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Functional evidence for NO-synthase activation by substance P through a mechanism not involving classical tachykinin receptors in guinea-pig ileum *in vitro*

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1 This study tested the hypothesis that a nitric oxide synthase (NOS) was activated in guinea-pig ileum *in vitro* in response to substance P (SP), and attempted to characterize the tachykinin receptor involved in this activation by the use of selective receptor agonists and antagonists.

2 Strips of guinea-pig ileum (8 × 2 mm) were superfused (Krebs, 37°C, 2 ml min^{-1}) with: (i) tachykinin receptor agonists: SP, GR 73,632 (NK_1), GR 64,349 (NK_2), senktide (NK_3), and neuropeptide (NP)γ; (ii) tachykinin receptor antagonists: CP 99,994 (NK_1), SR 48,968 (NK_2), SR 142,801 (NK_3); (iii) nerve-related agents: carbachol (CCh), atropine, tetrodotoxin (TTX), hexamethonium; (iv) NOS inhibitors: N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), N^{ω}-monomethyl-L-arginine (L-NMMA) and aminoguanidine (AG); (v) NO-related agents, L-arginine (L-Arg), D-arginine (D-Arg), sodium nitroprusside (NaNP) and methaemoglobin. Muscle contractility was recorded isometrically and quantified as integrated area of activity.

3 SP, tachykinin receptor agonists and NPγ (10 pM to 10 μM), produced concentration-dependent contractions of ileal strips, with EC_{50}s in the nanomolar range, and maximal responses (E_{max}) attained at 0.1 μM for SP and 1 μM for the other agonists. The E_{max} response to SP equalled that to KCl (60 mM) taken as a 100% control (99.3% [93.0–105.7]; mean and 95% CI; $n=12$); a comparable E_{max} contraction was obtained with the other tachykinin receptor agonists (1 μM) as well as with CCh (1 μM).

4 Under baseline conditions, L-NAME (1 μM), L-NMMA (1 μM) and AG (1 μM), failed to contract the muscle strip. In contrast, when superfused for 3 min, 10 min after SP (0.1 μM), they induced a transient contraction of the strip (e.g. for 1 μM L-NAME: 50 to 70 s duration; amplitude 73 ± 12%, $n=24$).

5 The NOS inhibitor-induced contractile response was not obtained after KCl (60 mM), GR 73,632, GR 64,349, senktide or CCh (all up to 1 μM). In contrast, this contractile response was obtained after NPγ (1 μM).

6 Blockade of tachykinin NK_1, NK_2 and NK_3 receptors by continuous superfusion of CP 99,994, SR 48,968 and SR 142,801 (1 μM) respectively, starting 5 min before SP, did not modify the response to L-NAME, superfused 10 min after SP (0.1 μM). The contractile response to L-NAME (1 μM) was blocked by atropine (1 μM), superfused either before or after SP. In contrast, it persisted after TTX or hexamethonium (1 μM) superfused in the same conditions.

7 The amplitude of NOS inhibitor-induced contraction (1 μM) was dependent on the concentration of priming SP (1 pM to 1 μM). In contrast, the contractile response to NOS inhibitors (1 nM to 10 μM) of the ileum strip primed with SP (0.1 μM) was not concentration-related.

8 L-NAME-induced contraction was prevented by continuous superfusion of L-Arg (1 μM), but not D-Arg (1 μM). In addition, the NO donor, sodium nitroprusside (1 μM) and the NO scavenger, methaemoglobin (10 μg ml^{-1}), both prevented the contractile response to L-NAME.

9 In summary, SP and to a lesser extent NPγ, exert a permissive action allowing contractile stimulating effects of L-NAME, L-NMMA and AG, in guinea-pig ileum *in vitro*, by a mechanism which apparently does not involve tachykinin NK_1, NK_2 and NK_3 receptors. This action is likely to result from the activation of a NO-synthase by SP in the vicinity of intestinal myocytes. Thus, L-NAME, L-NMMA or AG, by blocking this SP-induced NO production, unveiled a smooth muscle contraction which involves a cholinoceptor (atropine-sensitive) mechanism.

Keywords: Substance P; nitric oxide synthase; *in vitro* muscle; superfusion; guinea-pig ileum

Introduction

Tachykinins constitute a family of structurally-related endogenous neuropeptides, including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). These peptides interact with the tachykinin NK_1, NK_2 and NK_3 receptors, preferring SP, NKA and NKB, respectively (for review see Regoli *et al.*, 1989; Guard & Watson, 1991; Maggi *et al.*, 1993c; Hellstrom *et al.*, 1994). Histochemical, functional and radioligand binding studies have provided evidence for the presence of these receptors in neural and non-neural structures of the intestine in mammalian species, including guinea-pigs (Costa *et al.*, 1987; Maggi *et al.*, 1993c; Otsuka & Yoshioka, 1993). In addition, a tachykinin-related peptide, neuropeptide γ (NPγ), has been reported to interact with intestinal receptors, probably different from 'classical' NK receptors (Al-Saffar *et al.*, 1993; Rahman *et al.*, 1994). At the intestinal level, tachykinins may alter motility, secretion, blood flow and even immune functions in connection with gut inflammatory processes (Sharkey, 1992; Otsuka & Yoshioka, 1993). SP has been suggested as a major contractile stimulatory transmitter of the non-adrenergic non-cholinergic (NANC) system controlling gastrointestinal motility (Nakanishi, 1991; Maggi *et al.*, 1993b; Smits & Lefebvre, 1994). Pharmacological studies on the isolated ileum have demonstrated that SP exerts potent spasmo-

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genic effects, by interacting directly with both NK_1 and NK_2 receptors in the smooth muscle membrane, and indirectly via neurogenic atropine-sensitive pathways probably involving NK_3 receptors (Maggi *et al.*, 1993; Daniel *et al.*, 1995; Holzer-Petsche, 1995). Nevertheless, some studies have also reported SP-induced release of a putative NANC inhibitory transmitter, or inhibition of acetylcholine release, eventually leading to gastrointestinal smooth muscle relaxation (Fox & Daniel, 1986; Maggi *et al.*, 1993b; 1994).

Nitric oxide (NO) is a labile gaseous molecule generated from L-arginine by the action of nitric oxide synthases (NOS) of which constitutive (cNOS) and inducible (iNOS) isoforms exist (Knowles *et al.*, 1989; Marletta, 1993; Morris & Billiar, 1994). Although NO was initially identified as a major endothelium-derived relaxing factor (Palmer *et al.*, 1987), evidence has now accumulated, which suggests a more widespread distribution of both NO and its synthases (Moncada *et al.*, 1991). NO serves both as a neurotransmitter and an intracellular second messenger (Galmiche *et al.*, 1995). The identification of NOS immunoreactivity in neurones of the myenteric plexus (Llewellyn-Smith *et al.*, 1992), has suggested a role for NO in regulating intestinal muscle function (Calignano *et al.*, 1992). In fact, there are many neural and non-neural potential sources for NO at intestinal level: enteric nervous ganglia, but also interstitial and smooth muscle cells, express cNOS activity (Publicover *et al.*, 1993; Xue *et al.*, 1994). Thus, glial cells, mast cells, macrophages, neutrophils, endothelial cells, and smooth muscle cells, all have the potential of producing NO, upon stimulation by neurotransmitters which elevate cytosolic Ca^{2+}, which in turn, after binding to calmodulin, activates cNOS if present (Morris & Billiar, 1994). Several studies have demonstrated that NO is, together with the vasoactive intestinal polypeptide (VIP), a major inhibitory neurotransmitter, mediating NANC relaxation of the gastrointestinal tract (Bult *et al.*, 1990; Gustafsson *et al.*, 1990; Toda *et al.*, 1990; Boeckxstaens *et al.*, 1991; D'Amato *et al.*, 1992; Kanada *et al.*, 1992; Sanders & Ward, 1992; Stark & Szurszewski, 1992).

