Characterization and Autoradiographic Localization of Neurotensin Binding Sites in Human Sigmoid Colon

YAEI AZRIEL and ELIZABETH BURCHER
School of Physiology and Pharmacology, University of New South Wales, Australia

Received November 13, 2000; accepted January 30, 2001

ABSTRACT

Radioiodinated neurotensin (125I-NT) was used to characterize and localize NT binding sites in normal human sigmoid colon. Specimens were obtained from patients (30–77 years old) undergoing resection for colon carcinoma. Specific binding of 125I-NT to sigmoid circular muscle membranes was enhanced by o-phenanthroline (1 mM) but other peptidase inhibitors were ineffective. 125I-NT bound to a high-affinity site of KD = 0.88 ± 0.09 nM and Bmax = 4.03 ± 0.66 fmol/mg of wet weight tissue (n = 14), although in the majority of patients another site, of low but variable affinity, could also be detected. Specific binding of 50 μM 125I-NT was inhibited by NT(8-13) but variable affinity, could also be detected. Specific binding of 50 μM 125I-NT was enhanced by NT(8-13) but variable affinity, could also be detected. Specific binding of 50 μM 125I-NT was inhibited by NT(8-13) but variable affinity, could also be detected. Specific binding of 50 μM 125I-NT was inhibited by NT(8-13) but variable affinity, could also be detected. Specific binding of 50 μM 125I-NT was inhibited by NT(8-13) but variable affinity, could also be detected.

The brain-gut peptide neurotensin (NT) is a neurotransmitter and neuromodulator in the central nervous system, whereas it functions as a hormone in the gastrointestinal tract (Hermans and Maloteaux, 1998). NT is found in endocrine N cells of the distal jejunum and terminal ileum (Polak et al., 1977); exposure of these cells to ingested fats results in the release of NT, which acts postprandially to inhibit gastric acid secretion and regulate gastrointestinal motility (Rosell, 1982).

In vitro, NT is a potent spasmogen of intestinal smooth muscle, inducing both contraction and relaxation, depending on the species and region of the intestine studied (for review, see Kitabgi, 1982). In rats and guinea pigs, these actions have been attributed primarily to NT receptors located on the smooth muscle and on enteric neurons (Kitabgi and Freychet, 1978, 1979; Huidobro-Toro and Zhu, 1984; Carraway and Mitra, 1994; Labbé-Jullié et al., 1994; Mulè et al., 1995, 1996). These conclusions are supported by autoradiographic data demonstrating localization of NT binding sites on smooth muscle as well as myenteric and submucosal ganglia in guinea pig ileum (Goedert et al., 1984) and porcine jejunum (Seybold et al., 1990). In vivo, NT causes an increase in colonic motility in humans (Thor and Rosell, 1986). Current evidence suggests that NT-induced contraction of human colon circular muscle in vitro is mediated directly by NT receptors on the smooth muscle and therefore does not involve a neuronal component (Croci et al., 1999).

To date, three NT receptors with distinct functional and pharmacological properties have been identified: NT1, NT2, and nts3 (for review, see Le et al., 1996; Vincent et al., 1999). NT binds to all three receptors via its C-terminal hexapeptide sequence Arg-Arg-Pro-Tyr-Ile-Leu (Vincent et al., 1999). Both the NT1 and NT2 receptors are coupled to G proteins, whereas nts3 is structurally unrelated and is identical to the previously cloned gp95/sortilin receptor, which binds NT with high affinity (Zsürger et al., 1994; Mazella et al., 1998). NT has lower affinity for the NT2 receptor compared with the NT1 receptor (Schotte et al., 1986; Mazella et al., 1996; Vita et al., 1998).

