Colonic Motility

Interstitial cells of Cajal are involved in the afferent limb of the rectoanal inhibitory reflex

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Background and aims: Interstitial cells of Cajal (ICC) have been shown to be involved in nitrergic neurotransmission of the lower oesophageal sphincter and pylorus. Here we studied the role of ICC and nitric oxide (NO) in the inhibitory neurotransmission of the murine internal anal sphincter (IAS).

Methods: The rectoanal inhibitory reflex, rectal compliance, and relaxation of the isolated IAS to electrical stimulation were measured in controls, KIT W+/KIT Wv mice, and neuronal NO synthase (nNOS) deficient mice. In addition, we evaluated the effect of blockade of nNOS using N-nitro-L-arginine methyl ester. Distribution of nNOS positive neurones and ICC in the IAS was assessed immunohistochemically.

Results: KIT positive ICC were present in a dense network in the IAS of controls but not in KIT W/KIT Wv mice. Relaxation of IAS muscle strips induced by electrical stimulation was diminished in nNOS −/− mice but not in KIT W+/KIT Wv mice. Blockade of NOS reduced the relaxation of IAS muscle strips in both mice. Relaxation of the IAS to rectal distension was significantly diminished in KIT W/KIT Wv mice and nNOS deficient mice. In concert, in vivo blockade of NOS attenuated the relaxation of the IAS in controls. No significant difference in compliance was found.

Conclusion: The inhibitory innervation of the IAS and the rectoanal inhibitory reflex are mediated by NO and the rectoanal inhibitory reflex requires an intact network of ICC in the IAS. Thus both loss of nitrergic innervation and deficiency of ICC lead to impaired anal relaxation and may play an important role in rectal evacuation disorders.

Materials and Methods

Animals

Adult KIT W+/KIT Wv mice (20–30 g) and their wild-type (KIT W/+ ) controls, as well as neuronal nitric oxide synthase (nNOS) deficient mice (18–28 g) and their respective wild-type controls (site bred C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). For immunohistochemical studies, transgenic mice were used that carry the KIT W−/− allele (“lacZ positive controls”). KIT W−/−/KIT Wv lack ICC, similar to the more extensively studied KIT W+/KIT Wv animals, and will be referred to as “ICC deficient animals”.

All animals were maintained under controlled conditions and were used at 8–12 weeks of age. Experiments were approved by the Ethical Animal Research Committee of the University of Amsterdam, the Netherlands, and by the Faculty Committee for Use of Laboratory Animals of the Faculté de Médecine, Université Libre de Bruxelles, Belgium.

Immunohistochemistry

Fresh frozen anorectal specimens of control and KIT W+/KIT Wv mice were harvested for longitudinal cryosections, while paraformaldehyde fixed specimens of control (KIT W+/lacZ ) and KIT W−/−/KIT Wv mice were harvested for transverse and longitudinal cryosections as well as for whole mount preparations, as described previously.

Whole mounts: preparation and immunohistochemistry

After preincubation with 10% normal mouse serum, samples were incubated overnight with the KIT goat and the nNOS rabbit antisera (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted in Tris buffered saline-Trion X (TBS-TX) containing 1% normal horse serum (NHS). Samples were then rinsed in TBS, incubated in the dark for one hour at room temperature in TBS containing donkey antirabbit antiserum coupled to FITC (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) and biotinylated donkey antigoat antiserum (Jackson).

Abbreviations: IAS, internal anal sphincter; ICC, interstitial cells of Cajal; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; RAIR, rectoanal inhibitory reflex; L-NAME, N-nitro-L-arginine methyl ester; LOS, lower oesophageal sphincter; TBS-TX, Tris buffered saline-Trion X; NHS, normal horse serum; NANC, non-adrenergic non-cholinergic; SNP, sodium nitroprusside; ATP, 5′-adenosine triphosphate