Thus, both SP and NO have been shown to be active, though opposite neurotransmitters of the NANC system regulating intestinal smooth muscle contractility. Some time ago, SP-induced release of a putative NANC inhibitory transmitter, was suggested as a likely mechanism whereby SP could in certain cases cause relaxation of intestinal muscle (Fox & Daniel 1986; Maggi *et al.*, 1993b; 1994). Since then, evidence has accumulated which indicates that this NANC inhibitory mediator, released by the action of SP on presynaptic sites, is actually NO itself (e.g. Bult *et al.*, 1990; Sanders & Ward, 1992). Now, immunohistochemical and electro-microscopic evidence, showing close apposition of SP and NOS immunoreactivities (Li *et al.*, 1993), has strengthened the idea of functional relationships between these two mediators, especially during the neuroimmune response of the gut to intestinal inflammation. The aim of the present work was to address functionally, the issue of the relationship between SP and NO, as well as its consequences on intestinal contractility. To this end, we tested the specific hypothesis that a NOS could be activated in the guinea-pig ileum *in vitro*, in response to SP-induced stimulation. We have used an *in vitro* muscle superfusion system (Coleman & Nials, 1989; Garcia-Villar *et al.*, 1995), and tested several specific NOS inhibitors for their effects on guinea-pig ileum contractility. In addition, by using relatively selective tachykinin receptor agonists and antagonists, we attempted to determine which of the 'classical' tachykinin receptors so far described (i.e. NK_1, NK_2 and NK_3) was involved in the eventual activation of this enzyme by SP.

Methods

Animals

Healthy adult male guinea-pigs, weighing 300–400 g (Interfauna, Loches, France) were housed in individual cages and fed standard chow and water *ad libitum*. They were kept under conditions of constant temperature (22°C) and illumination (12 h light, 12 h dark), and were allowed to acclimatize for at least one week before being included in an experiment. On the day of experiments guinea-pigs were killed by a blow on the neck and exsanguinated. All procedures were approved by the local INRA Animal Care and Use Committee.

Strip preparation

A 5 cm-segment of ileum was excised between 10 and 5 cm proximal to the ileocaecal junction, opened lengthwise and rinsed in ice-cooled Krebs buffer of the following composition (mM): NaCl 118.0, $NaHCO_3$ 25.0, KCl 5.0, KH_2PO_4 1.0, $MgSO_4$ 1.0, glucose 11.0 and $CaCl_2$ 1.3. Eight whole thickness ileum strips (8×2 mm) were cut with a scalpel blade in the direction of the longitudinal muscle fibres. A long cotton thread was sutured to the upper end of each strip for attachment to a force transducer (UF1, Pioden Controls Ltd, Canterbury, U.K.), and the lower end was anchored to the bottom of a superfusion chamber. Care was taken not to allow tissues to dry during the preparation.

Superfusion system

The *in vitro* muscle superfusion system was similar to those described previously for tracheal and myometrial smooth muscle (Coleman & Nials, 1989; Garcia-Villar *et al.*, 1995). In the present study eight strips of ileum were simultaneously placed in individual chambers. Oxygenated (95/5% O_2/CO_2) Krebs buffer (35–37°C) of the composition described above, was superfused onto the muscle strips at a flow rate of 2.0 ml min^{-1}. Drugs were added to the superfusion buffer; rate of flow for drug solutions was one fiftieth of that of the buffer. This 1/50 dilution was taken into account when determining the final concentration of drug which actually came in contact with the superfused tissues.

Contractility data acquisition

After the ileum strips had been placed in the superfusion chamber, a passive optimal tension of 0.3 g (as determined in pilot tension-response studies) was applied to each strip which was then allowed to equilibrate for 1–2 h. Tension changes produced during the study ranged from 0.1 to 2.5 g, and were noted each second, quantified and stored by a microcomputer-driven data acquisition system, and displayed on a multichannel recorder (AstroMed MT59000, West Warwick, RI, U.S.A.).

Concentration-response curves

Concentration-response curves (CRCs) to both SP and NK-receptor agonists, were constructed from 10 pM to 10 μM. Each drug was superfused at regularly increasing concentration, for 3 min with a rinsing period of 20 min allowed between each concentration. For calculations, the activity level, expressed as mean integrated area of activity per minute, recorded during the 10 min preceding the superfusion of the lowest concentration of drug was used to determine baseline activity. The difference between activity levels obtained during drug superfusion and this baseline, was automatically calculated on line. All concentrations were expressed as final drug-concentrations actually in contact with intestinal tissue, and covered the full range from no effect to maximal contractile activity response. Response to KCl (60 mM) superfused for 3 min was used as 100% control.

Experimental schedule

The NO-synthase inhibitors used in the present study were tested both under baseline motility conditions i.e. 10 min before any other drug on a tissue which had equilibrated ap-

propriately for at least 2 h after being placed in the superfusion chamber, and then 10 min after the superfusion of a contractile agent (SP, NK agonists, NPγ, carbachol and KCl). SP was then chosen for convenience as a 'priming' contractile agent for time- and concentration-dependency studies of the response, if any, to NO-synthase blockers. Thus, L-NAME, L-NMMA and AG (1 μM) were superfused for 3 min repeatedly at 10 min-interval after SP, until the response disappeared. To study the influence of the concentration of SP on the response, if any, to NOS inhibitors, L-NAME, L-NMMA and AG (1 μM) were superfused for 3 min, 10 min after superfusion of SP at concentrations ranging from 0.1 nM to 10 μM; for these experiments the order of administration of the different concentrations of SP was randomized, and a rinsing interval of 60 min was allowed between each superfusion of contractile agent. All experiments were systematically performed in duplicate in each set of strips from the same animal and repeated at least four times in different animals. Drug effects were quantified for each strip as the integrated area under the tension *vs* time curve, and compared to the response of the same strip to KCl (60 mM) taken as 100% control.

Effects of tachykinin receptor antagonists

Blockade of NK-receptors was achieved by use of available selective antagonists for the NK_1, NK_2 and NK_3 receptors. These were, respectively, CP 99,994 (Pfizer Central, Groton, CT, USA), SR 48,968 and SR 142,801 (Sanofi Research, Montpellier, France). These antagonists were superfused continuously from 5 min before SP, until 10 min after the administration of NOS inhibitors, which, as a general rule, were superfused for 3 min starting 10 min after SP (0.1 μM). Drug dosage and superfusion procedure were determined in pilot studies designed to assess both the efficiency and selectivity of the tachykinin receptor antagonists eventually used in the final study.

Effects of NO-related and enteric nervous system-related agents

The effects of NO-related agents (0.1 to 1 μM), L-arginine (L-Arg), D-arginine (D-Arg), bovine methaemoglobin (NO-'scavenger') and sodium nitroprusside (NaNP; NO-'donor'), and those of enteric nervous system-related drugs i.e. atropine, tetrodotoxin (TTX) and hexamethonium, were investigated upon the contractile response, if any, of the guinea-pig ileum to NOS inhibitors after stimulation by SP (0.1 μM). For these experiments test-agents were superfused either as described above (i.e. for 25 min, starting 5 min before SP) or for 3 min starting 5 min after SP; in either case the NOS inhibitors were superfused for 3 min, starting 10 min after SP.

