Cyclooxygenase-dependent Alterations in Substance P-Mediated Contractility and NK₁ Receptor Expression in the Colonic Circular Muscle of Patients with Slow Transit Constipation

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ABBREVIATIONS: ACh, acetylcholine; CP99994, (2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine; DMPP, dimethylphenylpiperazinium; EFS, electrical field stimulation; idRNA, internal deleted cRNA; IQR, interquartile range; NKA, neurokinin A; NKA analog, [Lys^5,MeLeu^9,Nle^10]NKA(4-10); PG, prostaglandin; QC-RRT-PCR, quantitative competitive reverse transcription-PCR; SP, substance P; STC, slow transit constipation; stdRNA, standard cRNA; SR140333, 1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl]-4-phenyl-1-azoniabicyclo[2.2.2]octane, chloride; TTX, tetrodotoxin;

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ABSTRACT

Tachykinins are important neurotransmitters regulating intestinal motility. Slow transit constipation (STC) represents an extreme colonic dysmotility with unknown etiology that predominantly affects women. We examined whether the tachykinin system is involved in the pathogenesis of STC. Isolated female sigmoid colon circular muscle from STC and control patients was studied using functional and quantitative RT-PCR methods. A possible alteration of neurotransmission was investigated by electrical field stimulation (EFS) and ganglionic stimulation by dimethylphenylpiperazinium (DMPP). Substance P (SP)-mediated contractions in circular muscle strips were significantly diminished in STC compared with age-matched control ($P<0.001$). In contrast, contractile responses to neurokinin A, the selective $\text{NK}_2$ receptor agonist, $[\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4-10)$, and acetylcholine were unaltered in STC. The reduced responses to SP in STC were fully restored by indomethacin, and partially reversed by tetrodotoxin (TTX), but unaffected by atropine or hexamethonium. The restoration by indomethacin was blocked by the $\text{NK}_1$ receptor antagonist CP99994 and TTX. In STC colonic muscle, there was a significant increase of $\text{NK}_1$ receptor mRNA expression, but no difference in $\text{NK}_2$ mRNA level. DMPP generated biphasic responses; relaxation at lower and contraction at higher concentrations. Although the responses to DMPP were similar in STC and control, an altered contractile pattern in response to EFS was observed in STC circular muscle. In conclusion, we postulate that the diminished contractile response to SP in STC is due to an increased release of inhibitory prostaglandins through activation of upregulated $\text{NK}_1$ receptors. Our results also indicate some malfunction of the enteric nervous system in STC.
Introduction

The tachykinins substance P (SP) and neurokinin A (NKA) are important neurotransmitters regulating many gastrointestinal functions, including motility (Holzer and Holzer-Petsche, 1997). SP and NKA occur primarily in intrinsic enteric neurons, where they are co-localized with acetylcholine. We have previously shown that these natural tachykinins are potent spasmogens in human intestine, although the selective NK₁ receptor agonist [Pro⁹]SP is an ineffective contractile agent (Warner et al., 2000; Liu et al., 2002). Using selective antagonists, we showed that NK₂ receptors on smooth muscle mediate contraction by SP as well as by NKA (Warner et al., 2000; Liu et al., 2002; Burcher et al., 2008). However, there are also facilitatory NK₁ receptors on cholinergic neurons which make a minor contribution to the SP-induced contraction (Liu et al., 2002). Although immunohistochemical and autoradiographic studies have shown that NK₁ receptors are localized to smooth muscle, interstitial cells of Cajal (ICCs) and enteric neurons (Goode et al., 2000; Liu et al., 2002; Boutaghou-Cherid et al., 2006), NK₁ receptors appear uncoupled to contractile mechanisms (Liu et al., 2002) and their roles in the control of human intestinal motility remain unclear. However, SP and NK₁ receptors are involved in gastrointestinal immune functions, with NK₁ receptors expressed on a variety of immune cells (Koon and Pothoulakis, 2006). The tachykinin system has also been implicated in the pathophysiology of functional gastrointestinal disorders. Furthermore, results from the clinical trials with the NK₂ receptor antagonist nepedutant have shown promise in reducing gut motility and pain in patients with irritable bowel syndrome (Lecci et al., 2006).

