Tachykinin NK₂ Receptor and Functional Mechanisms in Human Colon: Changes with Indomethacin and in Diverticular Disease and Ulcerative Colitis

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ABSTRACT

Neurokinin A (NKA) is an important spasmogen in human colon. We examined inflammatory disease-related changes in the tachykinin NK₂ receptor system in human sigmoid colon circular muscle, using functional, radioligand binding, and quantitative reverse transcription-polymerase chain reaction methods. In circular muscle strips, indomethacin enhanced contractile responses to NKA (p < 0.01) and to the NK₂ receptor-selective agonist [Lys⁵,MeLeu⁹,Nle¹⁰]-NKA(4–10) (p < 0.05) in both normal and acute diverticular disease (DD) specimens, indicating NK₂ receptor-mediated release of relaxant prostanoids. Contractile responses to both tachykinins were reduced in strips from DD (p < 0.001) and ulcerative colitis (UC) (p < 0.05) specimens. Responses to acetylcholine were no different in other strips from the same disease patients, demonstrating that the change in responsiveness to tachykinins in disease is specifically mediated by the NK₂ receptor. In membranes from UC specimens, receptor affinity for ¹²⁵I-NKA (median Kᵢ 0.91 nM, n = 16) was lower (p < 0.01) than that in age-matched control specimens (Kᵢ 0.55 nM, n = 40), whereas Kᵢ (0.65 nM, n = 28) in DD was no different from control. No disease-related changes in receptor number (Bmax) were found (mean, 2.0–2.5 fmol/mg of wet weight tissue), suggesting that the reduced contractile responses in disease are not due to a loss of receptor number. Different mechanisms may account for the reduced contractility in DD compared with UC. A gender-related difference in receptor density was seen in controls, with Bmax lower in females (1.77 fmol/mg, n = 15) than in males (2.60 fmol/mg, n = 25, p = 0.01). In contrast, no gender-related differences were seen in NK₂ receptor mRNA in control colonic muscle, indicating that the gender difference is a post-translational event.

In humans and animals, the tachykinin peptide family exerts its actions through three distinct receptors, NK₁, NK₂, and NK₃, that preferentially but not exclusively interact with the neuronal tachykinins substance P (SP), neurokinin A (NKA), and neurokinin B, respectively (Pennefather et al., 2004). Tachykinins are widely distributed and have a broad spectrum of actions, including smooth muscle contraction, neuronal excitation, endothelium-dependent vasodilatation, plasma extravasation, salivation, and nociception, as well as proinflammatory actions (Harrison and Geppetti, 2001). In recent studies, non-neuronal tachykinins, hemokinin and endokinins, with immune and paracrine functions have been described, and these show high affinity for the NK₁ receptor (Page, 2004; Pennefather et al., 2004). NKA is a potent smooth muscle spasmogen in man (Warner et al., 2000; Lecci et al., 2006). In the human intestine, NKA and SP are found in neurons innervating the circular muscle, myenteric plexus, submucous plexus, blood vessels, and lamina propria of the human colon; in intrinsic neurons of the myenteric and submucous plexus of the intestine; and in primary afferent nociceptive fibers (Wattchow et al., 1988). These tachykinins are involved in the ascending excitatory reflex and atropine-resistant peristalsis (Holzer and Holzer-Petsche, 1997). In human colonic circular muscle, NKA is several orders of magnitude more potent than SP, with NK₂ receptors predominant in mediating contraction of the circular muscle by tachykinins (Giuliani et al., 1991;...
Menzie et al., 2001; Liu et al., 2002). NK2 receptors are mainly expressed on smooth muscle, with autoradiographic and molecular studies showing dense localization over circular muscle and weaker binding over muscularis mucosae (Gates et al., 1988; Renzi et al., 2000; Warner et al., 2000).