Data analysis

The contractile responses of tissues were normalized to the maximal response obtained with KCl (60 mM). Data from concentration-response studies were determined after non-linear least square regression to a sigmoidal curve (InPlot 4.0, GraphPad software, San Diego, California, U.S.A.) and expressed as means and 95% confidence intervals (95% CI): EC_{50} values (i.e. the concentration of agonist producing 50% of the maximal response) were calculated using pD_2 values (i.e. the negative logarithm of EC_{50}) and compared by Student's *t* test. Statistical analysis of contractility data, expressed as mean ± s.d., was performed with one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test as appropriate (InStat 2.0, GraphPad software, San Diego, California, U.S.A.). Two-way ANOVA (Prism, GraphPad software, San Diego, California, U.S.A.) was used, where relevant, to compare CRCs. Differences with probability values of $P<0.05$ were considered significant.

Drugs

Substance P (SP), neuropeptide γ (NPγ; γ-preprotachykinin-(72-92)-peptide amide), carbachol (CCh), N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), N^{ω}-monomethyl-L-arginine (L-NMMA), aminoguanidine (AG), L-arginine (L-Arg), D-arginine (D-Arg), sodium nitroprusside (NaNP) and bovine methaemoglobin were from Sigma Chemicals (Saint Quentin Fallavier, France). The tachykinin peptide agonists (Hagan *et al.*, 1991) GR 73,632 (δ-amino valeryl{L-Pro^9,N-MeLeu^{10}}-substance P(7–11); NK_1 agonist) and GR 64,349 ({Lys^3, Gly^8-R-γ-lactam-Leu^9}neurokinin A(3–10); NK_2 agonist) were from RBI (Bioblock, Illkirch, France). Senktide (succinyl-{Asp^6, MePhe^8}substance P(6–11); NK_3 agonist) (Guard *et al.*, 1990) was from Bachem (Bale Biochimie, Voisins-le-Bretonneux, France). The nonpeptide NK_1 antagonist, CP 99,994 {(2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine} hydrochloride (McLean *et al.*, 1993) was a kind gift from Dr S.B. Kadin (Pfizer Central, Groton, CT, U.S.A.). The nonpeptide NK_2 antagonist, SR 48,968 {(S)-N-methyl-*N*-[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl) butyl]benzamide} (Emonds-Alt *et al.*, 1992; 1993; Maggi *et al.*, 1993a) and the novel NK_3 antagonist, SR 142,801 {(S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl)propyl)-4-phenyl piperidin-4-yl)-N-methylacetamide (Emonds-Alt *et al.*, 1994)}, were gifts from Dr Emonds-Alt (Sanofi Research, Montpellier, France). Stock solutions of peptides (1 mM) were prepared in phosphate buffered saline (PBS, Sigma) acidified to pH 5.5 with acetic acid. For non-peptide agents stock solutions were prepared in absolute ethanol, unless otherwise indicated, and stored at −20°C. Serial dilutions were done in PBS (pH 7.0), so that final concentrations of ethanol in contact with the muscle tissue in the superfusion chamber never exceeded 1% (v/v). Fresh dilutions were prepared each day, left in ice until use, and discarded after each experiment. Reagents for buffers were of high analytical grade.

Results

Effects of tachykinin receptor agonists on strip contractility

Superfusion of the tachykinin receptor agonists SP, GR 73,632, GR 64,349, senktide, and NPγ (all 10 pM to 10 μM) produced concentration-dependent contractions of guinea-pig ileum strips, with EC_{50}s in the nanomolar range (Table 1 and Figure 1). Maximal effects (E_{max}) were attained at 0.1 and 1 μM for SP and the other agonists, respectively. The amplitude and shape of the E_{max} contractions induced by 'classical' tachykinin NK_1, NK_2 and NK_3 receptor agonists, as well as by NPγ (all at 1 μM) were comparable to those of contractions induced by SP (0.1 μM). At this latter concentration, the SP-induced contraction consisted of a high-amplitude early phase (7–12 s), followed by a plateau of more moderate amplitude, maintained throughout the whole period of superfusion with the peptide. In addition, the area under the muscle-tension *vs* time curve, generated by 0.1 μM SP (99.3 [93.0–105.7]; mean and [95% CI]; $n=12$), equalled that of the response of the same strips to KCl (60 mM) under similar superfusion conditions, which was taken as 100% control (See e.g. Figure 4). A comparable contraction (97.0 [84.8–117.3]; $n=8$) was elicited by carbachol (1 μM).

Effects of L-NAME, L-NMMA and AG on ileal muscle contractility

Under baseline conditions (unstimulated muscle strip), L-NAME, L-NMMA and AG, superfused in the range 10 nM to 10 μM, did not induce any change in the contractile activity of the ileum. In contrast, these NO-synthase inhibitors provoked a transient contraction of the strip, when superfused at the same concentration range (10 nM to 10 μM), but 10 min after

Table 1 Concentration-response curve parameters for the various natural tachykinins and selective tachykinin receptor synthetic agonists tested on the *in vitro* contractility of guinea-pig ileum strips placed in the direction of the longitudinal muscle fibres

Agonist	EC_{50} (nM)	Slope	E_{max} (% of KCl control)	n
Substance P	0.8 (0.4–1.6)	0.82 (0.65–1.22)	99.3 (93.0–105.7)	12
Neuropeptide γ	5.7 (2.7–12.1)	0.85 (0.59–1.35)	94.5 (84.8–104.2)	6
GR 73,632	20.3 (8.9–46.2)	0.97 (0.54–1.36)	98.2 (85.9–110.5)	6
GR 64,349	48.7 (36.6–64.7)**	0.88 (0.69–1.06)	89.6 (85.2–94.1)	6
Senktide	14.9 (7.4–29.9)	0.91 (0.63–1.52)	97.5 (87.6–107.4)	6

Values are means (95% confidence interval in parentheses) calculated after nonlinear regression analysis of concentration-response curves constructed in duplicate in tissues from n animals. ** Significantly different from the value obtained with substance P (Student's t test; $P<0.01$); statistical differences were not found for E_{max} values of agonists compared to control KCl (100% effect); slopes for each agonist were found to be not different from unity.

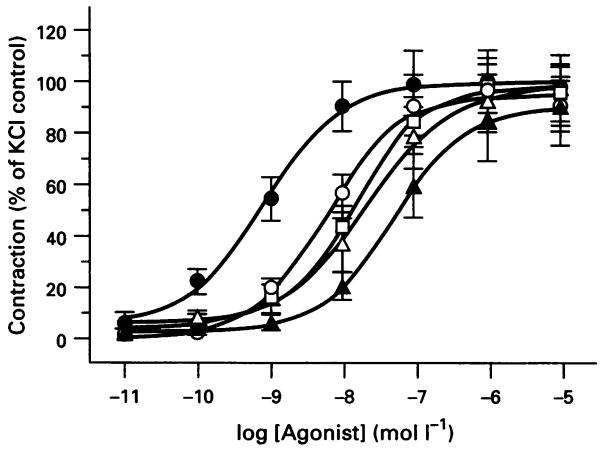

Figure 1 Concentration-response curves for tachykinin agonists on ileum strips from 6 guinea-pigs (except substance P: $n=12$). Tension (mean ± s.d.) is represented as a percentage of the maximum response obtained by superfusion of KCl (60 mM). Values from each animal were obtained from two replicates per data-point and per animal. The plotted curves are lines of best fit obtained by non linear regression analysis. (●) Substance P; (○) neuropeptide γ; (□) senktide; (△) GR 73,632; (▲) GR 64,349.

superfusion of SP (0.1 μM). At a concentration of 1 μM (conveniently chosen from concentration-response studies described below), either L-NAME ($n=24$; see e.g. Figure 2), L-NMMA or AG (both $n=6$; not shown), superfused 10 min after SP (0.1 μM), induced a transient (50 to 70 s) high-amplitude elevation of the tension baseline. The response to L-NAME (1 μM; Table 2) reached 73 ± 12% of the amplitude of the peak obtained in the same strips in response to KCl (60 mM; $n=24$). The duration of the contractile response was not increased when any of these NOS-inhibitors were superfused for periods of time longer than 3 min, (i.e. 5 min, $n=4$; 10 min, $n=4$) or at concentrations higher than 1 μM (i.e. 10 and 40 μM; $n=4$). Additional pilot studies in which L-NAME (1 μM; $n=6$; not shown) was superfused continuously from 10 min before, until 15 min after SP (0.1 μM), revealed that under these experimental conditions the contractile response to SP (0.1 μM) was not modified compared to controls, but there was no priming effect of SP as there was no increase in contractility during the superfusion of L-NAME after SP. We have no explanation for this latter observation.