Slow transit constipation (STC) is a chronic and debilitating functional colonic dysmotility that is unrelated to systemic disorders or pelvic floor dysfunction (Knowles and Martin, 2000; Knowles et al., 2001). The adult form of STC is found almost exclusively in young to middle-aged women (Lubowski et al., 1996; Knowles and Martin, 2000).
pathophysiological basis of STC is poorly understood. In pediatric STC, changes in NK₂ receptors but not in cholinergic transmission were reported (Stanton et al., 2003). However, the etiology and disease process of adult STC differs from pediatric STC – this occurs in males as well as females and the onset of the disease is typically in infancy to early childhood (Sutcliffe et al., 2004). In some studies, a reduction in the cell volume or number of ICCs has been reported in STC (He et al., 2000; Wedel et al., 2002), but others failed to detect any abnormalities in ICCs, despite the selection of highly symptomatic individuals (Toman et al., 2006). It was also suggested that decreased number and size of colonic enteric neurons may play a role (Knowles and Martin, 2000; He et al., 2000; Wedel et al., 2002). A recent study, using novel smooth muscle markers and transmission electron microscopy, revealed abnormalities linked to the smooth muscle contractile apparatus in patients with STC and idiopathic megacolon (Wedel et al., 2006). Most recently, there has been a report that abnormal levels of PGs and cyclooxygenase-2 (COX-2) may be associated with the impaired motor function in STC patients (Cong et al., 2007).

Results on involvement of tachykinin immunoreactivity in STC have been rather variable, with levels of SP like immunoreactivity increased (Sjolund et al., 1997), decreased (Tzavella et al., 1996; Porter et al., 1998) or unchanged (Dolk et al., 1990). Functional data investigating colonic motility and enteric signalling in STC are sparse. One study showed an increased contractility of isolated colon circular muscle to selective NK₂ receptor agonists in male STC patients compared to control, but the cholinergic component remained unchanged (Menzies et al., 2001). In contrast, other studies demonstrated a hyporesponsiveness to tachykinin and acetylcholine stimulation (Mitolo-Chieppa et al., 1998; Mitolo-Chieppa et al., 2001, Tomita 2008) and a reduced relaxation response to VIP (Tomita 2008). The aim of this study was to investigate whether there were changes in STC in tachykinin-mediated circular muscle contraction, and responses to electric field stimulation (EFS) and the ganglionic
nicotinic receptor agonist dimethylphenylpiperazinium (DMPP). Using the COX inhibitor indomethacin, we have provided insights into possible mechanisms underlying such changes.
Materials and Methods

Patients and specimens. Sigmoid colon segments were obtained from 27 female patients (median age 48.5, range 19-73 years) undergoing subtotal colectomy with ileorectal anastomosis for STC. These STC patients represented severe cases that were resistant to conventional laxative treatment (Wong and Lubowski, 2007). Control sigmoid colon segments (resected during surgery for carcinoma, taken 10-15 cm from the tumor) were obtained from 29 female patients (median age 48, range 30-72). Patients who had obstruction or had undergone radiation therapy or chemotherapy were excluded from the study.

Small pieces of specimens were collected directly into the RNAlater solution and used for RNA extraction. Other specimens (approximately 4 cm in length) were collected into carbogen-gassed ice-cold Krebs-Henseleit solution, transported to the laboratory on ice at the day of surgery and dissected within 4 h of arrival. The mucosa, submucosa and serosa were dissected away and the circular muscle bands were then separated from the taenia coli. The dissected circular muscle was placed in fresh Krebs-Henseleit solution, stored overnight at 4°C and used for functional studies the next day. This project was approved by the Human Ethics Committees of the University of NSW and the St George Hospital.

Functional Studies. Circular muscle bands were cut into 4 mm × 8 mm strips and suspended under 1 g tension in 2 ml siliconized glass organ baths containing Krebs-Henseleit solution at 37°C and aerated with carbogen. Muscle tension was recorded isometrically using Grass FTO3C force transducers and recorded by Polygraph (University of New South Wales, Australia). After 60 min equilibration with periodic washing, acetylcholine (ACh, 10 mM) was added into each bath to induce maximal muscle contraction and this was repeated at the end of each experiments. ACh was then washed out and the muscle strips were allowed to
rest 60 min before further experiments. Single concentration response curve was obtained from each strip, as described previously (Liu et al., 2002; Burcher et al., 2008). All muscle strips were weighed at the end of each experiment. The mean (+ S.E.M.) weights of strips used for SP studies were 54.9 ± 3.6 mg for control and 64.0 ± 3.5 mg for STC (P > 0.05, unpaired t-test).

