Interstitial cells of Cajal mediate nitrergic inhibitory neurotransmission in the murine gastrointestinal tract

Barbara Lies, Víctor Gil, Dieter Groveberg, Barbara Seidler, Dieter Saur, Erhard Wischmeyer, Marcel Jiménez, and Andreas Friebe


Gastrointestinal (GI) motility disorders such as gastroparesis, chronic constipation, or inflammatory bowel disease are often associated with a loss of interstitial cells of Cajal (ICC) (7, 25, 33). Animal models to study these illnesses and the underlying disorders in signaling are scarce. Up until now, W/Wv or Sl/Sld mutant mice (10, 21), detailed elimination of the cells challenge the interpretability of the results obtained from W/Wv or SI/SI mutant mice.

Electrophysiological studies have shown nitrergic and purinergic signaling to be the main inhibitory neurotransmission pathways in most GI tissues. For murine and human colon, it has been demonstrated adequately that postjunctional responses to enteric inhibitory neurotransmission are mediated by a two-component inhibitory junction potential (IJP) (11, 23, 29, 34). Pharmacological experiments revealed the first component to be dependent on activation of small-conductance, Ca$^{2+}$-activated K$^{+}$ channels and to be induced by purines (2, 10, 21, 35). The second component is blocked by inhibitors of nitric oxide synthase (NOS) and is therefore regarded as nitrergic (5, 23, 31). This characteristic biphasic IJP has been shown for several other regions of the GI tract of different species as well, such as antrum (15), small intestine (12), and internal anal sphincter (6, 30).

Although inhibitory purinergic signaling has been investigated recently using specific inhibitors for purinergic receptors and P2Y1 receptor knockout (KO) mice (10, 21), detailed investigation of the nitrergic signaling cascade is lacking. Its elucidation has been hampered by the fact that NO-sensitive guanylyl cyclase (NO-GC) is expressed in at least three different cell types, such as smooth muscle cells (SMC), ICC, and also fibroblast-like cells (FLC). The interplay between neurons and the different cell types leading to the formation of nitrergic postjunctional responses has not yet been clearly assessed.

Models we use are mice lacking NO-GC globally (GCKO) or specifically in SMC (SM-GCKO), ICC (ICC-GCKO), or both cell types (SM/ICC-GCKO). The use of these KO mice gives the opportunity to investigate nitrergic neurotransmission without lack of a whole cell type and thus to maintain the cellular assemblage intact in GI tissue. Previous studies in our group have shown that NO-induced relaxation of fundus tissue is mediated via a dual pathway: nitrergic smooth muscle relaxation can occur via NO-GC in SMC or ICC; only the deletion of NO-GC in both cell types abolishes NO-mediated smooth muscle relaxation (18).

The aim of this study was to gain a better insight into GI nitrergic neurotransmission by complementing our previous mechanical recordings with electrophysiological measurements. In a first approach, we used murine fundus tissue, based on its mainly nitrergic inhibitory neurotransmission seen under our conditions; furthermore, the electrophysiological responses of murine fundus resemble those observed in human esophagus and lower esophageal sphincter (28). Second, colon tissue showing purinergic and...
nitrergic cotransmission was used for the study of more complex neuroeffector interactions.

In summary, we demonstrate that NO-GC in both SMC and ICC participates in maintenance of resting membrane potential (RMP), suggesting a regulatory contribution of basal NO release. NO-GC in both SMC and ICC is necessary for the generation of a complete nitrergic IJP. Specifically in fundus, NO-GC in ICC is mandatory for the generation of nitrergic IJPs, whereas in the colon, the nitrergic IJP is carried by NO-GC in ICC and SMC and consists of two phases: a fast ICC-mediated and a slower SMC-mediated nitrergic hyperpolarization. NO-GC in SMC participates in strengthening/maintenance of the nitrergic hyperpolarization, resulting in a stronger IJP in fundus SMC and a prolonged IJP in colon SMC.