Effects of L-NAME after superfusion with tachykinin receptor agonists, NPγ, KCl or carbachol

L-NAME (1 μM) superfused for 3 min, 10 min after one of the contractile agents, failed to induce any contractility response when the NK_1-receptor agonist, GR 73,632, or the NK_2-agonist GR 64,349, or the NK_3-agonist senktide (all 1 μM) were superfused as 'priming' agents instead of SP (Table 2). Similarly, there was no contractile response to L-NAME (1 μM) when the muscle strip was primed with carbachol (0.1, 1 and 10 μM; $n=3$ for each concentration) or KCl (60 mM; $n=8$) instead of SP. In sharp contrast, superfusion of NPγ (1 μM; $n=12$) 10 min before L-NAME allowed a contractile response to L-NAME (1 μM) comparable to that obtained when the tissue was primed with 0.1 μM SP (Table 2).

Effect of tachykinin receptor antagonists on L-NAME-induced contraction after SP

Studies were designed to assess both efficiency and selectivity of the tachykinin NK_1, NK_2 or NK_3 receptor antagonists used. Thus, CP 99,994, SR 48,968 and SR 142,801 superfused onto the muscle strips at 1 μM for 5 min before challenge with the corresponding selective tachykinin receptor agonist (GR 73,632, GR 64,349 and senktide, respectively) given either at 0.1 or 1 μM ($n=4$), prevented the contractile response to their corresponding agonist. Lower concentrations (i.e. 1 and 10 nM; $n=4$) of the antagonists did not consistently prevent these contractile responses. Blockade of tachykinin NK_1, NK_2 or NK_3 receptors by continuous superfusion from 5 min before SP (0.1 μM) until 10 min after L-NAME (1 μM) of the selective antagonists CP 99,994, SR 48,968 or SR 142,801 (1 μM; $n=6$) respectively, did not significantly modify the contractile response to L-NAME (1 μM), superfused 10 min after SP, although each of these antagonists slightly reduced the SP-induced contraction ($P<0.05$; Table 2). To complete these data we further investigated whether different mixtures of two of the three tachykinin receptor antagonists (all 1 μM; Table 3), with or without atropine (1 μM) would be able to prevent the SP-induced priming. In all cases, the presence of atropine in the superfusion fluid diminished the SP-induced contraction and prevented the NOS inhibitor-induced contraction after SP (0.1 μM). The presence of either two or three antagonists dramatically reduced, but did not completely suppress, both the contractile response to SP and the priming effect of SP. These two effects, however, were fully prevented when the three antagonists were superfused in the presence of atropine ($n=4$; not shown).

Time-dependency study

When L-NAME, L-NMMA and AG (all 1 μM) were superfused repeatedly for 3 min at 10-min intervals after SP (0.1 μM), the contractility responses induced in the muscle strip decreased with time after SP. Thus, at 40 min after SP the amplitude of the response to L-NAME (1 μM) averaged only 42 ± 11% ($n=7$) of that obtained at 10 min after SP, and eventually disappeared in some strips within 60 to 80 min after SP. Nevertheless, responsiveness to L-NAME (1 μM) resumed with similar kinetic characteristics in the same ileum strip, if a second priming superfusion of SP (0.1 μM) was performed (Figure 2). The kinetics of the responses to L-NMMA and AG

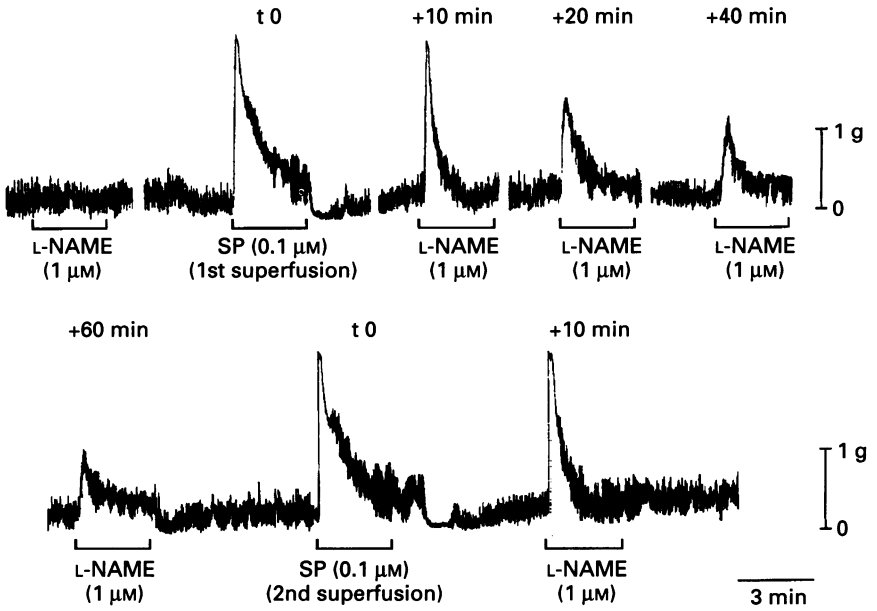

Figure 2 Effects of L-NAME (1 μM) on the ileum both before and after SP superfusion. Priming of the muscle strip with SP (0.1 μM) unveils a transient contractile response to L-NAME which is not obtained before superfusion of SP; note the decrease with time after SP of the contractile response to L-NAME (1 μM) superfused repeatedly for 3 min at times 10, 20, and 40 min after SP (upper trace). The L-NAME-induced response was dramatically reduced at 60 min after SP, but resumed in the same ileum strip after a second superfusion of SP (0.1 μM) with similar characteristics to those observed for the first SP superfusion (lower trace). The present traces are isometric tension mechanograms from a single experiment representative of similar experiments performed in duplicate in at least 6 animals. Horizontal bars represent duration of superfusion of each agent.

