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In vitro functional evidence of different neurotensin-receptors modulating the motor response of human colonic muscle strips

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1 The newly developed non-peptide neurotensin (NT)-receptor antagonists SR 48692 and SR 142948 were used to challenge NT responses of human colonic circular smooth muscle strips *in vitro*. The presence of NT_1 and NT_2 receptor transcripts in this tissue was tested by reverse transcriptase polymerase chain reaction (RT–PCR) analysis.

2 NT potently and dose-dependently contracted muscle strips, with significant regional differences in potency and efficacy between the transverse and distal colon: EC_{50}, 3.6 and 7.5 nM; the maximal effect was 70 and 55% of 0.1 mM carbachol. Colonic responses to NT in both segments were virtually the same in the presence of atropine (1 μM), levocabastine (10 μM) or tetrodotoxin (1 μM).

3 SR 142948 (10 nM–1 μM) competitively antagonized NT responses in the transverse and distal colon with similar affinities: pA_2 values 8.71 and 8.45, slopes 0.98 and 0.99. SR 48692 (10 nM–10 μM) antagonized the NT response competitively in the distal colon (pA_2 6.55, slope 0.79) and non-competitively in the transverse colon (pA_2 8.0, slope 0.51). Neither compound had any agonist effect.

4 The fact that the specific antagonists prevented NT-evoked atropine- and tetrodotoxin-insensitive mechanical responses of colonic muscle strips is highly consistent with the presence in these tissues of non-neuronal NT receptors, whose heterogeneity in the transverse segment is supported by the non-competitive antagonism of SR 48692. The finding of NT_1 receptor transcript in both transverse and distal colon suggests its identity with the lower affinity site disclosed functionally by SR 48692 in these segments.

Keywords: Human colon; neurotensin; NT_1 receptor; NT_2 receptor; SR 142948; SR 48692; gut

Abbreviations: CHO, Chinese hamster ovary cells; DMSO, dimethylsulphoxide; DTT, dithiothreitol; NT, neurotensin; RT–PCR, reverse transcriptase polymerase chain reaction

Introduction

Neurotensin (NT) has a potent spasmogenic effect on the mammalian gut through activation of specific receptors (Labbé-Jullié *et al*., 1994; Mulé & Serio, 1997). Its action on intestinal smooth muscle is complex and has not been fully investigated. *In vitro*, NT elicits contractions but also relaxation of rat colonic preparations depending on the experimental conditions (Mulé & Serio, 1997); it acts either directly on smooth muscle or indirectly through the neuronal release of acetylcholine and substance P (Mulé *et al*., 1996).

The heterogeneity of NT receptors has been suggested on functional grounds by *in vivo* rodent studies showing that SR 48692 antagonized some of the neuropeptide's effects (i.e. hypomotility and behavioural excitation), but not its ability to lower body temperature, raise pain threshold and produce some neurochemical changes (Dubuc *et al*., 1994; Steinberg *et al*., 1995). The cloning of rodent (Tanaka *et al*., 1990) and human NT-receptors from the HT 29 cell line (Vita *et al*., 1993) resulted in sites with high-affinity *in vitro* specific binding (NT_1); a low-affinity site (NT_2) was also cloned from rat brain (Chalon *et al*., 1996) and micromolar concentrations of the histamine H_1 receptor antagonist levocabastine completely prevented binding at this site (Gully *et al*., 1993; 1997). Levocabastine and SR 48692 reportedly have an agonist effect (stimulation of intracellular Ca^{2+} mobilization) in Chinese hamster ovary cells (CHO) expressing rat NT_2 receptors (Yamada *et al*., 1998).

Recently the human NT_2 receptor was also cloned from a brain cortex cDNA library and stably expressed in CHO cells (Vita *et al*., 1998). The structural homology between human and rodent NT_2 receptors is extensive, about 80%; the human NT_2 receptor also binds levocabastine but with lower affinity than the rodent receptor. However several other features apply to the human recombinant NT_2 receptor expressed in CHO cells: neither NT nor levocabastine evoked any response, but both inhibited the agonist responses to SR 48692 and SR 142948; both the latter enhanced inositol formation and Ca^{2+} mobilization; they also stimulated arachidonic acid release and mitogen-activated protein kinase activity. Thus a third NT receptor subtype (gP 95/Sertilin) has been cloned from a human brain cDNA library; its functional properties are currently unknown but it is the first transmembrane neuropeptide receptor that does not belong to the superfamily of G-protein coupled receptors (Mazzella *et al*., 1998).