MATERIALS AND METHODS

All experiments were conducted in accordance with the German legislation on the protection of animals and were approved by the local Animal Care Committee.

Animals

Mice (C57BL/6 background) were housed in standard mouse cages (267 × 207 × 140 mm; maximally three animals/cage) with woodchip bedding material and under conventional laboratory conditions (constant room temperature, 22°C; humidity level, 55%; 12-h light: 12-h dark cycle (lights on at 6 AM), either a standard rodent diet or a fiber-reduced diet (Altromin, Lage, Germany), and water available ad libitum). Animals of either sex were killed at the age of 8–16 wk by isoflurane overdose, and tissues were isolated. A total of 55 animals was used.

Generation of SM-GCKO, ICC-GCKO, and SM/ICC-GCKO Mice

SM-GCKO and ICC-GCKO mice carry a floxed exon (exon 10 of the β1 subunit of NO-GC) (8) and are transgenic for the inducible Cre recombinase in SMC (smooth muscle myosin heavy chain-CreERT²) (39) and ICC (cKIT-CreERT²) (24). SM/ICC-GCKO mice were generated by crossing SM-GCKO with ICC-GCKO mice. Mice from all three KO lines, aged 6–8 wk, were injected with tamoxifen (dissolved in Miglyol 812; 1 mg ip) on 5 consecutive days to remove all three KO lines, aged 6–8 wk, were injected with tamoxifen generated by crossing SM-GCKO with ICC-GCKO mice. Mice from (39) and ICC (cKIT-CreERT²) (24). SM/ICC-GCKO mice were

Intracellular Microelectrode Recordings

Animals were killed by isoflurane overdose. The abdomen was opened, and the fundus and colon were removed quickly and transferred to Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, pH 7.4, 7.5 mM glucose), bubbled with 95% O₂/5% CO₂. The fundus was opened by a short, perpendicular cut above the lower esophagus sphincter, allowing a plane fixation to a Sylgard base. Colon tissue was opened along the mesenteric border and likewise fixed to a Sylgard base. Mucosal and submucosal layers from both tissues were removed meticulously. Fundus and mid-colon muscle strips (8 mm × 4 mm) were then pinned with the circular smooth muscle layer facing upward in Sylgard-coated chambers and perfused continuously (3–4 ml/min) with carbogenated Krebs-Henseleit solution at 37 ± 1°C. The tissues were allowed to equilibrate for at least 1 h. Circular SMC were impaled with glass microelectrodes (≥25 MΩ) filled with KCl (3 M). Membrane potential was recorded using a standard electrometer (Duo 773; World Precision Instruments, Sarasota, FL). Tracings were displayed on a 40-MHz digital storage oscilloscope (Uni-T, Kwun Tong, Hong Kong, China) and digitalized (1,000 Hz) with a PowerLab 4/25 system and Chart 7.3.1 software for Windows (ADInstruments, Castle Hill, NSW, Australia). To sustain stable impalement, all experiments were carried out in the presence of nifedipine (1 μM). IJPs were elicited by electrical field stimulation (EFS) with train stimuli of 1 s at 90 V, a pulse duration of 0.9 ms, and a frequency of 10 Hz. This stimulation setting was used, since trains of lower frequencies have been shown to elicit mainly purinergic responses, whereas higher frequencies result in a more pronounced nitrergic response (9). Responses to EFS were abolished by TTX (1 μM). The SD of a stable, 30-s tracing in SMC was calculated to analyze electrical activity. Nitrergic IJP amplitude was measured and expressed in millivolts. Nitrergic IJP duration was determined in the presence of the P2Y₁ inhibitor MRS 2500 (1 μM) as the time from the start of the hyperpolarization to the point of RMP recovery. All experiments were conducted in the presence of nifedipine (1 μM). Nonadrenergic, noncholinergic (NANC) conditions were applied using atropine, phentolamine, and propranolol (1 μM, respectively), except for in-
vestigation of GCKO and ICC-GCKO fundus. Here, atropine was omitted to allow for depolarization with carbachol.