Table 2 Effects of the priming treatment on the contractile response of ileum strips to tachykinin receptor agonists and L-NAME superfused 10 min later

	Contractile response (% of KCl control)		
Priming treatment	*Tachykinin receptor agonist*	*L-NAME* (1 μM)	n
Control:			
Substance P (0.1 μM)	97.6 ± 7.4	73.0 ± 12.0	24
GR 73,632 (1 μM)	90.7 ± 10.4	6.1 ± 2.4***	6
GR 64,349 (1 μM)	83.6 ± 7.3	5.2 ± 1.3***	6
Senktide (1 μM)	96.2 ± 6.9	6.7 ± 1.8***	6
Neuropeptide γ (1 μM)	90.6 ± 6.4	66.4 ± 17.1	12
Substance P (0.1 μM) + CP 99,994 (1 μM)	69.2 ± 6.6***	74.4 ± 12.5	6
Substance P (0.1 μM) + SR 48,968 (1 μM)	74.3 ± 9.8***	65.0 ± 19.6	6
Substance P (0.1 μM) + SR 142,801 (1 μM)	79.0 ± 6.5***	70.3 ± 22.3	6

Values are means ± s.d. for determinations made in duplicate in *n* animals. Selective tachykinin receptor agonists (see text for explanations) induced ileal contractions that were not different from those induced by substance P, but did not produce, except for neuropeptide γ, the priming effect that allowed the muscle strips to contract in response to L-NAME. Note that neuropeptide γ (1 μM) allows a muscle response to L-NAME similar to that induced by a tenfold lower concentration of substance P (0.1 μM). Tachykinin receptor antagonists significantly diminished the contractile response of the strip to substance P, but not the subsequent response to L-NAME. ***Significantly different from the response obtained in the respective control conditions (Bonferroni multiple comparison test; $P<0.001$).

(both 1 μM; $n=3$; not shown) were similar to those to L-NAME. Furthermore, as there were no significant differences in the responses elicited by each one of the three NOS inhibitors tested, the rest of the study was pursued only with L-NAME as a NOS inhibitor.

Concentration-response studies with L-NAME

The contractility responses of the muscle strip to L-NAME (1 μM) superfused 10 min after SP (1 pM to 1 μM) were directly dependent on the concentration of the priming peptide (Figure 3). In sharp contrast, when L-NAME was superfused over a large range of concentrations (10 pM to 10 μM; $n=6$) 10 min after SP (0.1 μM), the contractile response obtained with L-NAME was not concentration-dependent: rather, it was an all-or-none response with a threshold around 0.1 – 1.0 nM. The CRCs for both the direct 'contractile' effect of SP ($n=12$) and 'priming' effect of SP ($n=6$), as indirectly revealed by the response of the muscle strip to 1 μM L-NAME (Figure 3) were compared. The EC_{50}s (0.8 [0.4 – 1.6] *vs* 9.1 [5.2 – 14.1] nM) were not significantly different for both effects of SP respectively (means and [95% CI]; $P>0.05$; Student's *t* test). In contrast E_{max} (99.3 [93.0 – 105.7] *vs* 77.6 [69.5 – 86.3]% of KCl control) were found to be significantly different; $P<0.001$. In addition, two-way ANOVA for curve comparisons showed that the difference between contraction values resulting from both 'contractile' and 'priming' effects of SP, was the same at all concentrations of SP; this difference being highly significant ($P<0.0001$).

Effects of NO-related agents on L-NAME-induced contraction after SP

NO-related agents were continuously superfused from 5 min before SP, until 10 min after L-NAME, i.e. a total superfusion time of 25 min. Under these conditions the contractile stimulatory action of L-NAME (1 μM) superfused 10 min after SP (0.1 μM) was inhibited by L-Arg (1 μM), but not by D-Arg (1 μM), as shown in Figure 4. Moreover, the NO donor sodium nitroprusside (NaNP; 1 μM), but also the NO-scavenger methaemoglobin (10 μg ml^{-1}), both abolished L-NAME-induced response (Figure 5). Additional studies in which NaNP (1 μM; $n=4$) or methaemoglobin (10 μg ml^{-1}; $n=4$) were superfused for 3 min starting 10 min after SP (0.1 μM), never produced a contractile response of the guinea-pig ileum, similar to that produced in the same conditions by NOS inhibitors.

Table 3 Effects of tachykinin NK_1, NK_2 and NK_3 receptor antagonists and atropine (all 1 μM) on the contractile response of ileum strips to substance P (0.1 μM) and L-NAME (1 μM) superfused 10 min later

	Contractile response (% of KCl control)		
Priming treatment	*Substance P*	*L-NAME*	n
Control: Substance P (alone)	97.6 ± 7.4	73.0 ± 12.0	24
Substance P + CP 99,994 + SR 48,968	37.3 ± 4.3***	12.1 ± 2.5***	4
Substance P + CP 99,994 + SR 142,801	44.7 ± 5.4***	10.8 ± 3.2***	4
Substance P + SR 48,968 + SR 142,801	56.5 ± 8.3***	16.5 ± 4.2***	4
Substance P + CP 99,994 + SR 48,968 + SR 142,801	18.5 ± 6.6***	10.2 ± 2.8***	4
Substance P + atropine	78.2 ± 6.9***	No effect	6

Values are means ± s.d. for determinations made in duplicate in n animals (control values are the same as in Table 2). *** Significantly different from respective control responses (Bonferroni multiple comparison test; $P<0.001$).

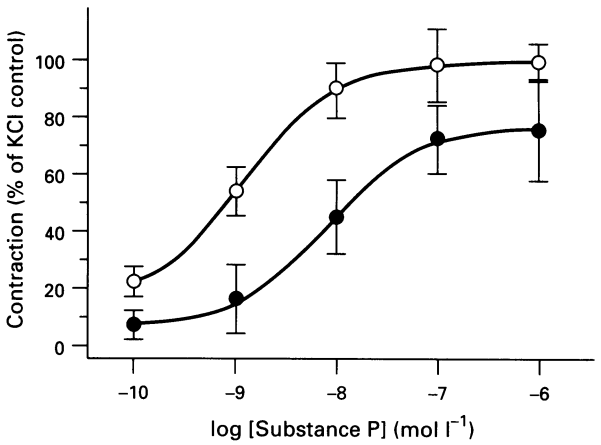

Figure 3 Concentration-response curves (CRCs) corresponding to both direct 'contractile' effect of substance P (○; $n=12$; as in Figure 1) and 'priming' effect of SP, indirectly revealed by the contractile response of the ileum strip to 1 μM L-NAME (●; $n=6$) superfused 10 min after SP. Values are means ± s.d. The respective EC_{50}s were not significantly different (Student's t test). In contrast the difference in tension values between both curves was constant at all concentrations of SP and highly significant ($P<0.0001$; two-way ANOVA; see text for explanations).

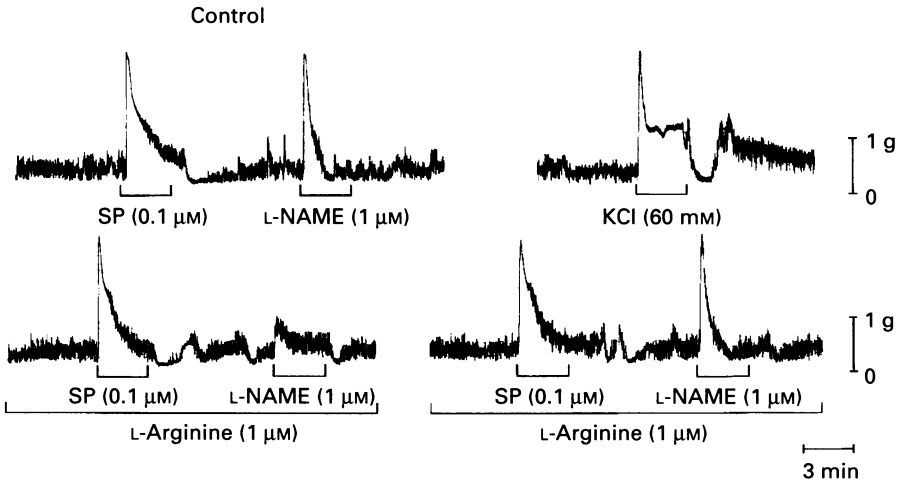

Figure 4 Effects of the NO-related agents L- and D-arginine on the contractile effect of L-NAME (1 μM) superfused for 3 min, 10 min after SP (0.1 μM). Control: L-NAME induced a contraction of the ileum strip when superfused after SP; the response of the strip to KCl (60 mM) is shown for comparison. L-NAME effect was abolished by L-arginine (1 μM), but not by D-Arginine (1 μM). Tension charts are from a single experiment representative of similar ones performed in duplicate in at least 6 animals. Horizontal bars represent duration of superfusion of each agent.