Since species differences have been noted for NT receptors (Cusack *et al*., 1995), functional studies to assess the affinity of the novel non-peptide specific antagonists for native human receptors are needed to clarify their therapeutic potential.

We recorded the mechanical responses of human colonic circular smooth muscle preparations elicited *in vitro* by NT, to

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investigate the pharmacological properties of its receptors, including their susceptibility to appropriate antagonists. A preliminary account of this work was presented at the DDW, May 11–14, 1997, Washington DC, U.S.A. (Croci *et al*., 1997). This study was approved by the ethics committee of the San Raffaele Hospital, Milan.

Methods

Tissue preparation

Specimens of human tranverse or distal colon from macroscopically normal regions were obtained from patients (16 males and 18 females, aged 55–78) undergoing surgery for colonic cancer (60% rectum, 40% transverse) at the San Raffaele Hospital, Milan. Patients had not received radiotherapy and had not been treated chronically with steroids or chemotherapeutics. However, two were taking enalapril for anti-hypertensive therapy, two a calcium antagonist and three the H_2-receptor antagonist ranitidine. Uncontrolled medication history, standard anaesthesia and other possible differences between samples apparently had no influence on the NT response from the colon of different patients in this study. Specimens were available at the operating theatre, each consisting of a whole colon segment; they were washed in saline and immediately placed in a cold (4°C) pre-aerated (95% O_2, 5% CO_2) Krebs' solution (composition mM, NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7) and transported to Sanofi laboratories within about 30 min.

Mucosa and sub-mucosa were gently removed and muscular regions between the taenia coli were cut into strips approximately 3 mm wide along the circular axis (total length of each preparation 2 cm). Smooth muscle strips stored overnight (16–18 h) in cold (4°C) pre-aerated Krebs' solution to reduce spontaneous phasic contractions and tonus maintained their full sensitivity to different peptide and non-peptide stimulants, consistent with previous reports (Couture *et al*., 1981; Croci *et al*., 1998). Under our experimental conditions, the pattern of spontaneous activity was reduced but still present in some tissue and strips from the same colon specimen. However, the low degree of phasic activity did not hamper assessment of tonic contraction. Strips from transverse or distal colon were stored at −80°C for later RT–PCR assay.

Experimental conditions

Eighteen to twenty-four strips were dissected from each specimen, allowing direct comparison of agonist and antagonist activities. A few responses were checked in duplicate, in which case the results were always averaged and considered as one from the same specimen.

Colonic strips were mounted in a 20-ml organ bath containing warm (37°C) aerated (95% O_2, 5% CO_2) Krebs' solution and stretched with 1–1.5 g; they were washed and allowed to equilibrate for 1 h, then challenged with a primer concentration (1 μM) of NT and washed five times. Contractions were recorded isotonically. About 2 h later, a cumulative agonist concentration-response curve (contact time 5–10 min) was plotted, followed – after a similar interval – by a second one; results were always expressed as a percentage of the maximal contraction given by the first reference curve. Five washout procedures were done immediately after the first cumulative agonist concentration curve. The response to carbachol (0.1 mM) was determined at the end of the experiment. For each specimen, at least one preparation that had given two reproducible cumulative concentration-response curves was used as control; this confirmed that desensitization did not occur under our experimental conditions. Only one NT-receptor antagonist was tested on each strip.

Antagonist incubation times were: 1 h for SR 48692, SR 142948, indomethacin; 30 min for atropine and levocabastine, 15 min for tetrodotoxin. Control tissues were incubated only with the drug vehicles (NT antagonists were dissolved in 2% DMSO (dimethylsulphoxide), NT in distilled water). Four to