It has been suggested that peptidergic systems such as the tachykinins may have a pronounced role in the pathophysiology of acute and chronic intestinal disorders (Holzer, 1998; Renzi et al., 2000). In particular, tachykinins have been implicated in inflammatory bowel disease (IBD), acute diverticulitis (an inflammatory disorder unrelated to IBD), and irritable bowel syndrome (Lecci et al., 2006). Studies by Menzie et al., 2001; Liu et al., 2002). In particular, tachykinins may have a pronounced role in the pathophysiology of acute and chronic intestinal disorders (Holzer, 1998; Renzi et al., 2000). In particular, tachykinins have been implicated in inflammatory bowel disease (IBD), acute diverticulitis (an inflammatory disorder unrelated to IBD), and irritable bowel syndrome (Lecci et al., 2006). Studies by in situ hybridization and immunohistochemistry revealed that ulcerative colitis (UC) and Crohn’s disease are associated with up-regulation of NK1 and NK2 receptors (Goode et al., 2000; Renzi et al., 2000).

Using radioligand binding, we have previously demonstrated the existence of distinct high-affinity NK2 (Warner et al., 1999) and NK binding sites (Liu et al., 2002) in human colonic circular muscle homogenates. The aim of this study was to study disease-related changes in the tachykinin NK2 receptor system in human sigmoid colon circular muscle of specimens from patients with UC and diverticulitis disease (DD). We compared the number and the affinity of 125I-NKA binding sites (NK2 receptors) using radioligand binding, measured the expression of NK2 receptor mRNA by quantitative reverse transcription-PCR (QC-RT-PCR), and examined the functional responses to NK2 agonists using smooth muscle strip pharmacology. We also examined any age- and gender-related differences. Possible involvement of the cholinergic system and cyclooxygenases was addressed by investigating the effect of atropine and indomethacin on responses in normal and disease preparations.

### Materials and Methods

**Patients and Specimens.** Colon ring segments approximately 3 cm in length were obtained from male and female patients undergoing total or partial colectomy for adenocarcinoma (age range 25–93 years), acute DD (age range 32–82 years), and UC (age range 21–79 years). The DD and UC colon specimens used in this study represented examples of acute disease, although strips were taken from regions of less inflamed tissue. Most segments were taken from the sigmoid colon, and a minority was also obtained from descending and ascending colon. The carcinoma patients constituted the control group and, as much as possible, were age-matched and region-matched with patients with DD and UC. Normal colon from patients with carcinoma was taken 10 to 20 cm from the tumor. Any control specimen that appeared to be inflamed or macroscopically abnormal in any way was discarded. Colon specimens from carcinoma patients with obstruction or who had undergone radiation therapy or chemotherapy were excluded from this study. This project was approved by the human ethics committees of the University of New South Wales and St George Hospital.

Immediately after collection, specimens were placed in ice-cold Krebs-Henseleit solution previously gassed with carbogen (95% O2 and 5% CO2) and transported to the laboratory on ice. For binding studies, most specimens were dissected within 4 h of removal, but others were stored overnight at 4°C. The mucosa, submucosa, and serosa were first removed, and then the taenia coli, leaving the circular muscle bands. Part of this muscle was used for functional studies (see below), and the remainder was frozen in liquid nitrogen and stored at −70°C for use in radioligand binding experiments. This “circular muscle” also contained a thin layer of longitudinal muscle, the myenteric plexus and small blood vessels (verified microscopically in initial studies).

**Functional Studies.** Sigmoid colon specimens only were used for these studies. Circular muscle strips were dissected and mounted in organ baths as described previously (Warner et al., 2000; Liu et al., 2002).

Discrete concentration-response curves to NKA and the NK2 receptor-selective analog [Lys5,MeLeu9,Nle10]NKA(4–10) were constructed, using increasing concentrations up to 10 μM. Each concentration was left in contact with the tissue for 4 to 6 min before washing. Addition of peptide was made at 60 to 90-min intervals to avoid tachyphylaxis, as described previously (Warner et al., 2000; Liu et al., 2002). Up to eight circular muscle strips were examined from each specimen. Responses were obtained in the absence and presence of atropine (1 μM) (normal colon and UC) or indomethacin (1 μM) (normal colon and DD), and a paired design was used wherever possible. Only one concentration-response curve was obtained from each strip. Because previous experiments had shown that contractile responses to NKA were not significantly enhanced by peptidase inhibitors (Warner et al., 2000), these inhibitors were omitted from the functional studies. In parallel experiments, responses to acetylcholine (ACh) were elicited in adjacent strips from the same specimens.