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Normal defecation depends on the complex interplay between colonic motility, rectal sensation, and anal sphincter function. When stool arrives in the rectum, filling of the rectum leads to stretch of the rectal wall with subsequent triggering of a transient relaxation of the internal anal sphincter (IAS), also called the rectoanal inhibitory reflex (RAIR). Abnormalities in the RAIR have been shown to be involved in defecation disorders and severe constipation, such as in Hirschsprung’s disease. The RAIR is a motor pattern mediated by intrinsic enteric nerves. Previous animal studies have shown that the inhibitory neurones relaxing the IAS mainly release nitric oxide (NO). Blockade of NO biosynthesis reduced the relaxation of muscle strips of the IAS and impaired the RAIR. Recently, evidence was provided that the nitrergic innervation is dependent on the presence of an intact network of interstitial cells of Cajal (ICC). ICC function as pacemaker cells coordinating the electromechanical activity of the gut. In addition, ICC located in the muscular layer have been shown to mediate nitrergic neurotransmission in the stomach and lower oesophageal sphincter (LOS). To what extent ICC are also involved in the nitrergic innervation of the IAS is unknown. Therefore, the present study was designed to evaluate the role of ICC in the nitrergic innervation of the IAS and in the triggering of the RAIR.
diluted 1/200 and 1/100 (v/v), respectively, rinsed in TBS, and finally incubated in the dark for one hour at room temperature with streptavidin Texas red (Jackson) diluted 1/400 (v/v) in TBS. Specimens were then transferred onto a glass slide coated with 0.1% poly-L-lysine and were cut into two equal parts. Coverslips were mounted with “Slow Fade Light” antifade mounting medium (Molecular Probes, Eugene, Oregon, USA) before viewing on a confocal microscope (MRC 1024; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) fitted on an inverted microscope equipped with a Plan-Neofluar 40x/1.3 oil immersion objective (Axiovert 100; Zeiss, Oberkochen, Germany). As KIT-ir appeared fairly weak in ICC deficient animals with the fluorescent detection system, the more sensitive detection system using peroxidase was additionally applied.

Sections: preparation and immunohistochemistry
Specimens were fixed overnight in fresh 4% paraformaldehyde solution in phosphate buffered saline, pH 7.4, at 4°C, cryopreserved in graded solutions of sucrose (10–30%), embedded in Tissue-Tek OCT compound (Miles, Elkhart, Indiana, USA), oriented either transversely or longitudinally, and snap frozen in 2-methyl butane at −80°C. Sections (15 mm thick) were cut on a cryostat and mounted on slides coated with 0.1% poly-L-lysine.

After preincubation with NHS, sections were exposed overnight at room temperature to KIT goat antiserum (Santa Cruz Biotechnology) diluted 1/500 in TBS containing biotinylated donkey anti-goat antiserum (Jackson) diluted 1/200 (v/v), followed by incubation for one hour at room temperature in a solution containing avidin-biotin complex (ABC Vectastain, Vector, Burlingame, California, USA). The immunoreaction was visualised with the chromogen 3,3′-diaminobenzidine (Dako, Denmark).

xgal histochemistry on KIT W−/−, KIT W+/+ and KIT W−/−, KIT W+/+ transgenic mice was performed as previously reported.10

In vitro studies
Organ bath experiments
KIT W+/+ W−/−, mice, nNOS−/−, and control mice were killed by cervical dislocation. The perianal skin was excised and the anus and terminal rectum were quickly removed and placed on a ring in Krebs-Ringer solution (KRS) (mmol/l: NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O2 and 5% CO2. A ring segment comprising 2 mm of the terminal rectum, including the anal orifice, was removed and opened. One circular muscle strip was cut and mounted in an organ bath (25 ml) filled with Krebs-Ringer’s solution. Each muscle strip was connected to a metal rod and attached to a strain gauge system using peroxidase was additionally applied.

In a second series, the effects of N-nitro-L-arginine methyl ester (L-NAME 200 μmol/l), the blocker of neuronal conduction tetrodotoxin (2.0 μmol/l), the blocker of P2 purinergic receptors suramin (100 μmol/l), and the combination of L-NAME and suramin were investigated on the responses to electrical impulses, to ATP (1.0 μmol/l), and to SNP (2.5 mmol/l). L-NAME and suramin were added at least 20 minutes before the experiments.