**Responses to agonists.** Discrete concentration-response curves were constructed for ACh, SP, NKA, the NK₂ receptor selective analogue [Lys⁵,MeLeu⁹,Nle¹⁰]NKA(4-10), and the ganglionic nicotinic receptor agonist DMPP, with ascending concentration sequences. Each concentration was left in contact with the tissue for 4-6 min before washing and a 30-60 min agonist-free rest period was given to avoid tachyphylaxis.

**Responses to SP in the presence of inhibitors.** In order to determine mechanisms underlying changes seen in STC tissues in response to SP, discrete concentration-response curves to SP were constructed in the presence of NK₂ receptor antagonist SR48968 (0.1 μM), NK₁ receptor antagonist CP99994 (0.1 μM), atropine (1 μM), hexamethonium (100 μM), tetrodotoxin (TTX, 1 μM), indomethacin (1 μM), indomethacin (1 μM) plus CP99994 (0.1 μM) or indomethacin (1 μM) plus TTX (1 μM). All inhibitors were added to the bath 30 min prior to the addition of SP, except SR48968 which was added 2 h before tissues were exposed to SP. These experiments were performed using a paired design.

**Activation of enteric nerves by electric field stimulation (EFS).** Circular muscle strips were positioned between two platinum rings in 2 ml organ baths and equilibrated under a rest tension of 1 g for 60 min. Muscle tension was recorded isometrically as described above. The maximal contraction was induced with 10 mM ACh. After wash-out of ACh, the muscle
strips were allowed to equilibrate for a further 60 min. EFS was conducted at supramaximal voltage with pulses of 1 ms duration at frequencies of 0.5, 1, 2.5, 5, 10, 20 and 40 Hz in trains lasting 10s. In some strips, EFS was carried out in the presence of atropine (1 μM) and/or tetrodotoxin (TTX, 1 μM).

**Data analysis.** Contractile responses to agonists or to EFS were measured in grams, and then expressed as a percentage of the maximal response to 10 mM ACh in each strip. The agonist potencies were expressed as pEC$_{50}$ (= -log EC$_{50}$) and the maximal response (E$_{\text{max}}$) to NKA and [Lys$^5$,MeLeu$^9$,Nle$^{10}$]NKA(4-10) were defined as the response achieved at 10 μM. The maximal responses to ACh and SP did not appear to have been reached at the highest concentration of agonist used; the apparent E$_{\text{max}}$ for ACh was taken at 10 mM, and that for SP at 100 μM for SP. The pEC$_{50}$ and E$_{\text{max}}$ values were expressed as mean ± S.E.M. and analyzed using Student’s paired or unpaired t-test. Concentration-response data were subjected to non-linear regression analysis and compared using 2-way ANOVA. The $n$ value represents the number of patients in each group. Age-related differences were examined using Pearson’s correlation analysis. All data analyses were carried out using Prism 5.0 (GraphPad Software, Inc.).

**Quantitative competitive RT-PCR.** Specimens stored in the RNAlater solution were dissected and the circular muscle samples (200 mg) were subjected to RNA extraction using the Trizol method followed by a DNase treatment (3U at 37°C for 20 min) to remove contaminating DNA.

The expression of NK$_1$ and NK$_2$ receptor mRNA was quantified by quantitative competitive RT-PCR (QC-RT-PCR) (Burcher et al., 2008). In brief, the oligo primers used for amplification of NK$_1$ receptor mRNA were: 5’-GTCGTGTGCATGATCGAATG-3’
(sense) and 5'-GTGCACACCACGACAATCATCA-3' (antisense), and NK2 receptor mRNA were: 5'-GGTAATGCCATCGTCATCTGGA-3' (sense) and 5'-ATGGTGACGGTGGAGTAGAAG-3' (antisense). The standard cRNA (stdRNA) and internal deleted cRNA (idRNA) were constructed by T7 RNA polymerase (Burcher et al., 2008). For QC-RT-PCR, standard curves were generated with an increasing amount of stdRNA (3-300 fg NK1 and 0.03-10 pg for NK2, respectively) co-amplified with a fixed amount of idRNA (30 fg for NK1 and 0.3 pg for NK2, respectively) by RT-PCR using the Access RT-PCR system. Expression of NK1 and NK2 receptor mRNA in colon circular muscle was determined in replicates by co-amplifying 100 ng sample RNA with fixed amount of idRNA and performed in parallel with the standard curve. The RT-PCR conditions were one cycle at 48°C for 45 min, one cycle at 94°C for 2 min, followed by 33 (for NK1) or 29 (for NK2) subsequent cycles at 94°C for 30 s, 55°C for 1 min and 70°C for 1 min, and a final synthesis at 70°C for 10 min.