Muscle strips were weighed at the end of the experiment. Contractile responses to tachykinins were measured in grams and then expressed as a percentage of the supramaximal response to 10 mM ACh in each strip. Data were plotted and analyzed using Prism 3.0 (GraphPad Software Inc., San Diego, CA). Because the maximal response of every strip may not have been achieved by the highest concentration (10 μM) of tachykinin, the agonist potencies were expressed as −logEC50, and the maximal response to each agonist was defined as the response achieved at 10 μM. Differences in concentration-response curves were analyzed using two-way ANOVA. The −logEC50 values and maximal responses were normally distributed and then analyzed using Student’s paired t test (for paired studies using atropine or indomethacin) or unpaired t test (normal versus disease). Responses in disease colon were compared with responses in age-, region-, and gender-matched normal colon. The n value represents the number of patients in each group.

**Radioligand Binding Studies.** Circular muscle was thawed and cut into small pieces on ice. Crude membranes (2% w/v) were prepared as described previously (Warner et al., 1999). Membranes were homogenized, centrifuged, and finally resuspended in incubation buffer consisting of 50 mM Tris HCl (pH 7.4, 25°C), 0.02% bovine serum albumen (BSA), 5 mM MnCl2, and chymostatin 4 μg/ml, as described previously for 125I-NKA binding in human colon circular muscle (Warner et al., 1999).

On each experimental day, membranes from at least one “normal” specimen patient were prepared, and “cold” saturation experiments were carried out in parallel with membranes from aged-matched DD and UC patients to determine the affinity (Kd) and binding capacity (Bmax) of 125I-NKA. In this study, increasing concentrations of NKA were coincubated with the membrane suspensions containing 70 pM 125I-NKA for 60 min at 25°C. Nonspecific binding was defined in replicate tubes by coincubation with 1 μM NKA (Warner et al., 1999).

In all experiments, the binding was terminated by rapid filtration and washed (3 x 3 ml) with ice-cold 50 mM Tris buffer containing 3 mM MnCl2 and 0.02% BSA, using Whatman GF/B glass fiber paper (presoaked in 0.5% BSA overnight) with a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). Filter-bound radioligand was quantified using a Wallac Wizard Gamma counter (>78% efficiency). Raw binding data were processed by Prism 3.0 and analyzed using single- and multiple-site models, and the F test was used to determine the most appropriate model. A total of p < 0.05 was considered statistically significant. Most data were best fitted to a single-site model; however, when a two-site analysis was preferred, the high-affinity values were selected. Unless otherwise stated, data
are expressed as the mean ± S.E.M. Binding affinity (Kd) values were not normally distributed and are therefore presented as median [interquartile range (IQR)]. Values were compared using one-way ANOVA followed by an appropriate multiple comparison test.

**QC-RT-PCR**. Small sigmoid colon specimens were collected directly into the RNAlater solution in the operating room and stored at 4°C overnight to allow the solution to thoroughly penetrate the tissue. The circular muscle was dissected free from the mucosa, submucosa, and taenia coli, and total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA) followed by a DNase treatment (3 U at 37°C for 20 min) to remove contaminating DNA.