Effects of atropine, TTX and hexamethonium on L-NAME-induced contraction after SP

Neurotropic agents were continuously superfused from 5 min before SP, until 10 min after L-NAME, i.e. a total superfusion time of 25 min. Figure 6 shows that under these conditions, only atropine (1 μM) blocked the contractile effects of L-NAME superfused 10 min after SP (0.1 μM). Conversely the L-NAME-induced response was not prevented by TTX or hexamethonium (both 0.1 to 10 μM). Similar effects were observed in a second series of experiments in which atropine, TTX or hexamethonium (all 1 μM; $n=3$ for each compound), were superfused for 3 min starting 5 min after SP.

Discussion

The present study, using an *in vitro* smooth muscle superfusion system (Coleman & Nials, 1989; Garcia-Villar *et al.*, 1995), confirms the already well-documented stimulatory action of SP on intestinal contractility (Maggi *et al.*, 1993c, for review; Suzuki *et al.*, 1994; Daniel *et al.*, 1995). It also recalls that comparable stimulating effects can also be induced in the guinea-pig ileum with selective tachykinin NK_1, NK_2 and NK_3 receptor agonists, as well as by NPγ. This latter natural neuropeptide was proposed to interact at the intestinal level with a putative 'NPγ receptor', different from the three 'classical' tachykinin receptors (Al-Saffar *et al.*, 1993; Rahman *et al.*, 1994). The most striking result of the present study, however, was that SP and to a lesser extent NPγ, were able to unveil the potential of the NOS inhibitors, L-NAME, L-NMMA and AG, to contract the guinea-pig ileum. In sharp contrast, these NOS inhibitors were devoid of any contractile stimulating efficacy, when superfused onto strips that either were not previously contracted by SP, or were contracted by any of the selective tachykinin receptor agonists, or by other contractile agents such as KCl or carbachol. Thus, it appears that this 'permissive' effect did not result from the mechanical muscle con-

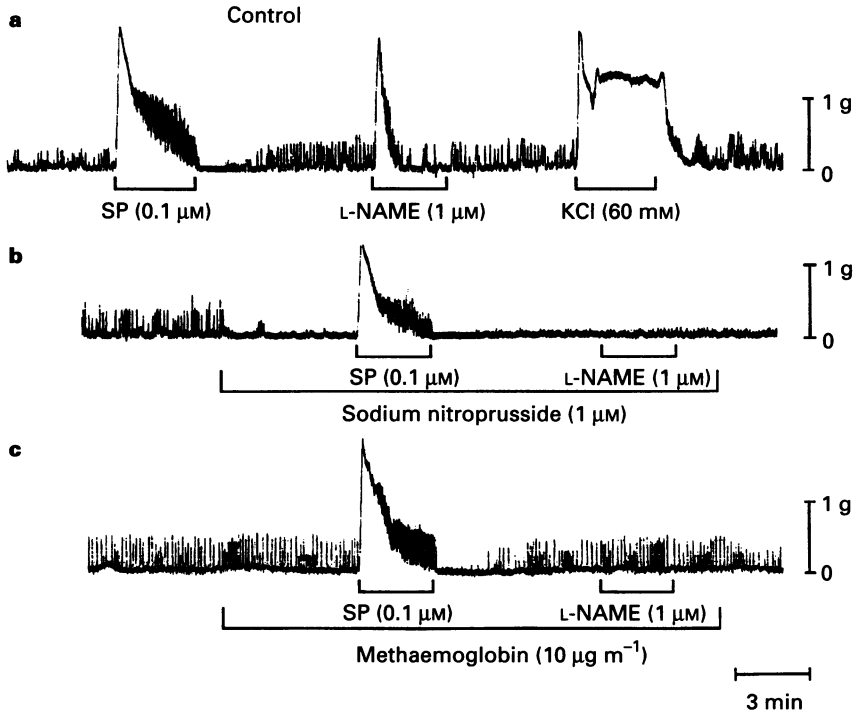

Figure 5 Effects of the NO-related agents sodium nitroprusside and methaemoglobin on the contractile effect of L-NAME (1 μM) superfused for 3 min, 10 min after SP (0.1 μM). (a) L-NAME-induced contraction after SP was abolished by sodium nitroprusside (1 μM; b), and methaemoglobin (10 μg ml^{-1}; c). Tension charts are from a single experiment representative of similar experiments performed in duplicate in at least 6 animals. Horizontal bars represent the duration of superfusion of each agent.

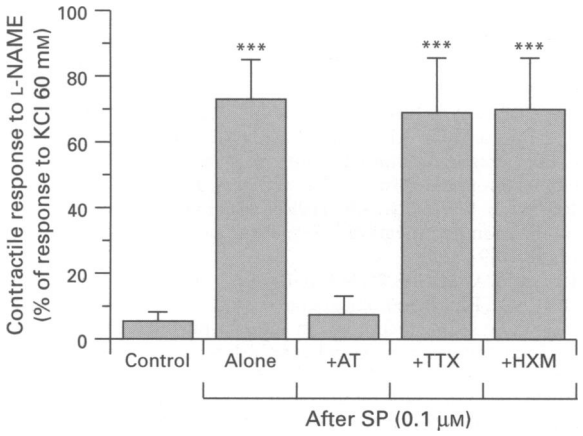

Figure 6 Effect of L-NAME (1 μM) on the motility of ileal strips both before (Control) and 10 min after superfusion of SP (0.1 μM), and influence of enteric nervous system-related agents on L-NAME-induced response (AT: atropine; TTX: tetrodotoxin; HXM: hexamethonium; all 1 μM). Tension values (mean ± s.d.) were from four animals with two replicates per data-point and per animal. ***Significantly different compared to the response in the absence of SP (Bonferroni multiple comparison test; $P<0.001$).

traction itself, but was specifically induced by SP, by a mechanism which remains to be elucidated.

Thus, SP exerted the observed permissive effect, through a mechanism which most probably does not involve an interaction with the tachykinin receptors thus far described. Indeed, neither selective tachykinin receptor agonists reproduced, nor antagonists were able to prevent this SP-induced contractile response to NOS inhibitors. NPγ was the only one of the tachykinin agonists used in this study which was able to mimic the observed permissive effect of SP. Thus NPγ, just like SP, allowed NOS-inhibitors to induce a transient contraction of ileum strips. Therefore, it can be suggested that a NPγ-preferring binding site could be involved in this permissive action of SP. It is also possible that SP interacted with an as yet undetermined, species-specific, isoform of one of the tachykinin NK receptor subtypes, but this hypothesis cannot be supported by the present data. Other hypotheses, currently under investigation in our laboratory, address the issue of an interaction of SP with intestinal mast cells. Indeed, it has been shown that SP may influence intestinal secretion in mice by promoting the release of mast cell mediators (Wang *et al.*, 1995). In this respect, a receptor-independent action of SP could be proposed to explain our findings, the mechanism of which could be a direct activation of G proteins in the membrane of intestinal mast cells, comparable e.g. to that reported for the SP-induced activation of rat peritoneal mast cells (Mousli *et al.*, 1990).