The PCR products were visualized by gel electrophoresis and quantified by densitometry. The NK1 and NK2 receptor expression was then normalized to the expression of β-actin in the same sample. The data did not fit the normal distribution, so were expressed as median (inter quartile range, IQR) and analyzed by the Mann-Whitney test.

Materials. SP, NKA, [Lys⁵,MeLeu⁹,Nle¹⁰]NKA(4-10) were purchased from Auspep (Melbourne, Australia). CP99994 was obtained from Dr M. Snider (Pfizer, Groton, CT, USA), SR48968 from Dr. X. Emonds-Alt (Sanofi-Synthélabo Recherche, Montpellier, France). Stock solutions of peptides were made in 0.01 M acetic acid containing 1% β-mercaptoethanol and stored in aliquots at -20°C. ACh, indomethacin, atropine, TTX, DMPP, and hexamethonium were purchased from Sigma Chemical Company (Sydney, Australia).
AmpliScribe T7 High Yield Transcription Kit was purchased from Astral Scientific (Sydney), Trizol reagent from Invitrogen (Sydney, Australia), and the Access RT-PCR system from Promega (Sydney, Australia).
Results

Functional studies in sigmoid colon circular muscle strips.

Response to agonists. Contractile responses to ACh, SP, NKA and [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4-10) were examined in sigmoid colon circular muscle. Figure 1 shows typical recording traces of contractile response to SP. In terms of potency and efficacy (Table 1), responses to ACh were unaltered in STC compared to control, and the two concentration responsive curves were superimposed (Fig 2A). For SP, the potency (pEC₅₀) and efficacy (Eₘₐₓ) were significantly reduced in STC compared with the matching control data (Table 1, Fig 2B). In contrast, contractile responses to NKA and [Lys⁵,MeLeu⁹,Nle¹⁰]NKA(4-10) were unaffected in STC (Table 1, Fig 2C,D).

Age-related changes. There were no age-related changes in the maximal response to SP in either control or STC circular muscle strips (Fig 3). Furthermore, age-related changes were completely absent with respect to maximal responses to ACh, NKA and [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4-10) (data not shown).

Response to SP in STC, in the presence of inhibitors. To determine mechanisms underlying reduced circular muscle contractile responses to SP in STC (Fig 2B), the effects of various inhibitors were investigated in paired studies. The contractile responses to SP were almost abolished by pretreating tissues with the selective NK₂ receptor antagonist, SR48968 (0.1 μM, Fig 4A), but were unaffected by the NK₁ receptor antagonist, CP99994 (0.1 μM, Fig 4B). This was similar to our previously published observation in the control (Liu et al., 2002), suggesting an NK₂ (rather than NK₁) receptor-mediated contractile response to SP in this tissue.
Atropine (1 μM, Fig 4C) and hexamethonium (100 μM, Fig 4D) had no effect on responses to SP in STC. TTX (1 μM, Fig 4E) showed a small enhancement of responses at 10 μM SP. A notable result was the enhancement by indomethacin (1 μM, Fig 4F) of contractile responses to SP. In other words, responses to SP in STC were fully restored to the control level in the presence of indomethacin.

The mechanism of this effect of indomethacin was further studied. TTX (1 μM) abolished the potentiation by indomethacin at lower concentrations of SP (10⁻⁵ M and less) and partially inhibited its effect at the highest SP concentration (10⁻⁴ M) (Fig 4G). CP99994 (0.1 μM) totally abolished the indomethacin-induced increase in responses at all concentrations of SP (Fig 4H).

**Response to DMPP.** The ganglionic nicotinic receptor agonist, DMPP, produced a biphasic response in circular muscle strips. Responses were characterized by very small net relaxations at lower concentrations (10⁻⁷-10⁻⁴M); these were significantly different from baseline tension. At the highest concentration (10⁻³M), there was a net contraction, equivalent to 8% of ACh maximal response. Responses were no different between control and STC strips (Fig 5).