Two widely used nonpeptide NT receptor antagonists, SR48692 (Gully et al., 1993; Labbé-Jullié et al., 1994) and the
chemically related, more potent SR142948A (Gully et al., 1997), have been developed but selective antagonists that are capable of differentiating between all three NT receptors are currently unavailable. Levocabastine is selective for the NT2 receptor in murine species and in transfected Chinese hamster ovary cells (Schotte et al., 1986), but this histamine H1 receptor antagonist is unsuitable for use in functional studies. The NT2 receptor is located primarily in the brain, with transcripts also detected in peripheral organs such as the heart and lungs (Chalon et al., 1996; Vita et al., 1998). In contrast, the NT1 receptor is extensively distributed in the brain as well as in the periphery, in particular the gastrointestinal tract (Labbé-Jullié et al., 1994).

Although well studied in animals, the actions of NT have been poorly investigated in humans. In this study, we have used radioligand binding techniques in human sigmoid circular muscle membranes to characterize the NT receptors mediating these actions. We have also provided preliminary functional and autoradiographic evidence for a direct and a neurally mediated component of the actions of NT on the circular muscle of the human colon.

**Experimental Procedures**

**Materials.** NT was purchased from Auspep, Melbourne, Australia. Radiolabeled NT (125I-Tyr3-NT), initial specific activity 2200 Ci/mmol, was synthesized by PerkinElmer Life Science Products (Boston, MA). The two NT receptor antagonists 2-[(4-[(chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]-adamantyl-2-carboxylic acid (SR48692) (Gully et al., 1993) and 2-[5-[(3,5-dimethylaminopropyl)-N-methyleneacetamidol]-isopropyl-phenyl)-1H-pyrazole-3-carbonyl]-amino]- adamantane-2-carboxylic acid (SR142948A) (Gully et al., 1997) were gifts from Dr. D. Gully, Sanofi-Synthélabo Recherche, Montpellier, France. cFP [N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate] was a gift from Dr. R. Lew (Baker Institute, Melbourne, Australia) and other peptidase inhibitors were purchased from Sigma Chemical Co. (Sydney, Australia). Levocabastine was a gift from Dr. X. P. Zeng (Therapeutic Goods Dep., Canberra, Australia). All other chemicals used in this study were of high chemical grade quality.

**Specimen Collection.** Human sigmoid colon specimens were obtained from male and female patients (30–77 years old, n = 36) undergoing resection for adenocarcinoma. Whole ring segments (4 cm) of “normal” colon were taken, 10 to 20 cm from the tumor. These were placed immediately in cold carbogenated (95% O2, 5% CO2) Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 11.7 mM glucose) and the external fatty tissue removed. The macroscopic appearance was assessed on arrival at the laboratory and the specimen was rejected if any inflammation or other abnormalities were observed. This study was approved by the University of New South Wales Human Ethics Committee (no. 97139).

**Radioligand Binding Methodology.** Tissue was either stored overnight at 4°C in carbogenated Krebs-Henseleit solution or dissected immediately. The serosa, mucosa, and submucosal layers were removed, leaving the muscularis. The circular muscle was separated from the taenia coli and sectioned into 500-mg portions, snap frozen in liquid nitrogen, and stored at −70°C until the day of the experiment. This circular muscle preparation also contained a thin layer of longitudinal muscle and the myenteric plexus. Crude membrane homogenates were prepared from the stored frozen muscle, using a method described elsewhere (Lew et al., 1990). Membrane homogenates (final concentration 2%) were then suspended in incubation buffer, containing 50 mM Tris-HCl, 0.02% bovine serum albumin (BSA), 1 mM MgCl2, and 50 pM 125I-Tyr3-NT (125I-NT), and incubated for 60 min at 25°C, in duplicate. Nonspecific binding was defined with 1 μM NT (and was no different when SR48692 was used). Membrane-bound radioligand was separated from free ligand by rapid filtration under reduced pressure through Whatman glass GF/B filters (previously soaked in 0.1% polyethyleneimine), using a 48-well cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with cold Tris-HCl containing 0.02% BSA and 1 mM MgCl2, placed in glass tubes, and counted in a Wallac Wizard gamma spectrometer 1470 (>78% efficiency).