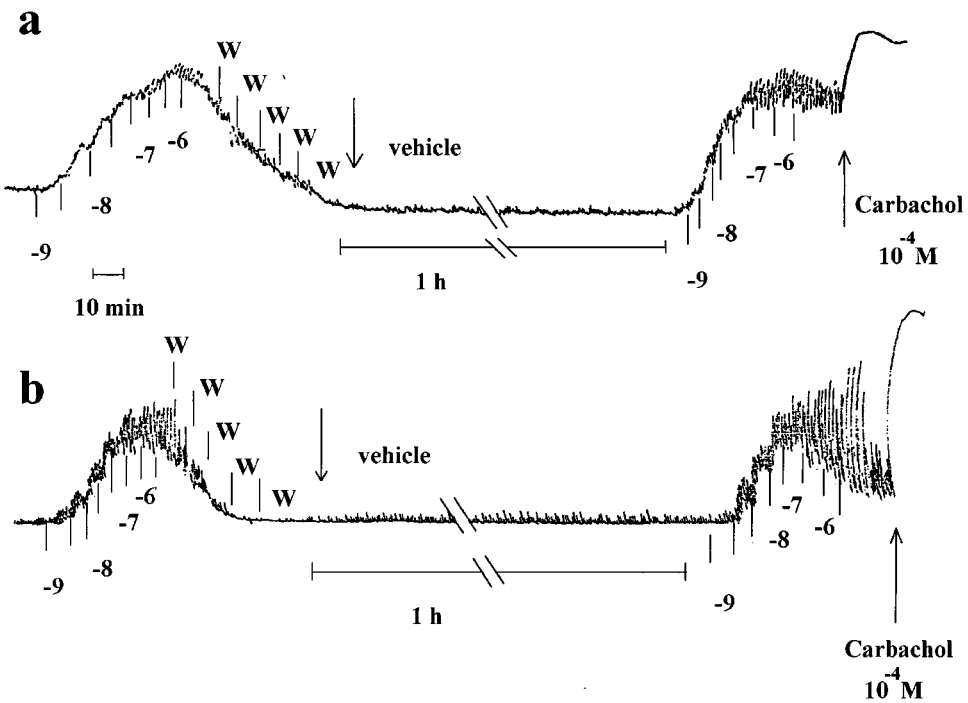

Figure 1 Representative tracings of the contractile response of human transverse (a) and distal (b) colonic circular smooth muscle strips to NT.

twelve different colonic preparations were used for each cumulative concentration-response curve. Figure 1 shows representative tracings of human transverse and distal colonic circular smooth muscle contracted by NT.

Transcript analysis of human $NT_1(hNT_1)$ and $NT_2(hNT_2)$ receptors by RT–PCR

Total RNA from circular muscle of human colon was prepared by the acid-guanidium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987).

cDNA synthesis was performed using 5 μg of total RNA incubated for 1 h at 37°C with 20 μl of a reaction mixture containing Tris-HCl (pH 8.3) 50 mM, DTT (dithiothreitol) 3 mM, KCl 10 mM, dNTP 0.5 mM, 40 U Rnasin (Promega, Madison, WI, U.S.A.), 200 U superscript II reverse transcriptase (Gibco–BRL, Paisley, U.K.).

PCR reactions were performed using 2 μl of the reverse transcriptase products in Tris-HCl (pH 9.2) 50 mM, $(NH_4)_2SO_4$, 16 mM $MgCl_2$ 1.75 mM, 10% DMSO, 0.3 mM of each primer and 3.5 μg of TAQ and PWO DNA polymerase (Boehringer Mannheim, Germany). The primers used were: 5′-GGTGACCAACGCACTCTT and 5′-GGGCCCCCAGCTTGCCAG for the hNT_1 receptor and 5′-CTGGCCCTCTGCTCCCAA and 5′-TCAGGTCCGGGTTTCTGGG for the hNT_2 receptor. The samples were denatured for 1 min at 95°C and subjected to 30 cycles of amplification in an automated thermal cycler. The cycling conditions were as follows: 1 min at 94°C, 1 min at 58°C (hNT_1-receptor) or 1 min at 55°C (hNT_2-receptor) and 1 min at 68°C. The final incubation step at 68°C was lengthened to 10 min.

The amplicons generated (800 pb for hNT_1-receptor and 510 pb for hNT_2-receptor) were analysed by electrophoresis using 1% agarose gels.

Calculations and statistical analysis

The agonist concentration with 50% of the maximal effect (EC_{50}) was calculated using a four-parameter logistic model according to Ratkovsky & Reedy (1986), with adjustment by non-linear regression using the Levenberg-Marquard algorithm in RS/1 software. The pA_2 for antagonists, as defined by Arunlakshana & Schild (1959), was obtained by linear regression of mean values of the log (dr−1) against the negative log of the antagonist concentration. When the Schild plot slope was not significantly different from 1, it was constrained to unity. Computer analysis was done as described by Tallarida & Murray (1987).