The expression of NK2 receptor mRNA in normal circular muscle was quantified using QC-RT-PCR, similar to the methods previously developed for muscarinic receptors (Mansfield et al., 2005). In brief, a NK2 cDNA fragment [483 base pairs (bp)] was generated by RT-PCR using human NK2 gene-specific primers [forward: 5'-GG-TAAATGCCATGTACACCTGGA-3' (5'-end of the primer included a T7 promoter sequence) and reverse: 5'-ATGGTGACGGTGGAGTAA-3'] (H11032). This NK2 cDNA fragment was then used as a template to perform a seminested PCR using the same forward primer (containing the T7 promoter) and a reverse primer that was designed to flank 140 bp internally. The standard cRNA (stdRNA) and the internally deleted or competitor cRNA were synthesized by T7 RNA polymerase (AmpliScribe T7 High Yield Transcription Kit; Astral Scientific, Sydney, NSW, Australia) using the NK2 cDNA fragment and internally deleted NK2 cDNA fragment as templates, respectively.

For QC-RT-PCR, a standard curve was constructed with an increasing amount of stdRNA (30 fg–10 pg) coamplified with a fixed amount of competitor RNA (0.3 pg) by RT-PCR using the Access RT-PCR system (Promega, Sydney, NSW, Australia). The expression of NK2 receptor mRNA in colon Circular muscle was determined in replicates by RT-PCR, coamplifying 100 ng of sample total RNA with 0.3 pg of competitor RNA and run in parallel with the standard curve. In addition to RNA, the RT-PCR mixture also contained 0.4 μM forward primer, 0.6 μM reverse primer, 0.4 mM dNTPs, 2.5 mM MgSO4, 2.5 U of AMV reverse transcriptase, and 2.5 U of Tfl DNA polymerase. The RT-PCR was performed at 48°C for 45 min and 94°C for 2 min, followed by 29 cycles of 94°C for 30 s, 55°C for 1 min, and 70°C for 1 min, and a final extension at 70°C for 10 min. A QC-RT-PCR was also developed for β-actin, which was used as the internal control. The stdRNA and competitor RNA for β-actin were produced in the same manner as that described for the NK2 receptor. The stdRNA (0.3–30 pg) or colon circular muscle total RNA (10 ng) was coamplified with 3 pg of competitor RNA by RT-PCR with the following primer set: forward 5'-ACGGGTCACCCAGACTGTCC-3' and reverse 5'-GTAGAAGCATTTGCGGTGGAC-3'. A PCR cycle of 25 amplifications was chosen, and other conditions were similar to that used for the NK2 receptor.

RT-PCR was performed for cyclooxygenase (COX)-1 and COX-2 gene expression with conditions similar to that used for the NK2 receptor. The primer sequences were as follows: COX-1, forward 5'-GGATGGTGGCAATGACCT-3' and reverse 5'-GCAAATGCCTCTTCCCTTGTGAC-3'; and COX-2, forward 5'-GGCTTTCCATGGACAGGAGC-3' and reverse 5'-GGCCCGGCTGTCTTACCCAGA-3'.

The PCR products were then visualized by gel electrophoresis and quantified by densitometry. For quantification, a standard curve (log stdRNA concentration versus log ratio of the intensity of PCR bands generated from stdRNA and competitor RNA) was plotted to determine the amount of sample mRNA (fmotogram stdRNA) and converted to copy numbers per microgram sample total RNA (1 fg of 483-bp stdRNA was calculated to be equivalent to 7.33 × 10^7 copies).

**Materials**. 125I-NKA (50 μCi, specific activity 2200 Ci/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA), reconstituted in distilled water, and stored in frozen aliquots at −70°C. GF/B glass filter papers were purchased from Biotab (Melbourne, NSW, Australia). NKA and [Lys5,MeLeu6,Nle10]NKA(4–10) were purchased from Auspep Pty Ltd. (Melbourne, NSW, Australia). Stock solutions of tachykinins were prepared in 0.01 M acetic acid with β-mercaptoethanol and stored as aliquots at −20°C. The peptidase inhibitors chymostatin, indomethacin, atropine, acetycholine, and BSA were purchased from Sigma Chemical Co. (Sydney, NSW, Australia). All other reagents were of analytical grade.

**Results**

**Functional Studies in Sigmoid Colon Circular Muscle—Normal Colon.** NKA and [Lys5,MeLeu6,Nle10]NKA(4–10) were potent agonists and contracted circular muscle in a concentration-dependent manner (Fig. 1, A and B). No significant difference in the potency and maximal response between these two agonists was observed in normal sigmoid colon. No gender or age differences were seen in responses to these agonists (Fig. 1, C and D) or to ACh (data not shown).