In preliminary experiments, the effects of various peptidase inhibitors 1,10-ortho-phenoanthrolene (1 mM), phosphoramidon (10 μM), bacitracin (40 μg/ml), z, z-prolinal (1 μM), cFP (25 μM), captopril (1 μM), benztin (50 μM), and EDTA (1 mM) were investigated. These concentrations were based on previous studies of NT metabolism in rat brain (Checher et al., 1986) and fundus (Checher et al., 1987).

“Cold” saturation studies were carried out on membrane preparations (2%) suspended in incubation buffer containing o-phenoanthrolene (1 mM). Varying concentrations of unlabeled NT (1 μM–1 μM) and a fixed concentration of 125I-NT (50 pM) were added to each tube. In competition studies, varying concentrations of unlabeled NT, the fragment NT (8–13), the biologically related peptide neuropeptin N, the nonpeptide NT receptor antagonists SR48692 and SR142948A, and the histamine H1 receptor antagonist levocabastine were coincubated with 50 pM 125I-NT in incubation buffer at 25°C for 60 min.

**Data Analysis.** All experiments investigating peptidase inhibitors were performed in triplicate, using specimens from four to eight patients. The specific binding in the presence of each compound was expressed as a percentage of control (no peptidase inhibitors). In preliminary experiments, o-phenoanthrolene, phosphoramidon, bacitracin, z, z-prolinal, cFP, captopril, and EDTA were compared with control, using ANOVA followed by Dunnett’s test. In subsequent experiments, bestatin, 1,10-ortho-phenoanthrolene, and a combination of inhibitors (o-phenoanthrolene, phosphoramidon, and cFP) were compared, using ANOVA followed by Bonferroni’s test.

In cold saturation experiments (patients aged 52–77 years, n = 14) the receptor binding constants Kd (receptor affinity) and Bmax (density of binding sites) were determined from the raw data generated using the analytical programs EDBA and LIGAND. Data were analyzed using single- and multiple site models and the F test was used to determine the most appropriate model. P < 0.05 was considered statistically significant. In competition studies, the inhibition constant Ki and the slope factors were calculated from competition curves generated by PRISM. Unless otherwise stated, data are expressed as the mean ± S.E.M. Data were depicted graphically using the program PRISM (GraphPad Software, San Diego, CA).

**Autoradiographic Studies.** Small segments of colon with all layers intact were mounted into small blocks containing octane compound (Tissue-Tek, Sakura Finetek, Torrance, CA). The blocks were snap frozen in liquid nitrogen and stored at −70°C. Transverse sections (10 μm) of sigmoid colon were cut on a cryostat and thaw mounted onto acid cleaned “subbed” microscope slides, air dried at −20°C, and stored desiccated at −70°C. Slide-mounted sections were preincubated (3 × 5 min washes) in buffer containing 50 mM Tris-HCl and 0.02% BSA (pH 7.4, 25°C). Sections were then incubated for 60 min at 25°C in 5 ml of incubation buffer containing 50 pM 125I-NT, 50 mM Tris-HCl, 0.02% BSA, 1 mM o-phenoanthrolene, and 1 mM MgCl2. Adjacent sections to illustrate nonspecific binding were coincubated with 1 μM NT or 100 μM levocabastine. Radiolabeling was terminated by washing sections (4 × 3 min) in ice-cold 50 mM Tris-HCl (pH 7.4, 4°C) containing 0.02% BSA and 1 mM MgCl2, followed by 2 × 10 s rinses in distilled water and rapid drying. Sections were fixed in paraformaldehyde vapor at 70°C for 30 min and then dipped in molten photographic emulsion (LM-1; Amersham Pharmacia Biotech, Buckinghamshire, UK). Emulsion-coated sections were exposed in the dark for 5 days before photographic development and fixation. The radiolabeled sections were then stained with pyronin Y. Adjacent slide-mounted sections were stained with

**Neurotensin Receptors in Human Colon**

1075
Fig. 1. Effect of peptidase inhibitors on specific binding of 125I-NT to membranes from human sigmoid circular muscle. Data represent specific bound cpm and are expressed as a percentage of control without peptidase inhibitors. Columns are mean ± S.E.M. from four to eight individual specimens. **P < 0.01 (ANOVA) for difference relative to control.