Materials

Nifedipine was purchased from Axxora (Lörrach, Germany). Atropine, phentolamine, propranolol, carbachol, and tamoxifen were from Sigma (Taufkirchen, Germany). MRS 2500 was purchased from Tocris (Wiesbaden-Nordenstadt, Germany), and L-N^G^-nitroarginine methyl ester (L-NAME) was purchased from Alexis (Lausen, Switzerland).

Statistical Analysis

For calculation of statistical tests, GraphPad Prism was used. For comparison of independent variables, GCKO, SM-GCKO, ICC-GCKO, and wild-type (WT) were compared by Kruskal-Wallis test. If \( P < 0.05 \) for the global test, then GCKO, SM-GCKO, and ICC-GCKO were each compared with WT by Mann-Whitney \( U \)-test (see Figs. 2 and 5). Comparisons of individual groups were only reported if global tests reached significance. Data are expressed as mean ± SE.

RESULTS

Fundus

Intracellular recordings in SMC. Intracellular microelectrode measurements were made in circular SMC of the gastric fundus. Figure 1 shows representative, original traces with unitary potentials in SMC from WT and SM- and ICC-GCKO, as well as global GCKO. Recordings were performed in the presence of the \( \text{Ca}^{2+} \) channel blocker nifedipine (1 \( \mu \)M), suggesting that unitary potentials do not depend on L-type calcium channels. The SD of stable, 30-s tracings did not differ significantly between WT and KO tissues. Similar values have been published for murine gastric antrum (36). Thus we conclude that NO-GC deletion does not impair electrical activity.

IJPs in NO-GC-deficient mice. EFS (90 V, 0.9 ms, 10 Hz) of smooth muscle was conducted under NANC conditions. In circular SMC from WT animals, EFS caused a hyperpolarization, which was abolished by L-NAME (200 \( \mu \)M), suggesting an exclusively nitrergic origin under the chosen conditions.

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Fig. 2. A: representative inhibitory junction potential (IJP) in SMC from WT fundus after electrical field stimulation (EFS; 90 V, 0.9 ms, 10 Hz). Addition of L-N^G^-nitroarginine methyl ester (L-NAME; 200 \( \mu \)M) completely abolished the IJP. B: representative membrane potential recordings from SM-GCKO, ICC-GCKO, and global GCKO after EFS (90 V, 0.9 ms, 10 Hz). IJP is abolished by deletion of NO-GC in ICC. C: statistical analysis of IJP in WT (23 cells from 7 animals), SM-GCKO (12 cells from 6 animals), ICC-GCKO (15 cells from 5 animals), and global GCKO (13 cells from 5 animals) after EFS (90 V, 0.9 ms, 10 Hz). *** \( P < 0.001 \) for SM-GCKO, ICC-GCKO, and GCKO vs. WT by Mann-Whitney \( U \)-test.
(Fig. 2A). Thus this stimulation protocol was optimal in our setup for the investigation of nitrergic neurotransmission in fundus, as a purinergic contribution was not observed. The EFS-induced IJP had an average amplitude of 15.9 ± 1.4 mV (Fig. 2C).

In contrast, EFS did not evoke a postjunctional inhibitory response in fundus from GCKO mice, supporting the complete deletion of NO-GC in GCKO animals (Fig. 2, B and C). The EFS-induced, postjunctional hyperpolarization was also abolished in fundus from ICC-GCKO, implying a major role of NO-GC in ICC for the generation of nitrergic inhibitory responses. The IJP in SM-GCKO tissue was preserved, although with a significantly reduced amplitude of ~3.8 ± 0.7 mV. Thus NO-GC in ICC is mandatory to initiate the nitrergic IJP, and the presence of NO-GC in SMC is necessary for the generation of a fully developed nitrergic IJP.

Colon

**Intracellular recordings in SMC.** To test nitrergic neurotransmission in another GI region, intracellular recordings were also conducted in circular SMC from mid-colon. As in fundus, unitary potentials were observed in the colon from WT and KO strains (Fig. 3). In addition, spontaneous IJPs occurred in all tissues. SD values of unitary potentials did not differ between WT and KO tissues, suggesting electrical activity to be similar.