Although the observed NOS inhibitor-induced ileum contraction was transient, and not consistently related to the concentration of NOS inhibitor, it appears to involve specifically NO-related mechanisms. The contractile response to NOS inhibitors observed after SP was prevented by L-Arg, and not by its stereoisomer D-Arg, which demonstrates that the response involves NOS-induced NO generation from the actual biological substrate which is L-Arg. Neither was the contractile response to NOS inhibitors after SP prevented by the blocker of neuronal conduction, TTX, indicating that axonal conduction was not involved in the observed effect of SP. In contrast, it was suppressed by the muscarinic receptor blocker, atropine. Thus, SP may release endogenous acetylcholine, which may then stimulate muscarinic receptors on myocytes, provided they are not 'protected' by an enhanced release of NO. It has been shown that acetylcholine can synergize with tachykinins in exciting gut muscle (Holzer & Maggi, 1994). Other studies have demonstrated NOS inhibitors-induced release of acetylcholine from guinea-pig myenteric plexus (Kilbinger & Wolf, 1994). Thus the atropine-sensitive contractile effect of NOS inhibitors observed after SP priming, may well be a postsynaptic phenomenon, depending on acetylcholine in a synergistic manner.

The NO-donor NaNP prevents the contractile response of the SP-primed ileum to L-NAME, L-NMMA or AG. Inter-

estingly, the NO scavenger methaemoglobin, which blocks NO effects by fixing this free radical to the haem core of its molecule, also prevented the muscle contractile response to NOS inhibitors. This is an apparently contradictory situation in which both a NO donor and a NO scavenger exert a similar final effect, namely, both prevented the intestinal muscle from contracting in response to NOS inhibitors. The contradiction is only apparent because the mechanisms involved in the unresponsiveness of the strip to NOS inhibitors are very different for the two compounds. On the one hand, NaNP generates NO independently of any NOS, and the amount of NO produced by 1 μM NaNP in our experimental conditions, most probably far exceeded that generated by SP-activated NOS in the same strip, and exerted by itself an inhibitory influence on muscle contractility. On the other hand, when methaemoglobin is superfused, any NO molecule generated under the effect of SP, is immediately scavenged, and then NOS inhibitors superfused after SP are unable to induce any contractile response. This observation actually strengthens the assertion that what is most probably involved in the process triggered by SP is the activation of a cNOS isoform, with subsequent *de novo* NO generation in intestinal tissues. The cellular source of this SP-induced NO generation cannot be specified from the present study, but could be either prejunctional neural structures or non-neural resident cells of the lamina propria of the intestinal wall.

In summary, we have shown that SP, and to a lesser extent NPγ, enabled NOS inhibitors to contract the guinea-pig ileum transiently *in vitro*, by exerting a priming effect which is unlikely to involve tachykinin NK_1, NK_2, or NK_3 receptors. This effect, may result from the activation by SP of an isoform of cNOS. Thus, *de novo* generated NO readily diffuses into intercellular spaces, and in a paracrine fashion, passes rapidly through biological membranes into smooth muscle cells, leading to intestinal relaxation. NO has a rather short, although not exactly known, biological half-life. Therefore, it has to be continuously generated to exert its effects. L-NAME, L-NMMA and AG, by interrupting this NOS-induced NO production, unveil an atropine-sensitive underlying contractile state of the SP-primed intestine, which could well result from the action of as yet unidentified inflammatory mediators released in the vicinity of intestinal smooth muscle cells under the stimulatory action of SP.

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References

AL-SAFFAR, A., RAHMAN, M. & HELLSTRÖM, P.M. (1993). Neurokinin receptor in motor responses to neuropeptide γ and electric field stimulation. *Acta Physiol. Scand.*, **151**, 497–505.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BOGERS, J.J., BULT, H., DE MAN, J.G., OOSTERBORSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Release of nitric oxide upon stimulation of inhibitory nonadrenergic noncholinergic nerves in the gastric fundus. *J. Pharmacol. Exp. Ther.*, **256**, 441–447.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VAN MAERCKE, Y.M. & HERMAN, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

CALIGNANO, A., WHITTLE, B.J.R., DI ROSA, M. & MONCADA, S. (1992). Involvement of endogenous nitric oxide in the regulation of rat intestinal motility in vivo. *Eur. J. Pharmacol.*, **229**, 273–276.

COLEMAN, R.A. & NIALS, A.T. (1989). Novel and versatile superfusion system. Its use in the evaluation of some spasmogenic and spasmolytic agents using guinea-pig isolated tracheal smooth muscle. *J. Pharmacol. Method*, **21**, 71–86.

COSTA, M., FURNESS, J.B. & LLEWELLYN-SMITH, I.J. (1987). *Histochemistry of the Enteric Nervous System*. New York: Raven Press.

D'AMATO, M., CURRO', D. & MONTUSCHI, P. (1992). Evidence for dual components in the non-adrenergic non-cholinergic relaxation in the rat gastric fundus: role of endogenous nitric oxide and vasoactive intestinal polypeptide. *J. Autonom. Nerv. Syst.*, **37**, 175–186.

DANIEL, E.E., PARRISH, M.B., WATSON, E.G., FOX-THRELKELD, J.E.T., REGOLI, D. & RAINSFORD, K.D. (1995). The tachykinin receptors inducing contractile responses of canine ileum circular muscle. *Am. J. Physiol.*, **31**, G161–G170.

EMONDS-ALT, X., ADVENIER, C., CROCI, T., MANARA, L., NELIAT, G., PONCELET, M., PROIETTO, V., SANTUCCI, V., SOUBRIÉ, P., VAN BROECK, D., VILAIN, P., LE FUR, G. & BRELIÈRE, J.C. (1993). SR48968, a neurokinin A (NK2) receptor antagonist. *Regul. Pept.*, **46**, 31–36.

EMONDS-ALT, X., BICHON, D., DUCOUX, J.P., HEAULME, M., MILOUX, B., PONCELET, M., PROIETTO, V., VANBROECK, D., VILAIN, P., NELIAT, G., SOUBRIE, P., LE FUR, G. & BRELIERE, J.C. (1994). SR 142801, the first potent non-peptide antagonist of the tachykinin NK3 receptor. *Life Sci.*, **56**, PL27–PL32.

EMONDS-ALT, X., VILAIN, P., GOULAOUIC, P., PROIETTO, V., VAN BROECK, D., ADVENIER, C., NALINE, E., NELIAT, G., LE FUR, G. & BRELIÈRE, J.C. (1992). A potent and selective non-peptide antagonist of the neurokinin A (NK2) receptor. *Life Sci.*, **50**, PL101–PL106.

FOX, J.E.T. & DANIEL, E.E. (1986). Substance P: a potent inhibitor of the canine small intestine in vivo. *Am. J. Physiol.*, **250**, G21–G27.

GALMICHE, A., ROZÉ, C., SCARPIGNATO, C. & GALMICHE, J.P. (1995). Monoxyde d'azote (NO), médiateur des influences non-adrénergiques non-cholinergiques du système nerveux entérique, et motricité oeso-gastrique. *Gastroenterol. Clin. Biol.*, **19**, 36–49.

GARCIA-VILLAR, R., GREEN, L.R., JENKINS, S.L., WENTWORTH, R.A., COLEMAN, R.A. & NATHANIELSZ, P.W. (1995). Evidence for the presence of AH-13205 sensitive EP2-prostanoid receptors in the pregnant baboon but not in the pregnant sheep myometrium near term. *J. Soc. Gynecol. Invest.*, **2**, 6–12.

GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor types: classification and membrane signalling mechanisms. *Neurochem. Int.*, **18**, 149–165.