In vivo studies
Anorectal manometry
Anorectal perfusion manometry was performed with a purpose built micromanometric anorectal catheter (outer diameter 0.8 mm). The polyimide catheter incorporated an array of four side holes spaced 0.5 mm apart for measurement of anal sphincter pressure and basal pressure within the rectum. All side holes were perfused with sterile degassed water at a rate of 0.02 ml/min. A polyethylene balloon was located proximally to the catheter to distend the rectum.

Protocol
Anorectal manometry was performed in anaesthetised mice. Mice were anaesthetised by intraperitoneal injection of a mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen Pharmaceuticals, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, the Netherlands) at 0.07 ml/10 g (fentanyl 0.32 mg/ml, fluanisone 10 mg/ml, midazolam 5 mg/ml). The catheter was positioned with at least two side holes straddling the anal sphincter high pressure zone and one side hole in the rectum. After positioning, basal anal sphincter pressure was recorded. Thereafter a polyethylene rectal balloon was introduced and positioned aside the manometric catheter. After an equilibration time of 15 minutes, graded rectal distensions of 10 seconds were applied using the polyethylene balloon (0.25–0.40 ml air insufflation) to evoke IAS relaxations. Consecutive rectal distensions were performed at one minute intervals and distensions were repeated three times at each volume. This distension protocol was repeated after addition of L-NAME (100 mg/kg intraperitoneally).16

In a separate series of experiments, rectal compliance was measured with a non-compliant (polyethylene) balloon with a maximum capacity of 0.45 ml. The balloon was fixed on a silicone catheter with a diameter of 1 mm. This catheter was connected to a pressure transducer measuring intraballoon pressure during stepwise volume controlled distension (0.04–0.40 ml).

Data analysis
Basal IAS pressures were obtained from the baseline period preceding the distension protocol. A drop in anal sphincter pressure of at least 5 mm Hg, over 2–5 seconds, was identified as a RAIR. If rectal distension resulted in a RAIR, the amplitude of each relaxation was determined as the percentage decrease in basal pressure. This was calculated from the mean anal sphincter pressure before distension to the minimal anal sphincter pressure during the reflex. Duration of the reflex was measured from the onset of the relaxation until the pressure returned to its basal level.

Chemicals
All chemicals were from Sigma (St. Louis, Missouri, USA), unless otherwise stated. The following drugs were used: ATP, L-NAME, suramin, tetrodotoxin, and SNP. All chemicals were dissolved in distilled water and diluted in Krebs-Ringer solution to the stated final concentration.
Statistical analysis
Group data are expressed as mean (SEM). Differences in data were evaluated using the Mann-Whitney U test or the Wilcoxon signed ranks test were indicated. A p value of <0.05 was considered statistically significant. The n values reported refer to the number of animals used in each protocol.

RESULTS
Immunohistochemistry
nNOS-ir in the region of the IAS
In both control (KIT W-lacZ/+ (not shown) and KIT W-lacZ/KIT Wv mice (fig 1A, B), a dense network of nNOS-ir nerve fibres was present in the circular and longitudinal muscle layers of the distal rectum, including the IAS. Within the intermuscular plane of the IAS, both nNOS-ir neurons and nerve bundles were present but myenteric ganglia contained fewer neurones and the nerve bundles were coarser when compared with the distribution in the proximal rectum (not shown). Conversely, the density of nNOS-ir nerve fibres and neurones of the

Figure 1  Neuronal nitric oxide synthase (nNOS)-ir distribution in the anorectum of a KIT W-lacZ/KIT Wv interstitial cells of Cajal deficient mouse. (A) An 8 μm thick projection of the myenteric region, showing a few nNOS-ir myenteric neurones and numerous large nerve bundles. (B) A 16 μm thick projection of the circular muscle layer and submucosal plexus, showing abundant nNOS-ir nerve fibres in the circular muscle layer and submucosal nNOS-ir fibres and neurones. Orientation: oral, left; aboral, right. Scale bars 50 μm.