**IJPs in WT.** For measurement of postjunctional inhibitory responses in colon-circular SMC, EFS was conducted under the conditions used for fundus (90 V, 0.9 ms, 10 Hz). In the colon, this stimulation protocol is also applicable as the nitrergic effect increases with longer pulses/higher frequencies (9, 12). EFS induced an apparently biphasic, postjunctional inhibitory response in SMC from WT animals, which consisted of a fast and a slow phase of hyperpolarization (Fig. 4, A and B). Addition of the specific P2Y1 receptor inhibitor MRS 2500 (1 μM) blocked the fast phase, showing its purinergic origin. The slow phase was abolished by the NOS inhibitor L-NAME (200 μM), which confirms this phase to derive from nitrergic neurotransmission.

**Nitrergic IJP in SMC from NO-GC-deficient mice.** Figure 5A shows representative tracings from colons of SM- and ICC-GCKO, as well as global GCKO animals. The nitrergic IJP in the WT colon was 5.3 ± 0.4 mV in amplitude and 5.0 ± 0.1 s in duration (Fig. 5, A–C). In the colon from GCKO animals, the nitrergic IJP was abolished completely (Fig. 5, A–C). This is in line with the results obtained from GCKO fundus (see Fig. 2B) and the experiments using L-NAME (see Fig. 4, A and B).

Much in contrast to fundus, nitrergic IJP in the colon from both SM- and ICC-GCKO was still measurable, albeit different from that seen in WT: the amplitude of nitrergic IJP in SM-GCKO was 5.2 ± 0.3 mV (Fig. 5B) and thus similar to that obtained in WT; however, nitrergic IJP duration was significantly shorter (3.1 ± 0.1 s; Fig. 5C). Vice versa, nitrergic IJP duration in colon from ICC-GCKO was as long as in WT (5.4 ± 0.1 s; Fig. 5C) but exhibited a strongly reduced amplitude (1.3 ± 0.1 mV; Fig. 5B). Thus the IJP in murine colon is actually three phasic. Figure 5D shows a model of the EFS-induced IJP with an initial, fast purinergic hyperpolarization (I), followed by a slow nitrergic hyperpolarization that can be subdivided into an ICC-mediated (IIa) phase, followed by an SMC-mediated (IIb) phase.

To test for a possible interference by purinergic signaling, we repeated the IJP measurements in the presence of MRS 2500 (1 μM). Figure 6A shows the isolated nitrergic IJP from the WT, SM-GCKO, and ICC-GCKO colon. The merge of the three original traces (Fig. 6B) clearly underscores the biphasic nature of the nitrergic signal to be independent from purinergic influence.

Taken together, the results from fundus and colon suggest that initiation of the nitrergic IJP is dependent on NO-GC in ICC. NO-GC in SMC, however, seems to reinforce/maintain the nitrergic response, resulting in a stronger IJP in fundus and a prolonged nitrergic IJP in colon.

**DISCUSSION**

Purinergic and nitrergic signaling is the main pathway for GI inhibitory neurotransmission. Recently, purinergic postjunctional responses have been investigated using specific inhibitors for purinergic receptors and P2Y1 receptor KO mice (10, 21). These data suggest that FLC (also known as PDGF receptor α-positive cells) are capable of transducing purinergic inputs and consequently, are candidates for the integration of
nerve-mediated purinergic responses (1). Up until now, however, the precise mechanism of nitrergic signaling leading to hyperpolarization of SMC has not been fully elucidated. The fact that NO-GC, the main effector for NO, is expressed in at least three GI cell types complicates the analysis of nitrergic signaling.