H&E to demonstrate histological features. Slides were photographed under bright and/or dark field.

**Functional Studies.** After overnight storage of the specimen at 4°C in carboxenated Krebs-Henseleit solution, circular muscle was carefully separated from the mucosal, submucosal, and serosal layers of the colon. Circular muscle strips (10 × 3 mm) were cut along the circular axis. Strips also contained a thin layer of longitudinal muscle and the myenteric plexus remained intact in these preparations. Strips were mounted in 2-ml organ baths containing Krebs’ solution (37°C) gassed with carbogen, and the activity of the muscle strips was measured using Grass FT03 (Quincy, MA) isometric force displacement transducers. Organ baths were siliconized to prevent peptides adhering to the glass surface. Strips were adjusted to 1-g tension and allowed to equilibrate for 1 h. Following equilibration, acetylcholine (Ach; final concentration 10 mM) was added to each organ bath and the maximal contraction of each strip recorded. Strips were then washed several times and allowed to equilibrate further (1 h) before commencing the experiment.

Concentration-response curves were constructed by the sequential addition of NT in concentrations ranging from 100 pM to 10 μM. To avoid tachyphylaxis, each concentration of NT was added at discrete time intervals, ranging from 15 to 90 min, depending on the concentration and size of contraction. Contact of the tissue with NT was allowed to continue until the response induced by NT had reached a maximum (usually 5–8 min) before washing. Concentration-response curves for NT, NT (8-13) and neuromedin N were constructed and the potency and efficacy was compared with NT.

The direct action of NT on the smooth muscle was investigated by the addition of a submaximal concentration of NT (100 nM), at 60- to 90-min intervals until reproducible responses were observed. The neuronal toxin tetrodotoxin (TTX, 1 μM) was added to the preparation and incubated for at least 15 min before two to three further responses to NT (100 nM) were obtained. A time interval of at least 1 h was allowed before each addition of NT. In certain preparations, electrical field stimulation (0.5–40 Hz, 0.1-mV pulse width, 70 V, and 10-s duration) was also performed to confirm neuronal viability. At the completion of the experiment, Ach (10 mM) was again added to each preparation and the maximum contractile response recorded.

Fig. 2. Representative saturation (A) and Scatchard (B) plot calculated from cold saturation experiments with 50 pM 125I-NT in circular muscle membranes. Scatchard plot suggests binding to two separate sites: a high-affinity site (Kd = 1.3 nM and Bmax = 1.7 fmol/mg of wet weight tissue) and a site of lower affinity (Kd = 50 nM and Bmax = 7.4 fmol/mg of wet weight tissue). Fourteen independent experiments were performed in duplicate. Nonspecific binding was defined with 1 μM NT.

Isometric tension was recorded using a computer program (Polygraph; E. Crawford, University of New South Wales, Sydney, Australia). The contractile responses (in grams) were measured from baseline to the maximum height of the tonic contraction. Phasic contractions were not included in these measurements. Responses to NT were expressed as a percentage of the maximum response induced by Ach (10 mM). Final analyses of the data (calculations of EC50, pD2 and statistical tests) were performed using PRISM.

**Results**

**Radioligand Binding: Effect of Peptidase Inhibitors.** Of the peptidase inhibitors tested, only o-phenanthroline significantly increased specific binding of 125I-NT to sigmoid circular muscle membranes by 99 ± 19% (n = 8, ANOVA, P < 0.01). The remaining seven peptidase inhibitors investigated did not significantly enhance or reduce specific binding (Fig. 1). The combination of o-phenanthroline, cFP, and phosphoramidon also resulted in a significant increase in specific binding by 90 ± 19% compared with control (n = 4, ANOVA, P < 0.01), but this was no different to that observed with o-phenanthroline alone. Therefore, in subsequent binding experiments, o-phenanthroline was the only inhibitor incorporated into the incubation system to protect the radioligand from proteolytic degradation.