The mechanism of contraction was studied in paired strips. Responses to these agonists were unaltered in the presence of 1 μM atropine (Table 1). However, responses to both NKA and [Lys5,MeLeu6,Nle10]NKA(4–10) were enhanced by 1 μM indomethacin (Fig. 2, A and B; two-way ANOVA). The pD2 value for [Lys5,MeLeu6,Nle10]NKA(4–10) but not NKA was increased in the presence of indomethacin (Table 1).

**Functional Studies in Sigmoid Colon Circular Muscle—Disease Groups.** The median ages (IQR) of the patient age. No correlation was seen: C, Pearson r = 0.095, p = 0.616; D, Pearson r = −0.198, p = 0.322.

**Fig. 1.** Concentration-response curves to NKA (A) and to the NKA analog [Lys5,MeLeu6,Nle10]NKA(4–10) (B) were obtained in isolated strips of male and female human sigmoid colon circular muscle. Points represent the mean ± S.E.M. of n individual patients. Each point was calculated as a percentage of the maximal contraction to ACh (10 mM). No significant gender differences were seen (A, p = 0.669; B, p = 0.755, two-way ANOVA). The individual Emax values (at 10 μM) for NKA (C, n = 30) and for [Lys5,MeLeu6,Nle10]NKA(4–10) (D) (n = 27) are plotted as a function of patient age. No correlation was seen: C, Pearson r = 0.095, p = 0.616; D, Pearson r = −0.198, p = 0.322.
tients contributing to these groups were similar: normal, 51 years (46–62, n = 41); DD, 61 years (50.5–72, n = 13); and UC, 46 years (36.5–68, n = 9) (p = 0.412, Kruskal-Wallis test). There were no gender-related differences in responses to either NK₂ agonist in strips from the three experimental groups (data not shown).

In circular muscle strips from patients with DD, responses to both NKA and \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4–10)\) were significantly less than those from age- and gender-matched normal colon (Fig. 2, C and D). The maximal responses to both agonists were also reduced in DD, but potencies were unaffected (Table 1).

In paired strips from patients with DD, responses to NKA and \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4–10)\) were significantly enhanced by 1 μM indomethacin (Fig. 2, E and F). Thus, although the “direct component” of the response was reduced in DD, the indomethacin-sensitive response or “indirect component” of responses to these agonists was diminished (if not increased) in DD.

Responses were also examined in circular muscle strips from patients with UC. Here, there was a significant reduction in responses to both NKA and \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4–10)\) compared with responses in normal colon (Fig. 2, G and H). The maximal responses to \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4–10)\) showed a trend toward significant reduction in UC (p = 0.052), but the \(E_{\text{max}}\) of NKA and potencies of both agonists were unaffected (Table 1). In other strips from the same UC patients, atropine pretreatment did not significantly alter responses to either NKA or \([\text{Lys}^5,\text{MeLeu}^9,\text{Nle}^{10}]\text{NKA}(4–10)\) (Table 1).

Reductions in responses to tachykinins in DD and UC might have been due to a general disease-related reduced responsiveness of smooth muscle to agonists. Thus, responses to ACh (expressed as an increase in grams of tension) were elicited in adjacent circular muscle strips from the same patients. However, responses were different in strips from patients with DD or UC compared with those from normal colon (Fig. 3, A and B).

Another possible explanation of differences might be related to different strip weights from the different disease groups. The circular muscle strips (mean ± S.E.M.) weighed 58 ± 2 (control), 60 ± 3 (DD), and 56 ± 2 mg (UC) and were not significantly different between patient groups (p = 0.79, one-way ANOVA).