**Response to EFS.** EFS usually induced contraction, although in some control and STC strips, EFS caused an initial relaxation at low frequencies.

In control strips, EFS elicited frequency-dependent increases in contractions, ranging from 13.5 ± 4.32% of ACh maximal contraction at 0.5 Hz to 63.8 ± 9.76% at 40 Hz (n = 9, Fig 6A). The contractions were mainly mediated by ACh released from myenteric nerves –
pretreatment of circular muscle strips with 1 μM atropine inhibited a large part of the contraction, particularly at lower frequencies. TTX (1 μM) had no further effect.

In STC (Fig 6B), contractions in response to EFS (n=11) were unrelated to the stimulation frequency (22-33% of ACh maximal contraction). In STC, atropine and TTX almost completely abolished responses to EFS.

In comparison with control (Fig 6C), at lower frequencies (0.5-5 Hz) the contractile amplitudes were higher in STC than in control, but contractile responses of STC to higher frequency stimulation (10-40 Hz) were greatly reduced compared with control.

**QC-RTPCR**

NK₁ and NK₂ receptor mRNA expression levels were measured in the sigmoid colon circular muscle of STC patients, compared with age matched controls. As shown in Fig 7A, there was a 2.6-fold increase in NK₁ receptor mRNA expression in STC. The NK₂ receptor mRNA was expressed at similar levels in STC and control (Fig 7B). No age-related differences in expression of NK₁ or NK₂ receptor mRNA were seen, for either control or STC data.
Discussion

Several important findings have resulted from this study. Firstly, SP-mediated contractions of female colonic circular muscle were significantly diminished in STC, compared with age-matched control. Secondly, this reduction was reversed by indomethacin in STC, suggesting changes in COX in this disorder. Thirdly, NK₁ receptors appear implicated, since (a) CP99994 antagonized the effect of indomethacin and (b) NK₁ receptor mRNA was upregulated in STC. Finally, neural mechanisms are also likely to be involved, since TTX also antagonized the effect of indomethacin, and responses to EFS were markedly altered in STC.

The reduction of contractility to SP seen in STC was not due to its action on NK₂ receptors, which are primarily responsible for mediating the direct component of SP-induced contractile responses (Warner et al., 2000; Liu et al., 2002; Burcher et al., 2008). Contractile responses to NKA and to the selective NK₂ receptor agonist, \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4-10)\), were unaltered in STC. Furthermore, ACh-induced contractions also remained unchanged, indicating that the attenuation of SP-responses was unrelated to non-specific diminished responsiveness of smooth muscle. A minor component(s) of the SP contractile response is independent of NK₂ receptors and involves NK₁ receptors associated with neuronal and/or immune cell mechanisms (Fig 8). We suggest that this indirect NK₁ receptor-mediated component is unmasked and/or amplified in STC and its activation by SP results in a net relaxation of colonic smooth muscle (Fig 8). This hypothesis is supported by the increased expression of NK₁ receptor mRNA in STC, whereas NK₂ receptor mRNA remained unchanged.

Constitutive COX-1 and COX-2 are widely expressed in normal human colon smooth muscle, myenteric ganglia, and ICCs, as well as in the mucosa (Fornai et al., 2005; Bernardini et al., 2006 Burcher et al., 2008). Prostanoids play many roles in normal gut
function, being involved in mucosal protection and repair, regulation of blood flow, mucosal secretion as well as motility. We previously reported that in normal human colon circular muscle, contractile responses to SP were unaffected by indomethacin (Liu et al., 2002). However, a novel finding of the present study was that the mechanism of the attenuated response to SP in STC appears to be related to the COX system. This result is consistent with a recent study (Cong et al., 2007) showing that abnormal levels of PGs and COX-1 and COX-2 occur in female patients with STC. They found that the reduced spontaneous motility in STC was associated with a reduction of contractile PGs thromboxane A₂ and PGF₂ (Cong et al., 2007), and an increase of PGE₂, known to relax colonic circular muscle (Bennett et al., 1981; Botella et al., 1995).