**Radioligand Binding: Cold Saturation Studies.** Cold saturation studies were performed with 125I-NT in sigmoid colon circular muscle membranes. Results from a typical experiment are shown in Fig. 2. For most experiments, curvilinear Scatchard plots were obtained and the binding could be resolved into two sites, even when a one-site fit was
A value of 0.88 ± 0.09 nM (n = 14) was obtained for the higher affinity site, using computer-assisted analysis, with a corresponding B_max of 4.03 ± 0.66 fmol/mg of wet weight tissue (n = 14). The K_d values obtained for the second site were extremely variable, ranging from 3 nM to 12 μM, and therefore a reliable estimate of the affinity of this site could not be obtained.

Radioligand Binding: Competition Studies. The pharmacological properties of the 125I-NT binding site were characterized in sigmoid circular muscle membranes using biologically active agonists and antagonists at the NT1 receptor. The most potent competitor for the 125I-NT binding site was NT(8-13). SR142984A was considerably more potent than SR48692. The overall rank order of affinity was NT(8-13) > NT > SR142984A > neuromedin N > SR48692 (Fig. 3). Levocabastine did not compete for 125I-NT binding sites in these membrane preparations. In contrast to the results obtained in cold saturation studies, the slope factor determined for each competitor was high (close to unity), indicating the presence of only one binding site (Table 1). Binding was sensitive to GTPγS (Fig. 3).

Autoradiographic Localization of NT Receptors. The specific binding of 125I-NT in all autoradiographic experiments was high (98 ± 0.86% of total binding). In transverse sections of sigmoid colon (n = 4), 125I-NT binding sites were densely and uniformly distributed over the circular muscle (Figs. 4 and 5). Specific binding over the longitudinal muscle was considerably less than that observed for circular muscle. The highest density of binding was observed on myenteric ganglia (Fig. 5). Dense specific binding was also observed on ganglia of the submucosal plexus, particularly on those adjacent to the circular muscle rather than those adjacent to the muscularis mucosae (Fig. 4). There was no specific binding associated with blood vessels or with the mucosa (Fig. 4). Coincubation with levocabastine had no effect.

TABLE 1

<table>
<thead>
<tr>
<th>Competitor</th>
<th>K_i (nM)</th>
<th>Relative Affinity</th>
<th>Slope Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotensin</td>
<td>0.89</td>
<td>100</td>
<td>0.90</td>
</tr>
<tr>
<td>Neurotensin (8-13)</td>
<td>0.63</td>
<td>141</td>
<td>0.90</td>
</tr>
<tr>
<td>Neuromedin N</td>
<td>2.65</td>
<td>34</td>
<td>0.81</td>
</tr>
<tr>
<td>SR142984A</td>
<td>1.55</td>
<td>57</td>
<td>0.95</td>
</tr>
<tr>
<td>SR48692</td>
<td>6.71</td>
<td>13</td>
<td>0.70</td>
</tr>
<tr>
<td>Levocabastine</td>
<td>&gt;10,000</td>
<td>&lt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Competition by NT and NT receptor agonists and antagonists for 125I-NT binding in circular muscle membrane membranes. (○) NT, dashed line; (■) NT(8-13); (○) neuromedin N, (△) SR142984A; (□) SR48692. Binding was GTPγS-sensitive, as shown by the decrease in binding when NT was coincubated with GTPγS (10 μM) (●). Curves represent mean values ± S.E.M. of 4 to 14 independent experiments performed in duplicate.