Chemicals

SR 48692 2-{[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino}-adamantyl-2-carboxylic acid (Gully *et al*., 1993) and SR 142948 (2[(5-(2,6-dimethoxyphenl)-1-(4-(N-(3-dimethylaminopropyl)-N-methylcarbamoyl)-2-isopropyl-phenyl)-1H-pyrazole-3-carbonyl)-amino)-

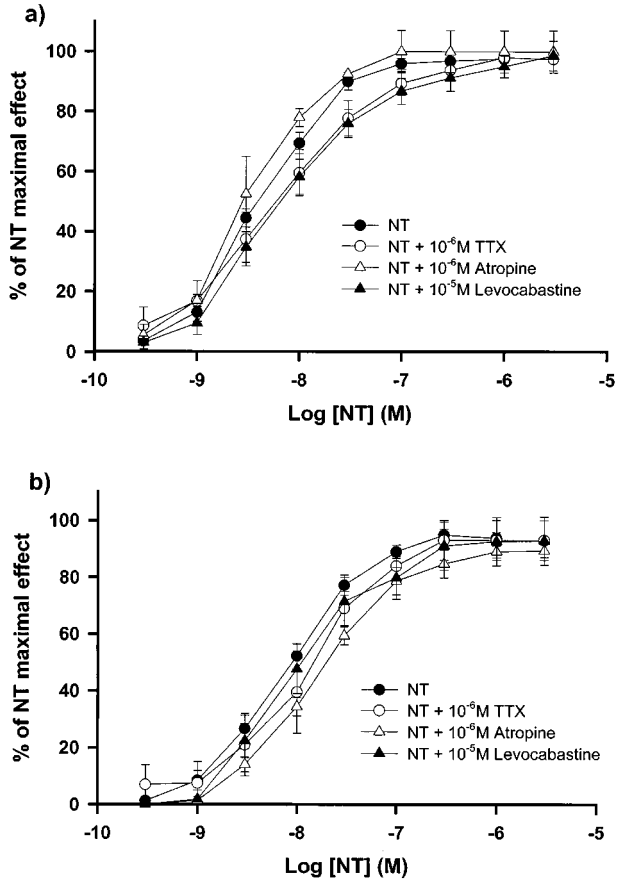

Figure 2 Contraction induced by NT in circular smooth muscle from human transverse (a) and distal (b) colon *in vitro*. Concentration-response curves to NT in the absence or presence of atropine, tetrodotoxin (TTX) or levocabastine. Results are mean ± s.e.mean of 4–11 colonic preparations from different patients.

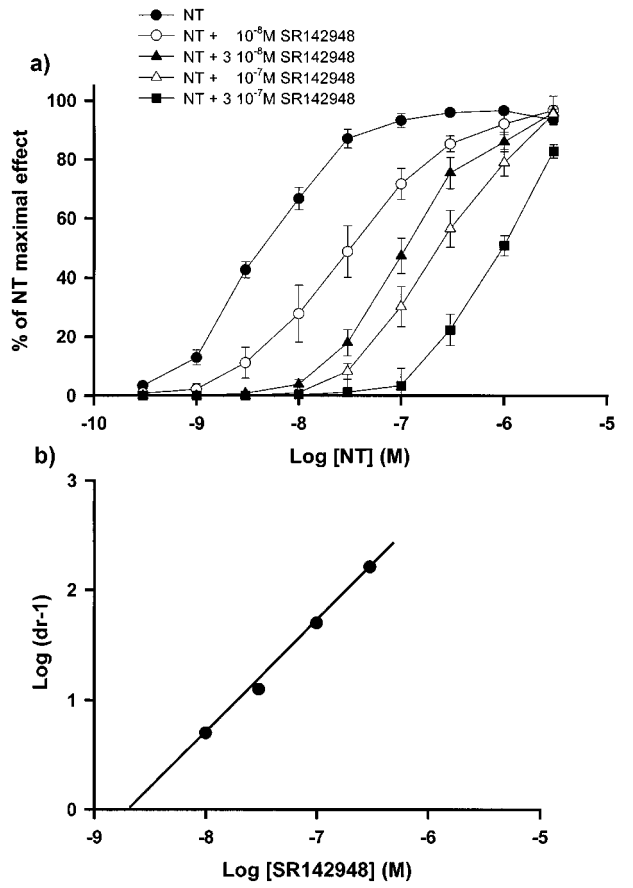

Figure 3 Effect of the NT receptor antagonist SR 142948 on contractions induced by NT in circular smooth muscle from human transverse colon *in vitro*. Concentration-response curve to NT in the absence or presence of SR 142948 (a) and the corresponding Schild plot (b). Results are mean ± s.e.mean of 5–10 colonic preparations from different patients.

adamantane-2-carboxylic acid) (Gully *et al*., 1997) were synthesized at Sanofi Recherche, Montpellier, France. The following chemicals were purchased from commercial sources: Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.): tetrodotoxin, carbachol, atropine sulphate, indomethacin, dimethylsulphoxide, dithiothreitol; Novabiochem (Laufelfingen, Switzerland) neurotensin and levocabastine.