Radioligand Binding Studies—Normal Colon. Binding of \(^{125}\text{I}\text{NKA}\) was highly specific (91–95%) in sigmoid colon circular muscle homogenates from normal colon. Binding of \(^{125}\text{I}\text{NKA}\) was saturable. \(^{125}\text{I}\text{NKA}\) bound to a single class of high-affinity binding sites (in 37 of 40 specimens). A gender-related difference in receptor density (\(B_{\text{max}}\)) was observed (Fig. 4); the \(B_{\text{max}}\) in female circular muscle was significantly lower than that in age-matched male muscle, but the affinity values (\(K_D\), 0.55 nM) in males and females were identical (Table 2). No age- or region-related differences in \(B_{\text{max}}\) and \(K_D\) were observed (data not shown).

Radioligand Binding Studies—Disease Groups. As seen for the control data, binding was of high capacity (Table 2). In 3 of 28 DD and 3 of 16 UC specimens, binding data were significantly better fitted to a two-site model compared with a one-site model. Unlike the normal colon, no gender-related differences in \(B_{\text{max}}\) were found for either DD or UC. No disease-related changes in \(B_{\text{max}}\) and \(K_D\) were observed (Table 2).

A disease-related difference in \(K_D\) was seen, with both control and DD groups significantly lower than UC, for both males and females (Table 2). \(K_D\) values in both DD groups were no different from their respective controls (Table 2).

Because the \(K_D\) values of males and females in each group were similar (Table 2), both gender groups were combined (Fig. 5). In this case, the affinity of all UC specimens (\(K_D\), 0.92, IQR 0.72–1.27, n = 16) for the NK₂ receptor was significantly reduced compared with controls (\(K_D\), 0.55, IQR 0.42–0.84, n = 40), but it was not different from DD (\(K_D\), 0.66, IQR 0.52–1.03, n = 28).

Although the median age of UC patients used for binding studies appeared lower than those of DD and control patients (Table 2), this was not significantly different. There were no significant correlations between age and binding parameters for any patient group (data not shown).

Molecular Studies—NK₂ Receptor QC-RT-PCR. QC-RT-PCR was carried out in normal colon to determine whether this gender-specific variation in NK₂ receptor protein expression corresponded to NK₂ receptor transcript levels. There were no gender- and age-related alterations in the expression of the housekeeping gene β-actin (Fig. 6A). NK₂
receptor mRNA expression (normalized to that of \( \text{H}9252/\text{actin} \)) in the same sample) was similar in males compared with age-matched females (Fig. 6B). The median (and IQR) expression level was 19.5 (8.8–37.6) \( 10^6 \) copies/g of total RNA in males and 16.7 (9.9–21.2) \( 10^6 \) copies in females (both \( n = 14 \), \( p = 0.05 \), Mann-Whitney test). The correlation analysis showed no age-related changes for males (\( n = 14 \), Spearman coefficient \( r = 0.24 \), \( p = 0.41 \), age range 32–93 years) or females (\( n = 14 \), \( r = 0.17 \), \( p = 0.58 \), age range 30–84 years) or in combined data from the two groups (\( n = 28 \), \( r = 0.16 \), \( p = 0.40 \)).

**Molecular Studies—COX RT-PCR.** In preliminary studies (\( n = 12 \)), strong constitutive expression of mRNA for both COX-1 and COX-2 was seen in normal circular muscle (Fig. 7). Expression of these two genes was also detected in the mucosa from the same specimens.

**Discussion**

In the human colon, contraction to NK\(_2\) agonists has been considered to be mediated directly rather than via neuronal or other mechanisms (Croci et al., 1998), although the presence of NK\(_2\) receptor immunoreactivity in myenteric neurons and nerve varicosities as well as smooth muscle (Jaafari et al., 2007; B. R. Southwell, unpublished data) suggests the
possibility of neuronal actions for NKA. The potentiation by indomethacin of contractile responses to NKA and [Lys5, MeLeu9, Nle10]NKA(4–10) seems to be a novel finding in the intestinal muscle, myenteric ganglia, and interstitial cells of Cajal (Bernardi et al., 2006). Our own preliminary data show mRNA for prostaglandin E2 receptors (EP1, EP2, EP3, and EP4) as well as COX-1 and COX-2 present in human colon muscularis (L. Liu, I. Markus, D. W. King, and E. Burcher, manuscript in preparation). Thus, we suggest that these NKA2 receptor agonists release prostanoids(s) from as yet unspecified sites, which may act to relax smooth muscle or to negatively modulate enteric cholinergic transmission in the normal sigmoid colon (Fig. 8). The latter mechanism is supported by our own observation in the human colon (L. Liu, I. Markus, D. W. King, and E. Burcher, manuscript in preparation) and by a recent finding in the rat colon that COX inhibitors potentiate muscle contraction evoked by nerve stimulation (Fornai et al., 2006).