Figure 2  Distribution of KIT-ir interstitial cells of Cajal (ICC) in the internal anal sphincter (IAS) region of control KIT W-lacZ/+ and ICC deficient KIT W-lacZ/KIT Wv mice. (A, B) Longitudinal sections across the IAS region of a control KIT W-lacZ/+ mouse (A) and an ICC deficient KIT W-lacZ/KIT Wv mouse (B). The arrows in the lumen indicate mucocutaneous transition. The various populations of KIT-ir ICC are abundant in the control (A) while KIT-ir ICC are essentially lacking in ICC deficient animals (B). (C, D) Whole mount preparations showing KIT-ir ICC in the submuscular plexus of a control KIT W-lacZ/+ mouse (C) and an ICC deficient KIT W-lacZ/KIT Wv mouse (D). KIT-ir ICC form chains running parallel to the circular muscle layer and interconnected by multiple processes in the submuscular plexus of the control KIT W-lacZ/+ mouse (C) while in the submuscular plexus of an ICC deficient KIT W-lacZ/KIT Wv mouse (D) chains of KIT-ir ICC appeared only faintly stained (arrowheads) and lack most longitudinal interconnecting processes. Orientation in (C, D): oral at left, aboral at right. CM, circular muscle layer; LM, longitudinal muscle layer; PF, striated muscle of the pelvic floor. Scale bars 50 μm.

Figure 3  Representative recordings showing the non-adrenergic non-cholinergic inhibitory responses to electrical impulses (0.25–8.0 Hz, 1 ms, pulse trains lasting 10 seconds), S'-adenosine triphosphate (ATP 1.0 mM), and sodium nitroprusside (SNP 2.5 mM) in muscle strips of a KIT W/+ control (A), KIT W/KIT Wv (B), neuronal nitric oxide synthase (nNOS) control (C), and nNOS-/- mouse (D).
submucosal plexus within the region of the IAS was similar to the proximal rectum.

KIT in the region of the IAS
In controls (fig 2A), spindle shaped KIT-ir ICC were present in both muscle layers, parallel to smooth muscle cells. Stellate KIT-ir ICC were abundant surrounding the myenteric ganglia. At the level of the submucosal plexus, KIT-ir ICC formed chains parallel to the circular muscle layer. These chains were connected to each other by numerous delicate longitudinally processes. ICC at the submucosal plexus exhibited a weaker KIT-ir compared with the other populations of KIT-ir ICC and were best viewed on whole mounts (fig 2C). In the jejunum of the same animals, KIT-ir was detected in ICC-MP and ICC-DMP, as expected (not shown).

In ICC deficient animals mice, KIT-ir ICC were lacking in the muscle layers and around the myenteric ganglia (fig 2B), while faint KIT-ir ICC at the level of the submucosal plexus were still present (fig 2D). However, they were less abundant and apparently lacked the normal branching pattern (compare with fig 2C). In the jejunum of these animals, a few ICC-DMP exhibited weak KIT-ir, as expected in these ICC deficient animals (not shown). Due to the extreme lack of KIT expressing ICC in the region of the IAS of ICC deficient animals, no quantification was attempted.

Xgal histochemistry in KIT W-lacZ/KIT Wv
While Xgal histochemistry readily detected nuclei of KIT expressing ICC-DMP in the jejunum, no Xgal deposit was observed in the proximal rectum, or in the region of the IAS (not shown).

Organ bath studies
Involvement of NO in the inhibitory innervation of the IAS
In the presence of atropine and guanethidine, electrical stimulation (0.25–8.0 Hz, 1 ms, pulse trains lasting 10 seconds) induced a frequency dependent relaxation of circular muscle strips of the murine IAS during the period of stimulation followed by an off contraction (fig 3A, C). Maximal relaxation of KIT W/+ controls was obtained at 4 Hz (0.16 (0.05) g) (fig 4A) and in nNOS controls at 2 Hz (0.32 (0.14) g) (fig 4B). The resting (active) tone of the muscle strips of KIT W/+ controls (0.5 g), KIT W/KIT Wv mice (0.5 g), nNOS controls (0.6 g), and nNOS W/ Wv mice (0.6 g) were comparable.