Here, we studied nitrergic hyperpolarization in GI smooth muscle tissue using a tamoxifen-inducible, cell-specific KO strategy. The estrogen receptor antagonist tamoxifen has been shown to induce GI side effects when given at high doses (e.g., 3 mg/20 g body wt); these include a rapid atrophy of the gastric epithelium (19), which reversed after 3 wk. To avoid these potentially interfering effects, we investigated tamoxifen-treated animals (1 mg/day on 5 consecutive days) at least 50 days after the last tamoxifen injection. We have not observed any obvious pathological changes in GI epithelium at this time point.

We are well aware of the fact that Cre-mediated exon deletion is hardly ever quantitative and in fact, a “knockout” much rather resembles a “knockdown.” As it is common to refer to Cre-dependent genetic models as KO models, however, we will use this term throughout our manuscript.

Global and cell-specific deletion of NO-GC did not alter electrical activity of SMC in fundus and colon. Furthermore, spontaneous IJPs occurred in the colons of all genotypes. These findings are in line with previous pharmacological studies showing that spontaneous IJPs are regulated by purinergic signaling (13).

In the absence of NO-GC, EFS-induced nitrergic IJPs were abolished in both fundus and colon. This clearly demonstrates that neuronally released NO induces IJPs only through the NO-GC, ruling out other targets. The individual role of NO-GC in ICC and SMC regarding nitrergic signaling, however, appears to depend on the GI region.

In fundus, nitrergic IJPs were abolished by ICC-specific deletion of NO-GC. Thus NO-GC in ICC is mandatory for the generation of nitrergic IJPs. Absence of NO-GC in SMC, though, did not impede the generation of a nitrergic IJP but greatly reduced its amplitude. Taken together, these data indicate that NO initiates the nitrergic hyperpolarization in ICC, while it enhances the ICC-induced hyperpolarization by activation of NO-GC in SMC.

In the colon, EFS has been described to evoke a biphasic IJP consisting of a purinergic and a nitrergic phase. MRS 2500, a P2Y1 receptor antagonist, was able to block quantitatively the purinergic phase in all KO animals (see Fig. 6). A possible qualitative change in purinergic signaling as a consequence of NO-GC deletion was not obvious; this topic will be the goal of future detailed studies. With regards to nitrergic signaling, we found that deletion of NO-GC in ICC significantly reduced the nitrergic IJP amplitude, whereas NO-GC KO in SMC led to a shorter IJP duration (see Fig. 5). Thus the nitrergic IJP can be divided into two phases: a fast ICC-mediated and a slower SMC-mediated phase. Our data from the colon indicate NO-GC-mediated effects to be mutually reinforcing in ICC and SMC; only with NO-GC present in both cell types could a fully pronounced

![Diagram](http://ajpgi.physiology.org/doi/10.220.33.2 on April 16, 2017)
nitrergic IJP be observed. Consequently, EFS of the colon actually leads to a triphasic IJP consisting of a fast purinergic and a slow nitrergic component that can be subdivided further into an ICC-mediated and a SMC-mediated phase. The more immediate EFS response of ICC (relative to SMC) may well be structurally based on the close apposition of ICC and nitrergic nerves (22, 37). Combined, these results emphasize the primary role of ICC as mediators of nitrergic neurotransmission.