Our studies in STC circular muscle found that the indomethacin-induced restoration of contractile responses to SP (Fig 4F) was blocked by the NK₁ receptor antagonist CP99994 (Fig 4H). Thus, we hypothesize that the STC-susceptible indirect component of the SP response is due to an increased release of inhibitory PGs, through activation of NK₁ receptors (Fig 8). Neuronal mechanisms may also be involved, since TTX reversed the attenuated responsiveness to SP in STC, and also (as well as CP99994) blocked the indomethacin-induced potentiation of contractile response to SP. We suggest that SP caused an indirect muscle relaxation, via activation of neuronal NK₁ receptors that mediate the release of an inhibitory mediator(s) (suggested to be PGs) from postganglionic enteric neurons and other cell types (Fig 8). This is consistent with literature findings that COX enzymes are expressed in myenteric ganglia and involved in inhibitory modulation of cholinergic motor functions in normal human colon (Fornai et al., 2005; Bernardini et al., 2006).

Although there is no functional evidence for the participation of NK₁ receptors in human colon circular muscle contraction, NK₁ receptors are present on the circular muscle, ICCs, enteric ganglia, nerve fibers and vascular endothelial cells of the human colon (Goode et al.,
We postulate that the NK₁ receptor is closely associated with COX enzymes in the human colonic myenteric ganglia and/or muscularis externa. The restoration of contractility by SP revealed that an imbalance in prostanoid production appears to be related to the pathophysiology of STC.

Our finding that EFS-evoked contractions were increased at lower frequency and reduced at higher frequency in STC compared to control may reflect an impaired enteric motor function in STC. Unlike the control, EFS-evoked contractions in STC were similar in amplitude and unrelated to stimulation frequency. Similar to the control, the EFS-induced contractions in STC were mainly cholinergic in origin, and in some strips, an initial relaxation was seen at low frequencies. In the intestine, the inhibitory motor neurons release a combination of at least three transmitters, nitric oxide (NO), ATP and VIP, but in circular muscle of the human colon, nitric oxide is the principal inhibitory transmitter (Keef et al., 1993; Boeckxstaens et al., 1993). It is possible that dysfunctions of both inhibitory and excitatory neurotransmission occur in STC. It has been shown that STC patients display a loss of enteric neurons, partially due to increased apoptosis (Bassotti et al., 2006). A reduced release of [³H]ACh in response to EFS in STC taenia coli muscle strips and decreased choline acetyltransferase in colonic myenteric neurons of STC have been demonstrated (Burleigh et al., 1988, Wattchow et al., 2008). Conversely, increased NO synthase-positive neurons and activities are associated with STC (Tomita et al., 2002, Wattchow et al., 2008). Our result is in agreement with the study by Mitolo-Chieppa and colleagues performed in male STC circular muscle, which shows that EFS-induced relaxation of SP-precontracted muscle strips is reduced at 0.5 Hz (ATP mediated) and enhanced at 4 Hz (NO) medicated compared to control, suggesting an abnormality in NO and ATP release (Mitolo-Chieppa et al., 2001). Therefore, we cannot rule out that the reduced contractile amplitudes at higher frequency may be attributed to (or partially attributed to) the excessive NO production in STC.
In animals, DMPP induces relaxation of rat distal colon by a purinergic and a nitrergic mechanism, and causes guinea-pig ileum contraction by increase of ACh output, through stimulation of nicotine receptors situated on myenteric ganglia (Borjesson et al., 1997; Galligan et al., 1999). In human colon, the dual effects of DMPP on circular muscle activity do not appear to have been reported. At lower concentrations, the net effect was a slow relaxation indicating the release of inhibitory neurotransmitters, whereas at the highest concentration, the output of excitatory neurotransmitters predominated. In contrast to our EFS results, responses to DMPP were unaltered in STC. The results are difficult to interpret since any alteration in enteric neurotransmission may be masked by opposing inhibitory and excitatory actions. One explanation is that DMPP has a relatively limited action; activating ganglionic motor neurons, whereas EFS activates mixed populations of all axons and nerve terminals.

A recent study suggests that a defective expression of proteins associated with the smooth muscle contractile apparatus may be involved in the pathogenesis of STC (Wedel et al., 2006). Immunostaining for smooth muscle myosin heavy chain, smoothelin and histone deacetylase 8 was homogenous in normal colon smooth muscle, but was either absent or greatly reduced in STC smooth muscle (Wedel et al., 2006). No abnormality of smooth muscle contractile apparatus was observed in our study, since the contractile responses to NKA and ACh, both of which are via direct myogenic mechanisms, were unchanged in STC. However, because both the receptor subtypes involved (tachykinin NK₂ and muscarinic M₃ receptors) use the same second messenger pathway: Gq-protein-coupled inositol trisphosphate and calcium, the occurrence of other specific defects of smooth muscle contractile mechanisms in STC cannot be excluded.