Results

Functional studies

As shown in Figures 1 and 2, neurotensin caused concentration-dependent contractions of human colonic circular smooth muscle with EC_{50}, nM (in parentheses 95% confidence limits) 3.6 (3.2–4.2) in the transverse and 7.5 (6.2–9.1) in the distal colon; the maximal effect (mean±s.e.mean) was 70±3% and 55±7% of 0.1 mM carbachol.

Atropine (1 μM), tetrodotoxin (1 μM) and levocabastine (10 μM) did not significantly inhibit the responses elicited by NT (Figure 2): EC_{50} nM 2.9 (2.1–3.9), 5.8 (3.9–8.2), 5.2 (3.8–6.7) transverse colon; 11 (8–16), 13 (8.3–21), 8.3 (5.3–12) distal colon. Indomethacin (0.01 mM) also had no effect on the NT response in the colonic segments (data not shown).

As shown in Figures 3 and 4 and Table 1, the NT-receptor antagonist SR142948 (10 nM–1 μM) produced a concentration-dependent rightward shift of the cumulative concentration-response curve to NT in the transverse and distal colon; the calculated pA_2 were 8.71 and 8.45, and the slopes (0.98 and 0.99) were not significantly different from unity, attesting to the competitive nature of the antagonism in both segments.

The NT-receptor antagonist SR 48692 also caused a concentration-dependent rightward shift of the concentration-response curve to NT in transverse and distal colon preparations. However, lower concentrations of SR 48692 (10 and 100 nM), that inhibited NT responses in the transverse

Table 1 Quantitative antagonism of NT-responses in human colonic circular smooth muscle preparations by their non-peptide antagonists

	Concentration range (nM)	*pA_2*	*Slope*
Transverse colon			
SR 48692	10–10,000	8.02±0.08	0.51±0.10**
SR142948	10–300	8.71±0.03	0.99±0.02
Distal colon			
SR 48692	300–10,000	6.55±0.06	0.79±0.09
SR142948	30–1000	8.45±0.05	0.98±0.03

Values are means±s.e.mean. The pA_2 was obtained from the concentration response curves shown in Figures 3–6. **$P<0.01$ significantly different from unity.

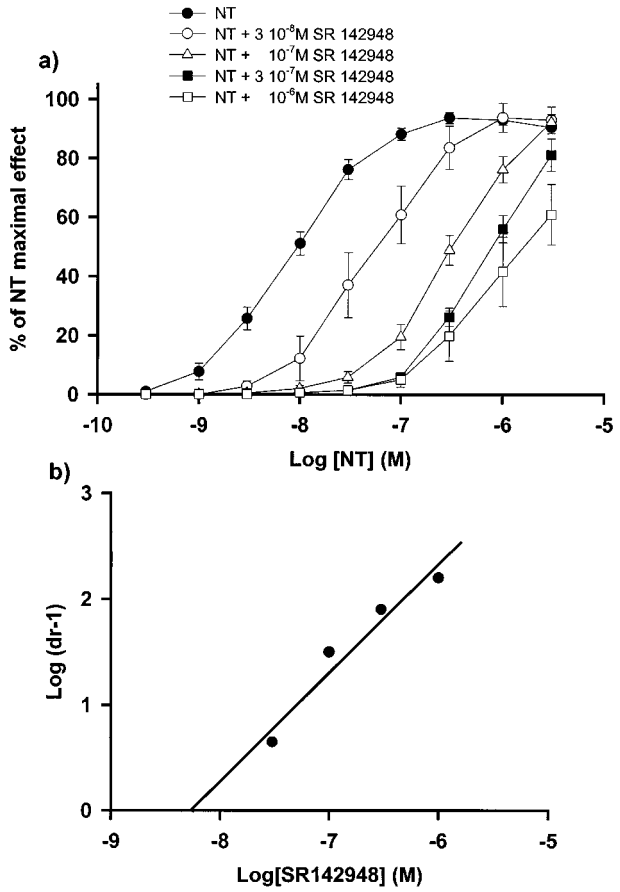

Figure 4 Effect of the NT receptor antagonist SR 142948 on contractions induced by NT in circular smooth muscle from human distal colon *in vitro*. Concentration-response curve to NT in the absence or presence of SR 142948 (a) and the corresponding Schild plot (b). Results are mean±s.e.mean of 5–10 colonic preparations from different patients.