Both relaxation and the off contraction were abolished by tetrodotoxin, a blocker of neuronal conductance. SNP induced sustained relaxation (0.15 (0.01) g for control mice) of IAS muscle strips. Relaxation to SNP was resistant to tetrodotoxin. In contrast with SNP, ATP failed to relax the IAS. Inhibition by L-NAME reduced relaxations in KIT W/+ controls but the amplitude of the RAIR was lower in nNOS W/+ mice compared with nNOS controls. In KIT W/KIT Wv mutant mice, aberrant RAIRs are only observed on distension volumes of 0.40 ml. Clear RAIRs are also seen in nNOS W/+ mice but the amplitude of the RAIR is lower in nNOS W/+ mice compared with nNOS controls.

Effect of L-NAME in control mice
Inhibition by L-NAME reduced relaxations in KIT W/+ controls (n = 8, fig 4A) and in nNOS control mice (n = 6, fig 4B) (p<0.001). L-NAME had no effect on relaxations to SNP. The combination of L-NAME and suramin did not further reduce relaxations (n = 4, p = 0.19) (data not shown).

Studies in nNOS W/+ mice
To further evaluate the role of nitricergic neurotransmission in the relaxation of the IAS, mice deficient in nNOS were
Studies in nNOS−/− mice
Basal IAS pressure in NOS−/− mice was comparable to their nNOS controls (21 (3) and 21 (2) mm Hg, respectively). However, the amplitude of the RAIR was significantly lower in nNOS−/− mice compared with nNOS controls (p<0.01) (figs 6, 7). Notably, the average duration of the RAIR was increased in nNOS−/− mice compared with their controls (24 (3) and 15 (1) seconds, respectively; p<0.01).

Evaluation of the role of ICC in the inhibitory innervation of the IAS

Basal IAS pressure in KIT W/+KIT Wv mice was comparable to controls (15 (2) and 21 (3) mm Hg respectively, n = 10) (fig 5). Although the magnitude of the basal pressure was comparable, KIT W/+KIT Wv mice showed a more irregular basal sphincter pressure pattern with twitch contractions compared with controls. Distension of the rectum elicited a volume dependent relaxation in KIT W/+KIT Wv mice. However, the RAIR amplitude was significantly attenuated in KIT W/+KIT Wv mice compared with KIT W/+ controls (figs 6, 7). At high distension volume, the RAIR could be observed in KIT W/+KIT Wv mice although with variable duration and amplitude (figs 6, 7).

Administration of intraperitoneal L-NAME to KIT W/+KIT Wv mice had no effect (fig 7).

Compliance in KIT W/+ control mice and in KIT W/+KIT Wv mice
As the differences in RAIR could result from changes in rectal compliance, we also determined this parameter in both control and KIT W/+KIT Wv mice. Stepwise rectal volume distension revealed a similar increase in rectal pressure, demonstrating that rectal compliance was similar in control and KIT W/+KIT Wv mice (fig 8).

DISCUSSION

NO is recognised as an important inhibitory neurotransmitter in the IAS. Previous studies showed that blockade of NO biosynthesis reduced the relaxation of muscle strips of the IAS induced by electrical stimulation and impaired the RAIR. We, in the present study, the role of NO as an inhibitory neurotransmitter to the murine IAS: blockade of NO biosynthesis resulted in a reduction of both the in vitro and in vivo relaxation of the IAS. These findings were further corroborated by experiments in NOS−/− mice. Isolated muscle strips relaxed significantly less whereas the RAIR was impaired compared with controls. It should be emphasised that the IAS still relaxed in NOS−/− mice and that blockade of NO biosynthesis in controls incompletely

Figure 7 Amplitude of the rectoanal inhibitory reflex (RAIR) in neuronal nitric oxide synthase deficient (nNOS−/−) mice and KIT W/+KIT Wv is significantly decreased compared with nNOS control mice and KIT W/+ control mice (n = 10; p<0.05, Wilcoxon signed ranks test). Treatment of wild-type mice with N-nitro-L-arginine methyl ester (L-NAME; 100 mg/kg intraperitoneally) reduced the occurrence of RAIR to a similar extent. Treatment of KIT W/+KIT Wv with L-NAME (100 mg/kg intraperitoneally) slightly reduced the occurrence of RAIR (NS). Values are mean (SEM).