In summary, SP-mediated circular muscle contractions were significantly diminished in STC, whereas contractile responses to NKA and ACh were unaltered. An important and
original finding is that the reduced contractile responses to SP in STC were restored by indomethacin to the normal control level, suggesting a role of prostanoids in the pathogenesis of STC. The restoration by indomethacin was blocked by the NK1 receptor antagonist CP99994 and by TTX. We also found that STC circular muscle strips showed an altered contractile pattern in response to electric nerve stimulation, supporting the theory of an impaired function of the enteric nervous system in STC. In conclusion, the reduction in magnitude of contractile responses to SP in STC may be due to an increased release of inhibitory PGs from possibly neuronal sites, via upregulated NK1 receptors.
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References


Footnotes

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Legends for Figures

**Fig 1.** Typical recording traces showing SP induced contractile responses of isolated colonic circular muscle strips from control (A) and STC (B) patients. Arrows denote addition of increasing concentration of SP. Solid dots denote addition of ACh (10^{-2}M) to produce maximal contraction. Asterisks denote washout of SP or ACh.

**Fig 2.** Concentration-response curves to ACh, SP, NKA and the NKA analog [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) were obtained in isolated strips of female human sigmoid colon circular muscle from control and STC patients. Responses were expressed as grams tension. (A), Responses to ACh were unaltered in STC. (B), SP-induced contractions in STC were significantly diminished compared to control (P < 0.0001, 2 way ANOVA for curve comparison; * P < 0.05, ** P < 0.01; *** P < 0.001, Bonferroni’s post-test for individual points). Responses to NKA (C) and [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) (D) showed no difference between control and STC. Points represent the mean ± S.E.M. of n individual patients.

**Fig 3.** Responses to SP at 10^{-4}M plotted against patient age, for control and STC patients. The lines show the linear regression (not significant). No age-related effects were seen (Pearson’s correlation coefficient of 0.145 and – 0.094 for control and STC, respectively).
**Fig 4.** Contractile responses to SP in isolated strips of sigmoid colon circular muscle from STC patients. Concentration-response curves in the absence and presence of inhibitors were constructed using a pairwise design, and compared using 2 way ANOVA. SP-induced contractions in STC were (A) virtually abolished by SR48968 (0.1 µM), 2 way ANOVA $P < 0.001$; (B) unaffected by CP99994 (0.1 µM); (C) unaffected by atropine (1 µM); (D) unaffected by hexamethonium (100 µM); (E) slightly enhanced by 1 µM TTX (only at SP $10^{-6}$ M, $P < 0.05$, Bonferroni’s post-test); (F) markedly enhanced by indomethacin (1 µM), 2 way ANOVA $P < 0.001$; (G) unaffected by indomethacin in combination with TTX; (H) unaffected by indomethacin in combination with CP99994. Responses were expressed as the maximal contraction to ACh (10 mM). Points represent the mean ± S.E.M. of $n$ individual patients. In each panel, the dashed curve represents the mean response to SP in strips from control patients. *, $P < 0.05$; ***, $P < 0.001$, paired t-test for individual points.

**Fig 5.** Responses of colon circular muscle strips to dimethylphenylpiperazinium (DMPP). DMPP relaxed muscle strips at lower concentrations ($10^{-7}$-$10^{-4}$ M) and contracted the tissue at the highest concentration ($10^{-3}$ M). No differences were observed between control and STC. Responses were expressed as gram tension. Points represent the mean ± S.E.M. of $n$ individual patients.
**Fig 6.** Responses of colonic circular muscle strips to electric field stimulation (EFS). Responses were expressed as the maximal contraction to ACh (10 mM). (A) Control circular muscle strips yielded frequency-dependent increases in tension, that were reduced by atropine (1 μM) but not further reduced by TTX (1 μM), *, $P < 0.05$, one way ANOVA; (B) EFS-evoked contractions in STC circular muscle were unrelated to stimulation frequency, and were reduced by atropine (1 μM); TTX (1 μM) showed no further significant reduction, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, one way ANOVA. (C) At lower frequencies (0.5-5 Hz), the contractile amplitudes were higher in STC than in control ($P < 0.05$, 2 way ANOVA). However, contractile responses to higher frequency stimulation (10-40 Hz) were greatly reduced in STC compared to control ($P < 0.01$). Points represent the mean ± S.E.M. of $n$ individual patients.