Fig. 4. Photomicrographs of 125I-NT binding sites in transverse sections of human sigmoid colon. Lightfield photomicrograph of histological section (A), total binding (B), and nonspecific binding (C) are darkfield photomicrographs of adjacent sections labeled with 125I-NT. D, high-power photomicrograph of submucosal ganglia (+) depicted in photomicrograph B, showing binding on submucosal ganglia from the outer submucosal plexus. Moderate specific binding of 125I-NT occurs over the circular muscle with dense binding over submucosal ganglia. There is an absence of specific binding on blood vessels (large arrow in D), the muscularis mucosae, and the mucosa. cm, circular muscle; g, submucosal ganglia; bv, blood vessel; mm, muscularis mucosae; mu, mucosa. Scale bar, 200 μm.
Functional Studies: Agonist Potency and Efficacy.
Contractile responses were elicited to NT, the C-terminal fragment NT (8-13) \((n = 5)\), and the related agonist neuromedin N \((n = 5)\) (Fig. 6). The potency of NT (8-13) and neuromedin N was similar, with \(pD_2\) values 6.4 ± 0.3 and 6.4 ± 0.2, respectively; these were not significantly different to that of NT \((pD_2 = 6.8 ± 0.2)\). Furthermore, the maximum responses induced by these agonists, NT (8-13) \((33 ± 6\%)\) and neuromedin N \((41 ± 6\%)\), were not significantly different to that of NT \((32 ± 4\%)\) (ANOVA).

Functional Studies: Effect of TTX. A representative experimental trace is shown in Fig. 7. Initially, at least three responses were obtained to NT \((100 \text{ nM})\) in the absence of TTX. The initial response was approximately 2-fold larger than the second response to NT, but there was no difference in size between the second and third responses. Consistent responses to NT were only achieved following three expo-

![Fig. 6. Concentration-response curves to NT (■), dashed line; NT(8-13) (□); and neuromedin N (○) elicited in circular muscle strips. Data show mean ± S.E.M. of 5 to 25 experiments. No differences in potency or efficacy were seen (ANOVA).](image)
sures to NT. Addition of TTX to the bath resulted in a slight increase in tension of the circular muscle, in most strips. The effect of TTX on responses to NT was variable and its overall effect \( (n = 8) \) is illustrated graphically in Fig. 8. In four patients, the magnitude of the NT responses after TTX was increased by approximately 80\%, relative to that of the response immediately before; that is, responses were restored to a level similar to that of the first NT response in that preparation (Fig. 7). In the remaining patients, TTX either caused an absolute potentiation of response relative to the initial response \( (n = 2) \) or had no effect \( (n = 2) \). A reduction in contractile response to NT after addition of TTX was never observed under our experimental conditions.

**Discussion**

The present study appears to be the first report of the characterization of \(^{125}\)I-NT binding sites in the human colon. Specific binding of \(^{125}\)I-NT was enhanced by the metalloproteinase inhibitor o-phenanthroline, which inhibits cleavage of NT at the Pro\(^{10}\)-Tyr\(^{11}\) bond by endopeptidase 3.4.24.16 (Checler et al., 1986, 1987). The degradation of NT in human sigmoid circular muscle membrane homogenates appears to be primarily mediated by this endopeptidase, as other inhibitors were ineffective.

Results from cold saturation experiments yielded curvilinear Scatchard plots, indicating the binding of \(^{125}\)I-NT to two sites, one of high affinity \( (K_d = 0.88 \text{ nM}) \) and one of lower affinity. The dissociation constant obtained for this high-affinity site in human sigmoid colon circular muscle is consistent with previously documented values \( (K_d = 0.6 \text{ nM}) \) for the human cloned NT1 receptor (Vita et al., 1993). Competition studies revealed a rank order of potency similar to that previously described for the human NT1 receptor (Ahmad and Daniel, 1991; Vita et al., 1993; Gully et al., 1997). The potency order for the NT receptor agonists was NT \( (8-13) \geq NT > \text{neuromedin N} \), and the NT receptor antagonist SR142948A was more potent than the antagonist SR48692. This correlates well with the potency observed in functional studies in the human colon (Croci et al., 1999) and in other systems (Gully et al., 1997). This suggests that the high-affinity binding site for \(^{125}\)I-NT corresponds to the NT1 receptor.