GUARD, S., WATSON, S.P., MAGGIO, J.E., TOO, H.P. & WATLING, K.J. (1990). Pharmacological analysis of [3H]-senktide binding to NK3 tachykinin receptors in guinea-pig ileum longitudinal muscle-myenteric plexus and cerebral cortex membranes. *Br. J. Pharmacol.*, **99**, 767–773.

GUSTAFSSON, L.E., WIKLUND, C.U., WIKLUND, N.P., PERSSON, M.G. & MONCADA, S. (1990). Modulation of autonomic neuroeffector transmission by nitric oxide in guinea pig ileum. *Biochem. Biophys. Res. Commun.*, **173**, 106–110.

HAGAN, R., IRELAND, S., JORDAN, C., BERESFORD, I., DEAL, M. & WARD, P. (1991). Receptor-selective, peptidase-resistant agonists at neurokinin NK-1 and NK-2 receptors: new tools for investigating neurokinin function. *Neuropeptides*, **19**, 127–135.

HELLSTROM, P.M., MURTHY, K.S., GRIDER, J.R. & MAKHLOUF, G.M. (1994). Coexistence of three tachykinin receptors coupled to Ca^{++} signaling pathways in intestinal muscle cells. *J. Pharmacol. Exp. Ther.*, **270**, 236–243.

HOLZER, P. & MAGGI, C.A. (1994). Synergistic role of muscarinic acetylcholine and tachykinin NK-2 receptors in intestinal peristalsis. *Naunyn-Schmiedeberg's. Arch. Pharmacol.*, **349**, 194–201.

HOLZER-PETSCHE, U. (1995). Tachykinin receptors in gastrointestinal motility. *Regul. Pept.*, **57**, 19–42.

KANADA, A., HATA, F., SUTHAMMATPONG, N., MAEHARA, T., ISHII, T. & YAGASAKI, O. (1992). Key roles of nitric oxide and cyclic GMP in nonadrenergic and noncholinergic inhibition of rat ileum. *Eur. J. Pharmacol.*, **216**, 287–292.

KILBINGER, H. & WOLF, D. (1994). Increase by NO synthase inhibitors of acetylcholine release from guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's. Arch. Pharmacol.*, **349**, 543–545.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 5159–5162.

LI, Z.S., MURPHY, S., FURNESS, J.B., YOUNG, H.M. & CAMPBELL, G. (1993). Relationships between nitric oxide synthase, vasoactive intestinal peptide and substance P immunoreactivities in neurons of the amphibian intestine. *J. Autonom. Nerv. Syst.*, **44,** 197–206.

LLEWELLYN-SMITH, I.J., SONG, Z.M., COSTA, M., BREDT, D.S. & SNYDER, S.H. (1992). Ultrastructural localization of nitric oxide synthase immunoreactivity in guinea-pig enteric neurons. *Brain Res.*, **577,** 337–342.

MAGGI, C.A., GIULIANI, S., QUARTARA, L., ROVERO, P., RENZETTI, A.R., MIZRAHI, J. & GIACHETTI, A. (1992). Heterogeneity of tachykinin NK-2 receptors in rabbit, guinea-pig and human smooth muscles. *Neuropeptides*, **23,** 181–186.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S. & GIACHETTI, A. (1993a). In vivo and in vitro pharmacology of SR 48,968, a non-peptide tachykinin NK2 receptor antagonist. *Eur. J. Pharmacol.*, **234,** 83–90.

MAGGI, C.A., PATACCHINI, R., MEINI, S. & GIULIANI, S. (1993b). Nitric oxide is the mediator of tachykinin NK3 receptor-induced relaxation in the circular muscle of the guinea-pig ileum. *Eur. J. Pharmacol.*, **240,** 45–50.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIACHETTI, A. (1993c). Tachykinin receptors and tachykinin receptor antagonists. *J. Autonom. Pharmacol.*, **13,** 23–93.

MAGGI, C.A., ZAGORODNYUK, V. & GIULIANI, S. (1994). Tachykinin NK3 receptor mediates NANC hyperpolarization and relaxation via nitric oxide release in the circular muscle of the guinea-pig colon. *Regul. Pept.*, **53,** 259–274.

MARLETTA, M.A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.*, **268,** 12231–12234.

MCLEAN, S., GANONG, A., SEYMOUR, P., SNIDER, S.M., DESAI, M.C., ROSEN, T., BRYCE, D.K., LONGO, K.P., REYNOLDS, L.S., ROBINSON, G., SCHMIDT, A.W., SIOK, C. & HEYM, J. (1993). Pharmacology of CP 99,994, a non-peptide antagonist of the tachykinin neurokinin-1 receptor. *J. Pharmacol. Exp. Ther.*, **267,** 472–479.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43,** 109–142.

MORRIS, S.M. & BILLIAR, T.R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.*, **266,** E829–E839.

MOUSLI, M., BUEB, J.L., BRONNER, C., ROUOT, B. & LANDRY, Y. (1990). G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. *Trends Pharmacol. Sci.*, **11,** 358–362.

NAKANISHI, S. (1991). Mammalian tachykinin receptors. *Annu. Rev. Neurosci.*, **14,** 123–136.

OTSUKA, M. & YOSHIOKA, K. (1993). Neurotransmitter functions of mammalian tachykinins. *Physiol. Rev.*, **73,** 229–308.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524–526.

PUBLICOVER, N.G., HAMMOND, E.M. & SANDERS, K.M. (1993). Amplification of nitric oxide signaling by interstitial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 2087–2091.

RAHMAN, M., LORDAL, M., AL-SAFFAR, A. & HELLSTROM, P.M. (1994). Intestinal motility responses to neuropeptide γ in vitro and in vivo in the rat: comparison with neurokinin 1 and neurokinin 2 receptor agonists. *Acta Physiol. Scand.*, **151,** 497–505.

REGOLI, D., DRAPEAU, G., DION, S. & D'ORLÉANS-JUSTE, P. (1989). Receptors for substance P and related neurokinins. *Pharmacology*, **38,** 1–15.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262,** G379–G392.

SHARKEY, K.A. (1992). Substance P and calcitonin gene-related peptide (CGRP) in gastrointestinal inflammation. In *Neuro-immuno-physiology of the Gastrointestinal Mucosa. Implications for Inflammatory Diseases.* ed. Stead, R.H., Perdue, M.H., Cooke, H., Powell, D.W. & Barrett, K.E. pp. 425–442. New York: Annals of the New York Academy of Sciences.

SMITS, G.J.M. & LEFEBVRE, R.A. (1994). Tachykinin receptors involved in the contractile effect of the natural tachykinins in the rat gastric fundus. *J. Autonom. Pharmacol.*, **14,** 383–392.

STARK, M.E. & SZURSZEWSKI, J.H. (1992). Role of nitric oxide on gastrointestinal and hepatic function and disease. *Gastroenterology*, **103,** 1928–1949.

SUZUKI, N., MIZUNO, K. & GOMI, Y. (1994). Tachykinin-induced contractions in the circular muscle of guinea-pig ileum. *Jpn. J. Pharmacol.*, **65,** 233–240.

TODA, N., BABA, H. & OKAMURA, T. (1990). Role of nitric oxide in non-adrenergic, non-cholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips. *Jpn. J. Pharmacol.*, **53,** 281–284.

WANG, L., STANISZ, A.M., WERSHIL, B.K., GALLI, S.J. & PERDUE, M.H. (1995). Substance p induces ion secretion in mouse small intestine through effects on enteric nerves and mast cells. *Am. J. Physiol.*, **269,** G85–G92.

XUE, C., POLLOCK, J., SCHMIDT, H.H.H.W., WARD, S.M. & SANDERS, K.M. (1994). Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. *J. Autonom. Nerv. Syst.*, **49,** 1–14.

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