**Fig 7.** Expression of mRNA from control and STC colonic circular muscle. Scatterplots represent values for (A) NK1 receptor and (B) NK2 receptor, from individual age-matched female control ($n=16$) and STC ($n=9$) patients; bars indicate the medians. (A), the median levels of NK1 receptor mRNA expression were 2.57 (IQR 1.82-3.74) $\times 10^5$ copies/μg RNA in control compared to 6.79 (IQR 3.48-13.5) in STC. NK1 receptor mRNA expression was significantly increased in STC ($P < 0.05$, Mann-Whitney test). (B), No difference was seen in NK2 receptor mRNA expression. The median NK2 receptor mRNA expression was 14.4 (IQR 7.41-22.8) and 17.5 (IQR 8.06-24.9) $\times 10^6$ copies/μg RNA, respectively for control and STC.
**Fig 8.** Proposed mechanism for reduction in SP-induced contractions in STC circular muscle, and the effect of indomethacin. (A), Suggested components of functional responses to SP in control and STC. (B), Schematic concentration response curves to SP in STC. The SP-mediated response in control muscle consists of a major component of NK₂ receptor-mediated contraction; this component is unchanged in STC. In control muscle, there is no net effect from NK₁ receptor mediated COX-dependent component as responses to SP remained unaltered in the presence of indomethacin (Liu et al., 2002). However, in STC, an NK₁-mediated COX-dependent relaxation is revealed by indomethacin, responsible for the reduced contraction in STC. (C), Proposed mechanism of action of SP in STC. SP acts (with low potency) at NK₂ receptors on smooth muscle. SP also stimulates NK₁ receptors on COX-containing colonic cells (neurons, muscle, mast cells and/or inflammatory cells) to release prostanoid(s). These relax smooth muscle and/or may negatively modulate enteric neurons. This relaxant mechanism, a minor component of the overall response, was unmasked by indomethacin and inhibited by NK₁ receptor blockade and by TTX. ▼, prostanoid receptor. Graph adapted from Burcher et al. (2008).
<table>
<thead>
<tr>
<th>Agonist</th>
<th>pEC$_{50}$ Control</th>
<th>pEC$_{50}$ STC</th>
<th>E$_{\text{max}}$ Control</th>
<th>E$_{\text{max}}$ STC</th>
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<tr>
<td>ACh</td>
<td>3.28 ± 0.34 (23)</td>
<td>3.22 ± 0.39 (14)</td>
<td>4.16 ± 0.57 g (23)</td>
<td>4.43 ± 1.02 g (14)</td>
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<td>SP</td>
<td>6.14 ± 0.13 (28)</td>
<td>5.35 ± 0.06*** (27)</td>
<td>62.8 ± 9.22% (18)</td>
<td>45.0 ± 2.30%*** (21)</td>
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<td>NKA</td>
<td>7.43 ± 0.09 (26)</td>
<td>7.36 ± 0.10 (14)</td>
<td>60.6 ± 3.47% (25)</td>
<td>58.0 ± 4.58% (13)</td>
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<tr>
<td>NKA analog</td>
<td>7.51 ± 0.12 (25)</td>
<td>7.31 ± 0.16 (15)</td>
<td>63.5 ± 3.57% (24)</td>
<td>57.9 ± 4.35% (15)</td>
</tr>
</tbody>
</table>

a, data are shown as mean ± S.E.M (n), where n represents the number of patients.

b, responses achieved at the highest concentration used. ACh E$_{\text{max}}$ is expressed as gram tension and E$_{\text{max}}$ values for tachykinins are expressed as percentage of ACh maximal response.

***, P < 0.001 compared to corresponding control data (unpaired t-test).
Figure 1

A

B
Figure 2.
Figure 3
Figure 4
Figure 5
Figure 7

(A) NK₁ mRNA (x10⁵ copies) in control vs. STC groups. *P < 0.05.

(B) NK₂ mRNA (x10⁶ copies) in control vs. STC groups.
Figure 8