Fig. 5. A: representative microelectrode recordings from the WT, SM-GCKO, ICC-GCKO, and global GCKO colon after EFS (90 V, 0.9 ms, 10 Hz). B: statistical analysis of nitrergic IJP amplitude in WT (12 cells from 5 animals), SM-GCKO (21 cells from 6 animals), and ICC-GCKO (13 cells from 6 animals). In the global GCKO, a nitrergic IJP was not measurable; on the contrary, the purinergic IJP was followed by a slight depolarization (10 cells from 5 animals). Significance was reached for ICC-GCKO and GCKO vs. WT by Mann-Whitney U-test (***(P < 0.001) but not for SM-GCKO vs. WT (P = 1.0; not significant (n.s.)); C: statistical analysis of nitrergic IJP duration in WT (17 cells from 7 animals), SM-GCKO (15 cells from 7 animals), and ICC-GCKO (16 cells from 7 animals). As a nitrergic IJP was not detectable (n.d.) in the global GCKO, the duration was not assessed (10 cells from 5 animals). Significance was reached for SM-GCKO vs. WT by Mann-Whitney U-test (***(P < 0.001) but not for ICC-GCKO vs. WT (P = 0.22; n.s.)); D: model of colonic IJP showing the sequence of fast purinergic (I) and slow nitrergic ICC-mediated (IIa) and SMC-mediated (IIb) hyperpolarizations.
Studies investigating the downstream targets of cGMP revealed the involvement of cGMP-dependent protein kinase I (PKG), but the identity of further downstream mediators is uncertain (14, 26, 32). In a recent publication, Klein et al. (24) proposed PKG to be the exclusive mediator of the nitrergic IJP in colonic ICC, based on an apparently abolished nitrergic hyperpolarization in ICC-specific PKG KO mice. These findings appear to be in contrast to our data, since our ICC-specific NO-GC-KO still showed a nitrergic hyperpolarization (see Fig. 5A). However, comparison of these findings is difficult to carry out, as the protocol by Klein et al. (24) used single-pulse stimulation (which does not allow for the summatory development of a clear NO signal), whereas this study used a repetitive stimulation to achieve a prominent nitrergic signal. Nonetheless, close examination of the data from Klein et al. (24) (their Figs. 4g and S5g) reveals a remaining nitrergic hyperpolarization corresponding to the slow, second SMC-mediated nitrergic IJP. This notion is supported in their own data (their Fig. S5g) by the fact that the small remaining nitrergic hyperpolarization was abrogated by addition of L-NG-nitroarginine. In conclusion, the data by Klein et al. (24) are in accordance with ours and support our concept of a biphasic nitrergic IJP in colon SMC.

To verify if SMC and ICC from the colon are the only determinants of nitrergic neurotransmission, we used double-SM/ICC-GCKO animals. Previous experiments showed an abrogated nitrergic relaxation in these animals, leading to an increased gut transit time (18). For that reason, we expected the nitrergic IJP to be abolished in the SM/ICC-GCKO colon. Yet, nitrergic IJP was abolished in some cells, whereas other cells of the same tissue exhibited a reduced nitrergic IJP similar to that in ICC-GCKO (not shown). Therefore, experiments investigating single-cell function (IJP) and tissue functions (relaxation and motility) produce divergent results with regard to the role of nitrergic transmission via ICC. This divergence may be based on the nonquantitative deletion of NO-GC in these cells: as shown previously, NO-GC is deleted effectively in >75% of ICC and >90% of SMC in fundus tissue by the cKIT-Cre recombinase (17, 18). The remaining few NO-GC-positive cells may only have a minor effect on a tissue response (relaxation or gut motility) that results from the integration of the signals from different cell types in GI smooth muscle. However, single-cell functions, such as the electrical behavior of a SMC, are likely to be influenced profoundly by residual NO-GC expression in either cell type.

In summary, we demonstrate that neuronally released NO targets solely NO-GC to mediate IJPs in murine fundus and colon. Furthermore, we were able to dissect the individual roles of NO-GC in ICC and SMC for nitrergic neurotransmission. In fundus, NO-GC in ICC is mandatory for the generation of the IJP, whereas NO-GC in SMC modulates its amplitude. In the colon, we have shown nitrergic hyperpolarization to be biphasic: a fast ICC-mediated and a slower SMC-mediated phase. Conceivably, NO-GC in ICC is responsible for initiation of the
nitrigic IJP, whereas NO-GC in SMC is important for its maintenance.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

B.L. and A.F. conception and design of research; B.L. performed experiments; B.L. and A.F. analyzed data; B.L. and A.F. interpreted results of experiments; B.L. and A.F. prepared figures; B.L. and A.F. drafted manuscript; B.L., V.G., D.G., B.S., D.S., E.W., M.J., and A.F. edited and revised manuscript; B.L., V.G., D.G., B.S., D.S., E.W., M.J., and A.F. approved final version of manuscript.

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