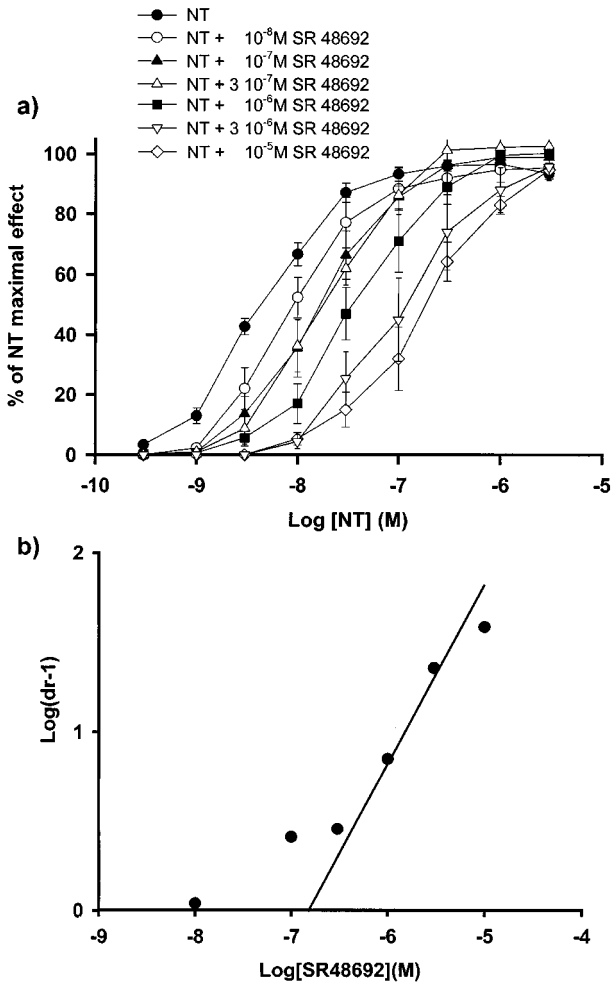

Figure 5 Effect of the NT receptor antagonist SR 48692 on contractions induced by NT in circular smooth muscle from human transverse colon *in vitro*. Concentration-response curve to NT in the absence or presence of SR 48692 (a) and the corresponding Schild plot (b). Results are mean±s.e.mean of 4–5 colonic preparations from different patients.

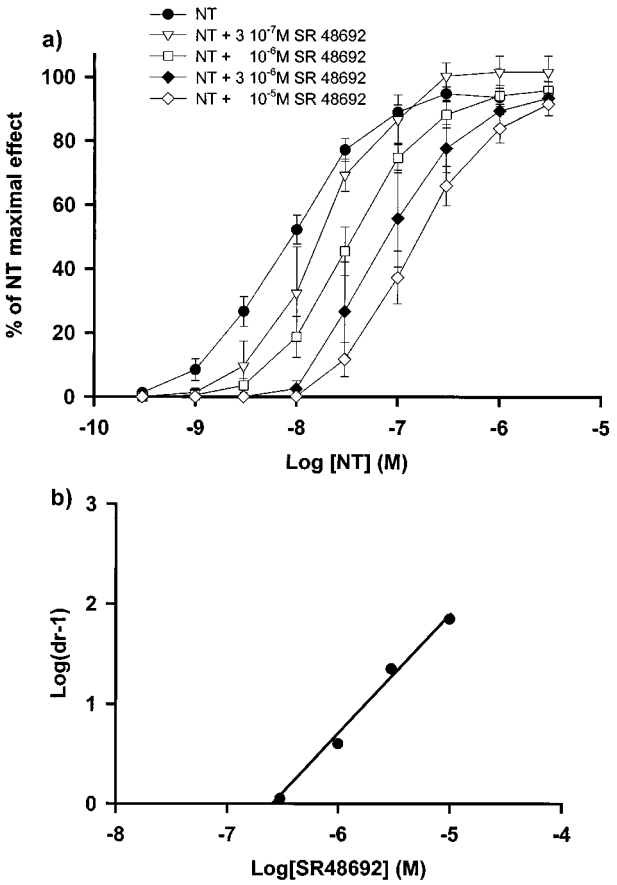

Figure 6 Effect of the NT receptor antagonist SR 48692 on contractions induced by NT in circular smooth muscle from human distal colon *in vitro*. Concentration-response curve to NT in the absence or presence of SR 48692 (a) and the corresponding Schild plot (b). Results are mean ± s.e.mean of 4–8 colonic preparations from different patients.