Membranes from rat brain typically contain two classes of binding sites: a high-affinity \( (K_d = \sim 0.5 \text{ nM}) \) site corresponding to the NT1 receptor, and a low-affinity \( (K_d = \sim 3 \text{ nM}) \), levocabastine-sensitive site corresponding to the NT2 receptor (for review, see Vincent et al., 1999). Recent pharmacological studies have demonstrated levocabastine-sensitive \(^{125}\)I-NT binding to the human (Vita et al., 1998) as well as the mouse NT2 receptor (Schotte et al., 1986). Although our data in the colon suggest binding to two sites, the concentration of ligand that we were able to use was inadequate in properly defining the low-affinity site. However, in both membrane binding and autoradiographic studies, levocabastine showed no affinity for \(^{125}\)I-NT binding sites. These results are in agreement with previous reports showing the absence of NT2 messenger RNA transcripts in circular muscle from human sigmoid colon (Croci et al., 1999). Thus, the low-affinity site indicated by our studies is unlikely to be the NT2 receptor.

Our autoradiographic studies represent important evidence for the presence of both neuronal and muscular NT receptors in human sigmoid colon. The localization of \(^{125}\)I-NT binding sites on ganglia and muscle is consistent with previous autoradiographic studies in guinea pig ileum (Goedert et al., 1984), porcine jejunum (Seybold et al., 1990), and also from human colon tissue directly surrounding carcinoid tumors (Reubi et al., 1999). In our study, the lower density of silver grains on the circular muscle compared with the myenteric ganglia is most likely due to a difference in receptor number. However the existence of two populations of receptors, with those of high affinity on enteric ganglia and those of moderate affinity on circular muscle, cannot be excluded.
Whether these might represent different receptor subtypes corresponding to the low- and high-affinity binding sites identified here could not be determined, as selective antagonists are currently unavailable.

A large body of evidence from rat, guinea pig, and canine isolated intestine has demonstrated both neuronal and direct actions of NT (Kitabgi and Freychet, 1978; Fox et al., 1987; Mulè et al., 1995). In rodents, NT induces contraction of intestinal smooth muscle via activation of NT receptors on nerves (primarily cholinergic) and to a lesser extent by a direct action of NT on the smooth muscle (Kitabgi and Freychet, 1978, 1979; Ohashi et al., 1994; Mulè et al., 1995). In the nonhuman intestine, NT also induces a direct myogenic relaxation (Kitabgi and Freychet, 1978, 1979; Huidobro-Toro and Zhu, 1984; Allescher et al., 1992; Ohashi et al., 1994; Mulè and Serio, 1997). These data are in contrast to results from limited studies using isolated smooth muscle preparations from human colon, which have indicated only direct, contractile actions of NT (Bennett et al., 1992; Maselli et al., 1998; Croci et al., 1999), which were insensitive to TTX (Croci et al., 1999). However, our studies with TTX (discussed below) clearly demonstrate a neuronal component as well as a direct contractile response. Therefore, the localization of 125I-NT binding sites to myenteric ganglia and submucosal ganglia as well as to circular muscle is consistent with both indirect (neuronal) and direct mechanisms of action of NT and suggests for the first time a role for NT in neuronal mechanisms in the human colon.

Our preliminary functional data indicate that NT induces multiple actions in the human sigmoid colon. Under our experimental conditions, both contraction and relaxation could be observed in response to NT in isolated circular muscle (Fig. 7). When several consecutive responses to the same submaximal concentration of NT were elicited, a diminution of the second response was nearly always observed, suggesting desensitization induced by the initial concentration. Following this, responses became stable. The subsequent addition of TTX resulted either in an absolute enhancement of the contractile response to NT or restoration of the response to the magnitude of the initial response (Figs. 7 and 8). These results are in contrast to a recent report that the contraction induced by NT was insensitive to TTX, in human sigmoid and transverse circular muscle strips (Croci et al., 1999). These differences observed with TTX may be due to the use of cumulative concentration-response curves by Croci et al. (1999) in contrast to the discrete responses used in our study, and also to their use of an initial maximum concentration of NT in each strip.