Figure 8 Rectal compliance in KIT W/+KIT Wv was not different from KIT W/+ control mice. Values are mean (SEM) (n = 6).
reduced electrical stimulation induced relaxation. These observations suggest that either nitrergic blockade was incomplete or, most likely, that another inhibitory neurotransmitter is involved. Previous studies in the rat IAS suggested ATP as a possible candidate. However, in our study, high concentrations of ATP failed to relax the IAS. In addition, suramin had no effect on relaxation, excluding the possible involvement of ATP. To what extent vasoactive intestinal polypeptide is involved remains to be investigated.

ICC have been suggested to play a crucial role in normal gastrointestinal motility. In addition to their role as pacemaker cells, previous studies have suggested that ICC function as an intermediate between nerve fibres and smooth muscle cells mediating the nitrergic inhibitory neurotransmission in the stomach, LOS, and pyloric sphincter. Due to the absence of the intramuscular-type of ICC, LOS, and pyloric sphincter, the differences in sensitivity of smooth muscle cells to NO, could be explained by the fact that in the stomach, ICC have been shown to be involved in nitrergic and cholinergic neurotransmission, most likely contributing to the described changes in compliance in mice lacking ICC. In our experiments, however, we were unable to confirm the involvement of ICC in the nitrergic neurotransmission of the IAS, which may explain why compliance in the anorectal area is not altered. Our finding that the RAIR is strongly diminished in KitW-kitW mice in the presence of an unaltered rectal compliance suggests the involvement of ICC in the triggering of the RAIR.

Theoretically, ICC could play a role in the neural pathway mediating the RAIR, either at the afferent limb in the detection of rectal distension or at the efferent part by mediating nitrergic neurotransmission. Our data favour involvement of ICC in the afferent limb, as both immunohistochemical and in vitro studies showed normal nitrergic neurotransmission in the IAS of KitW-kitW mice. One possible explanation could be that ICC act as mechanoreceptors detecting rectal distension. Previous studies indeed showed that ICC make direct functional contact with intramuscular arrays of enteric nerves and therefore could be involved in mechanical perception of distension. Alternatively, ICC may be required for the development of afferent nerves, especially as intramuscular arrays of vagal afferent fibres are significantly reduced in density in KitW-kitW mice compared with controls. This would suggest that abnormalities in the afferents secondary to the absence of a normal ICC network would underlie the impaired detection of stretch. However, the RAIR is considered to be mediated by a neural circuitry located within the enteric nervous system and, so far, no abnormalities in the enteric nervous system have been described in ICC-deficient mice. To what extent the RAIR is impaired in KitW-kitW mice can be explained by the fact that a deficiency of ICC impairs the RAIR, delayed maturation of ICC may lead to impaired anal relaxation and may play an important role in rectal evacuation disorders.

In conclusion, we showed that the inhibitory innervation of the IAS and the RAIR are mediated by NO. Moreover, a normal RAIR requires an intact network of ICC in the IAS. Thus both loss of nitrergic innervation and deficiency of ICC lead to impaired anal relaxation and may play an important role in rectal evacuation disorders.

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Conflict of interest: None declared.

REFERENCES

EDITOR’S QUIZ: GI SNAPSHOT

The radiologist made the diagnosis

Clinical presentation
A 59 year old man was referred with a one year history of change in bowel habit and a low folate level. He had been opening his bowels more frequently but there was no associated abdominal pain or weight loss. Previous medical history included uveitis. Medication consisted of folic acid and he smoked 40 cigarettes a day. Physical examination was unremarkable. Initial blood tests revealed a normal full blood count, urea and electrolytes, liver and thyroid function, glucose, and C reactive protein A double contrast barium enema was performed (fig 1).

Question
In fig 1, what abnormality is demonstrated and what diagnostic procedure was then performed?

See page 1168 for answer

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Figure 1 Double contrast barium enema.

Robin Spiller, Editor