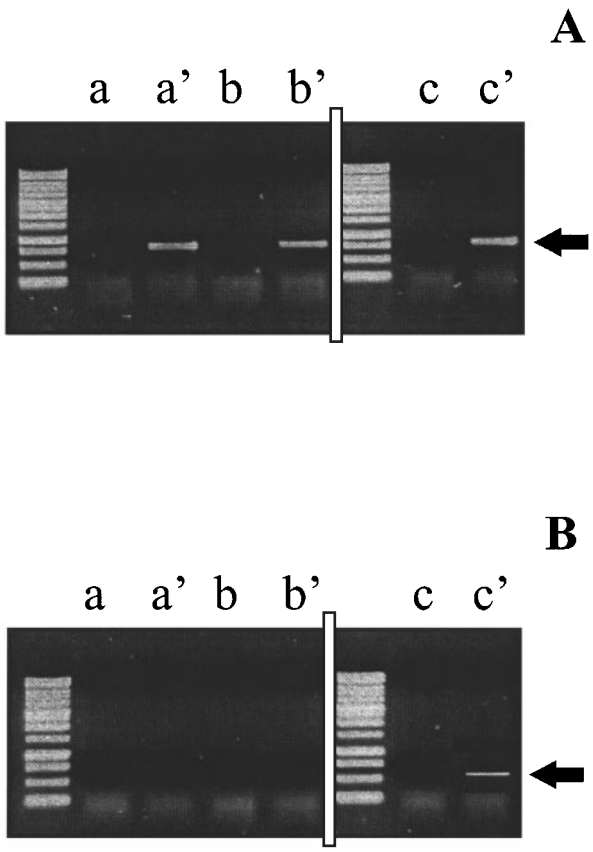

Figure 7 Expression pattern of hNT_1 and hNT_2 receptors mRNA in human colonic circular smooth muscle of transverse and distal colon. RNA was prepared from colonic strips of transverse (lanes a,a') and distal (lanes b,b') segments, or human brain as reference tissue (lanes c,c'). RNA samples were copied without (lanes a, b, c) or with (lanes a',b',c') reverse transcriptase and submitted to PCR using specific primers for hNT_1R (panel A) or hNT_2R (panel B) as described in Methods. The amplicons generated (800 b.p. for hNT_1R and 510 b.p. for hNT_2R) were analysed by electrophoresis using 1% agarose gels.

segment (Figure 5), were ineffective in the distal colon; NT EC_{50}, nM 7.7 (5.3–11) and 11 (8–14), not significantly differently from control.

The antagonism by SR48692 appeared to be non-competitive in the transverse segment, slope 0.51 (Table 1 and Figure 5) and competitive in the distal segment, pA_2 6.55, slope 0.79, not significantly different from unity; after constraining the slope to unity, the latter pA_2 became 6.3 (Table 1 and Figure 6). However, in the transverse segment, antagonism by SR 48692 became competitive between 0.3 and 10 μM (pA_2 6.7 ± 0.2 with slope constrained to unity).

SR 48692 and SR142948 up to the highest concentration tested did not inhibit carbachol (0.5 μM) contractions and showed no NT-like agonist activity (data not shown).

Transcript analysis of NT receptors by PCR

RT–PCR analysis (Figure 7) showed that transcripts of hNT_1-receptor are present in the human colon of the transverse and distal segments; conversely hNT_2-receptor transcripts were detected in the human brain (reference tissue), but not in the colon segments.

Discussion

The present results suggest that the human colon circular smooth muscle contains functional NT receptors. Regional differences were observed in colonic responses to NT, with slightly but significantly higher potency and efficacy in the transverse segment than the distal one. Our finding that NT elicits tonic contractions virtually insensitive to atropine and tetrodotoxin indicates that neuronal receptors are not involved. In isolated rat proximal colon, beside contractile effects, NT reportedly inhibits spontaneous phasic activity; it was suggested that the overall response to NT results from the summation of excitatory and inhibitory responses (Mulè & Serio, 1997; Mulè *et al*., 1995). In our preparations of human colonic circular smooth muscle, we only occasionally noted regular spontaneous phasic activity, like the one we had studied in the rat proximal colon (Bianchetti & Manara, 1990). Thus, under our experimental conditions NT had no inhibitory effect on human colon strips and always induced reproducible tonic and variable phasic contractions.

The novel non-peptide compounds SR 142948 and SR 48692 appear to be selective and potent antagonists of NT receptors *in vitro* and *in vivo* (Gully *et al*., 1993; 1997; Dubuc *et al*., 1994; Steinberg *et al*., 1994a,b; 1995 Schaeffer *et al*., 1998), although, as already mentioned, they may act as agonists under given experimental conditions (CHO cells transfected with rodent NT_2-receptors; Yamada *et al*., 1998). Differences in receptor structure and functional responses were observed within species (Gully *et al*., 1997); in these studies, SR 142948 was generally more potent than SR 48692.