DD is an inflammatory disorder that has a different etiology than UC and Crohn’s disease, and although DD has a much lower public profile, it is actually much more costly than IBD (Lewin Group; American Gastroenterological Association Report, 2001, http://www.gastro.org/user-assets/documents/burden-report.pdf). The most striking finding in DD was a large decrease in the direct component of the contractile responses to the tachykinin NKA2 receptor agonists NKA and [Lys5, MeLeu9, Nle10]NKA(4–10). Responses to SP were also attenuated in our earlier study using many of the same DD specimens (Liu et al., 2002). The reduced responsiveness to tachykinins in DD does not seem to be caused by a generalized reduction in responsiveness of smooth muscle, because responses to ACh were unchanged here and in other studies (Guagnini et al., 2006).

Reduced responsiveness to tachykinins in DD is unlikely to reflect an increased contribution from relaxant prostanoids, because responses to NKA and [Lys5, MeLeu9, Nle10]NKA(4–10) were affected (potentiated) by indomethacin to a degree similar to that seen in both control and DD; in other words,
NK2 receptor-stimulated prostanoid mechanisms remained unchanged in DD (Fig. 8). In normal human colon, contractile responses to SP are unaffected by indomethacin and are mainly mediated by NK2 receptors, but there is a small component of NK1 receptor-mediated atropine-sensitive contraction (Liu et al., 2002). Nevertheless, the role of COX in the normal and pathophysiological human intestine still remains unexplored. A recent study has shown that SP stimulates COX-2 expression and prostaglandin E2 production in human colonocytes transfected with human NK1 receptors (Koon et al., 2006). Abnormal levels of prostaglandins and COX enzymes have been linked to the impaired motor function in patients with slow transit constipation (Cong et al., 2007).

Motility changes have been reported in patients with DD (Bassotti et al., 2003) as well as UC (reviewed in Lecci et al., 2006). Bassotti et al. (2005) have reported a decrease in interstitial cells of Cajal and glial cells in DD, but no change in enteric neurons, and suggested that these changes might underlie the motor abnormalities found in DD. However, some striking alterations in neuronal mechanisms have been described in DD (Guagnini et al., 2006). The predominantly cholinergic responses to electrical field stimulation were inhibited by tetrodotoxin (TTX) in normal but not in DD colonic muscle (Maselli et al., 2004; Guagnini et al., 2006). The enhanced TTX-resistant contractions were reduced by SR140333, suggesting that, in DD, there is unmasking of the actions of TTX-resistant terminals that release tachykinin(s); alterations in cannabinoid mechanisms were also reported (Guagnini et al., 2006), suggesting that major changes occurred in enteric neuronal functions.

A diminution of functional responses to NKA and [Lys⁵, MeLeu⁶,Nle⁷]NKA(4–10) was also seen in UC, although the effect was not as great as that seen in DD. This decreased contractility to NKA and SP has been reported previously (Tomita et al., 2000; Menzies et al., 2001; Liu et al., 2002), and mechanisms have been explored. For example, in UC, there is evidence that interleukin-6 (Vrees et al., 2002) and hydrogen peroxide (Cao et al., 2004) contribute to the decrease of NKA-induced Ca²⁺ release from intracellular Ca²⁺ stores. There is also UC-induced remodeling of SP/NKA-ACh containing neurons of the enteric nervous system with an increased number of SP/NKA-immunoreactive neurons in UC patients (Neunlist et al., 2003). In this study, we found atropine to be without significant effect in modifying contractions to NK2 agonists in UC. The inter-relation between tachykinins and COX enzymes was not investigated in UC specimens. Nevertheless, a recent study in a rat model of colitis has shown that acute experimental inflammation impedes COX-1 activity and that COX-2 becomes the predominant isoform in maintaining an inhibitory control of colonic neuromuscular function (Fornai et al., 2006).

