotoxin GVIA (10⁻⁶ mol·l⁻¹), or ω-agatoxin IVA (10⁻⁷ mol·l⁻¹). Values are means ± SEM, n = number of cells tested. * p < 0.05 versus baseline before administration of GEA 3162. For the Δ fura-2 data, homogenous groups are indicated by identical letters (analysis of variances followed by test of Scheffé).