Functional experiments with TTX complement our autoradiographic data to support a neuronal component in the mechanism of action of NT. Our results with TTX suggest that NT is able to induce release of a relaxant neuronal mediator, perhaps nitric oxide and/or vasoactive intestinal peptide, but do not exclude the possibilities that NT has direct relaxant actions or induces the release of contractile neuronal mediators. The mechanism in humans appears unlike that in rodents, where the contractile response is primarily neuronal (Kitabgi, 1982; Mulè et al., 1995). Although acetylcholine appears to be an important neuronal mediator in guinea pig ileum and rat colon (Kitabgi and Freychet, 1978, 1979; Mulè et al., 1995), responses to NT in human sigmoid colon circular muscle appear insensitive to atropine, suggesting that acetylcholine is not involved (Croci et al., 1999). Tachykinins or even prostaglandins might also be involved as mediators following neuronal excitation, since there is evidence for this in the guinea pig ileum (Carraway and Mitra, 1994; Nguyen-Le et al., 1997). We are currently investigating some of these possibilities and our data suggest quite complex mechanisms, which will be the subject of a future report.

It was notable that the potencies of NT, NT(8-13), and neuromedin N were substantially lower in functional studies (Fig. 7) compared with competition binding studies (Fig. 3). Moreover, the potency order in functional studies (NT > NT (8-13) = neuromedin N) was somewhat different from that seen in binding studies. One possible explanation could be due to the use of the proteolytic enzyme inhibitor o-phenanthroline in binding studies but not in functional studies, contributing to the low functional potency of NT and NT1 receptor agonists. Widely differing EC50 values for the contractile responses to NT on colon sigmoid colon circular muscle have been reported, ranging from ~5 to 10 nM (Bennett et al., 1992; Croci et al., 1999) to ~1 μM (Maselli et al., 1998). Since peptidase inhibitors were not used in any of these studies, proteolytic degradation of NT may be partly responsible for the discrepancy observed between studies. However, the EC50 value that we report here (~160 nM) changes in the presence of a number of inhibitors (data not shown) and discrepancies between laboratories may also represent diverse experimental conditions that favor, to different extents, the involvement of various indirect components of the actions of NT. Similarly, the difference in Kd and functional potency of NT in this study may be at least partly attributed to the numerous components in the mechanism of action of NT. Comparable receptor affinity and potency values may be obtained once these separate components have been isolated with the use of TTX as well as other non-neuronal inhibitors. These studies are currently underway in our laboratory.

In conclusion, the results from autoradiographic and functional studies with TTX support the indirect and direct actions of NT and the involvement of complex mechanisms in human sigmoid colon circular muscle. Although there is evidence for NT receptors on myenteric neurons, the nature of the NT binding sites on nerves was not clarified in this study. Binding studies also confirm the presence of NT1 receptors in the human sigmoid colon circular muscle and provide a preliminary, rather weak indication of another NT receptor subtype in the human sigmoid colon. Further functional and binding studies involving all regions of the human colon and intestine are needed to provide more conclusive evidence. The development of antagonists or agonists with greater receptor selectivity will be invaluable for the further characterization of NT receptor systems in both the periphery and central nervous system.

Acknowledgments

We thank Dr. D. Z. Lubowski and Dr. D. W. King for supply of surgical specimens and Fei Shang for technical assistance.

References


Send reprint requests to: Professor E. Burcher, School of Physiology and Pharmacology University of New South Wales, NSW 2052, Australia. E-mail: e.burcher@unsw.edu.au