SR 142948 has been reported to inhibit, with nanomolar affinity close to NT, [^{125}I-Tyr^{3}]-NT specific binding to the human high-affinity NT receptor (NT_1) naturally present in the HT 29 cell line and stably expressed in transfected CHO cells (Gully *et al*., 1997). SR 48692 displaced human NT high-affinity binding with 20–100 times less potency than SR 142948 (Gully *et al*., 1997). Both antagonists were also reported to inhibit, with similar nanomolar affinity, labelled NT-specific binding to human NT_2 receptors recently cloned from a brain cortex cDNA library and stably expressed in CHO cells (Vita *et al*., 1998).

Heterogeneity of NT receptors has been described in rodent brain (Schotte & Laduron, 1987; Gully *et al*., 1993; 1997), containing a high- (NT_1) and a low-affinity (NT_2) levocabastine-sensitive NT-specific binding site, whose functional roles still remain to be determined. SR 142948, unlike SR 48692, did not distinguish the low from the high-affinity NT binding sites (Gully *et al*., 1997). The human recombinant NT_2 receptor also binds levocabastine, but with lower affinity than rodent receptors (Vita *et al*., 1998). Recently neurotensin NT_2 receptor transcripts were detected by PCR in different human tissues, i.e. brain, kidney, uterus, heart and lung (Vita *et al*., 1998). In isolated muscle strips from human colon, NT-responses were virtually insensitive to a micromolar concentration of levocabastine, suggesting that histamine is not involved and that NT_2-receptors are not present. This view is supported by the finding of NT_1 but not NT_2 receptor transcripts in specimens of transverse and distal segments belonging to the the same batches used for functional studies.

The fact that levocabastine did not affect the NT responses we recorded in the human isolated colon is consistent with results in NT-sensitive human umbilical vein endothelial cells (Schaeffer *et al*., 1995), whereas NT binding sites susceptible to levocabastine have only been found in rodent brain (Chalon *et al*., 1996; Mazella *et al*., 1996).

In our tests SR 142948 proved a potent and competitive antagonist of colonic NT receptors, with much the same affinity (pA_2) in transverse and distal segments. Conversely, SR 48692 antagonized NT responses competitively in the distal colon and non-competitively in the transverse segment, with substantially lower affinity in the former. Non-competitive antagonism by SR 48692 strongly suggests that more than one receptor subtype is involved; its segment-dependent antagonism is consistent with a substantial amount of each of two NT receptor subtypes in the transverse colon, whereas only the subtype with lower affinity for this antagonist is apparently also abundant in the distal colon.

The latter subtype may be tentatively identified with the cloned human NT_1 receptor (Vita *et al*., 1993) whose transcript we found in muscle strips from both colonic segments; the former subtype at present remains an assumption based on 'classical' pharmacological kinetic constants, as determined in our functional assays. The differences we report between the two antagonists tested in similar smooth muscle preparations of transverse and distal colon are unlikely to involve artefacts of the experimental condition (i.e. non equilibrium steady state between agonist, antagonist and receptor), but indicate that SR 48692 can evidence receptor multiplicity (Kenakin, 1997).

The slight but significant difference in potency of NT in eliciting colonic contractions in the different segments is consistent with similar findings by other investigators (Maselli *et al*., 1998) and would fit with a different abundance of two NT receptor subtypes along the G.I. tract, although assuming different receptors from agonist responses has inherent shortcomings. Our experiments with SR 142948 produced no evidence of multiple NT receptors in the human colon as it seemed to have high affinity for both the putative NT receptor subtypes SR 48692 disclosed therein. However, antagonism of functional responses to NT or inhibition of its specific binding by SR 48692 has not so far given any indication of NT receptor heterogeneity in other human tissues including adult and newborn brain, HT 29 cells (Gully *et al*., 1993) and umbilical vein endothelium (Schaeffer *et al*., 1995).

In conclusion, the present findings attest to the potency and selectivity of two non-peptide antagonists for NT receptors naturally expressed in the circular smooth muscle of the human colon. They also provide evidence of two NT receptor subtypes mediating intestinal smooth muscle contractility, since SR 48692 antagonizes NT responses distinguishing between a high- and a low-affinity site, the latter probably associated with the human NT_1 receptor we found by RT–PCR analysis in both colonic segments. The use of SR 48692 and SR 142948 seems warranted for studying the underlying receptor mechanisms in normal and altered gut function and NT receptor antagonists might offer a new therapeutic approach to the treatment of some intestinal motor disturbances.

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