The mechanism underlying decreased contractility in UC seems to be different from that in DD. Our binding study using 125I-NKA showed that colonic muscle from patients with UC but not DD had a significantly lower affinity for the radioligand compared with normal colon. What was the cause for this decrease in Kᵦ in UC, and does this result explain the functional data in circular muscle strips? Firm evidence now exists for genetic variations in IBD, with four linkages identified based on genome-wide scanning and candidate gene analysis (Watts and Satsangi, 2002). However, it is not clear whether this effect explains the decrease in functional responses. The reduction in binding affinity seen in UC patients might result from changes in NK2 receptor structure or conformation, mechanisms of G-protein coupling, and/or receptor phosphorylation. Another explanation for the reduced receptor affinity in UC may be the consequence of an increased proportion of NK1 receptors in the total receptor capacity. NK1 receptor binding sites are massively up-regulated in colon specimens from UC patients, particularly on blood vessels located in external circular muscle, longitudinal muscle, and serosa (Mantyh et al., 1995; Renzi et al., 2000).

Because 125I-NKA has a low but not negligible affinity for NK1 receptors (Warner et al., 2000), the Kᵦ value measured would reflect the contribution of both NK2 and NK1 receptors, as manifested by the presence of two sites binding in some samples. The nature of these changes is impossible to determine from the present study. Nevertheless, it is clear that the reduced responses are receptor- and disease-specific, rather than the result of nonspecific damage to the tissue by...
inflammation, because responses to ACh were no different between control and disease. Our study found no differences in $B_{\text{max}}$ between disease groups and controls. Note that the $B_{\text{max}}$ (maximal number of binding sites) cannot be compared with $E_{\text{max}}$ (maximal response), because spare receptors exist and only a minority of receptors is required to produce a maximal response. However, the gender differences seen with the control $B_{\text{max}}$ data were surprising. The differences may be related to post-translational processing of the NK$_2$ receptor, because we found no evidence for gender differences in NK$_2$ receptor mRNA. Anecdotal evidence suggests that females show more gastrointestinal symptoms than males, and there is evidence for such differences in irritable bowel syndrome (Chang et al., 2001). In healthy subjects, colonic intestinal transit times are slower in women than in men, with some data also influenced by body mass, hormonal, and smoking status (Meier et al., 1996; Graff et al., 2001).

Age-related changes in human gut function have been reported, although the effect of increased incidence of gastrointestinal and other diseases may be more important than aging itself (for review, see Salles, 2007). Colonic motility is slower in older subjects compared with younger ones (Graff et al., 2001). However, our functional, binding, and molecular data showed no statistical differences or even trends for age-related changes. Previous aging studies suggest that there are degenerative changes in the enteric nervous system with age (for review, see Wade, 2002), and, in very old individuals, this degeneration seems to be more critical than changes in smooth muscle function.

In summary, we found that contractile responses to tachykinins in normal colon were enhanced by indomethacin, which has not been reported previously. In normal colon, we also observed gender-related differences in NK$_2$ receptor expression at the protein level but not at the mRNA level. In DD, our most impressive finding was the marked reduction of contractile responses, but this decrease was not accompanied by major differences in NK$_2$ receptor affinity or density. The reduced contractile responses were also seen in UC, but to a lesser extent than DD. However, there was a decrease in receptor affinity in UC for both male and female subjects. The large number of specimens studied and the comparison with carefully age-, gender-, and region-matched normal colon strengthens our conclusion of a reduced contractile response in DD.

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