

Calcitonin gene-related peptide (CGRP)-enhanced non-adrenergic non-cholinergic contraction of guinea-pig proximal colon

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- 1 We have investigated the effect of calcitonin gene-related peptide (CGRP) on non-adrenergic, non-cholinergic (NANC) excitatory transmission to the longitudinal muscle of the guinea-pig proximal colon.
- 2 In the presence of atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM) and indomethacin (3 μM), electrical field stimulation (EFS, 1 Hz, 0.3 ms for 10 s) produced tetrodotoxin-(300 nM)-sensitive contractions which were reduced by the combined administration of FK 888 (10 μM) and MEN 10,376 (0.3 μM), to block tachykinin NK₁ and NK₂ receptors, respectively. Thus, the EFS-induced NANC contractions are a tachykinin-mediated response.
- 3 CGRP, at concentrations higher than 0.1 nM, caused an increase in the electrically-evoked, NANC contractions in a concentration-dependent manner and at 10 nM produced a maximal effect ($\text{pEC}_{50} = 9.20 \pm 0.17$, $n = 6$).
- 4 5-Hydroxytryptamine (5-HT, 1–100 nM) also caused an increase in the EFS-induced NANC contractions in a concentration-dependent manner and at 30 nM produced a maximal effect ($\text{pEC}_{50} = 8.06 \pm 0.09$, $n = 4$), but calcitonin (10–100 nM) failed to enhance the EFS-induced NANC responses. Moreover, a 5-HT₄ receptor antagonist, DAU 6285 (3 μM) abolished the enhancing action of 5-HT (30 nM).
- 5 The combined administration of FK 888 (10 μM) plus MEN 10,376 (0.3 μM) abolished the enhancement of EFS-induced NANC contractions by CGRP (10 nM), but DAU 6285 (3 μM) had no effect on the enhancement.
- 6 Human CGRP_{8–37} (1 μM), a CGRP₁ receptor antagonist had no effect on the submaximal enhancement of the electrically-evoked, NANC contractions by CGRP (1 nM).
- 7 CGRP (30 nM) had no effect on contractions evoked by exogenous substance P (0.3–1 nM).
- 8 These results indicate that in the guinea-pig proximal colon, CGRP produced an enhancement of NANC contraction induced by EFS through prejunctional mechanisms and that the enhancement is mediated by the stimulation of non-CGRP₁ receptors located on intramural tachykininergic neurones. Further, the possible contribution of 5-HT to the enhancing effect of CGRP appeared to be negligible.

Keywords: Calcitonin gene-related peptide (CGRP); non-adrenergic, non-cholinergic (NANC) transmission; proximal colon (guinea-pig)

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid residue neuropeptide that is widely distributed in the mammalian peripheral and central nervous system (Yamamoto & Tohyama, 1989). In the gut it has been shown to be present both in sensory and motor neurones (Rodrigo *et al.*, 1985). CGRP produces relaxation of intestinal smooth muscle (Maggi *et al.*, 1986), increases gastric mucosal blood flow (Holzer & Guth, 1991) and stimulates somatostatin release from antral D cells (Ren *et al.*, 1993).

A neuromodulatory role has also been proposed for CGRP. In the guinea-pig small intestine, the CGRP-induced contraction is significantly reduced by atropine suggesting that CGRP releases acetylcholine from cholinergic nerve terminals (Holzer *et al.*, 1989). In contrast, CGRP caused dose-dependent inhibition of [³H]-acetylcholine release from rat antral tissue fragments (Ren *et al.*, 1993). CGRP may also affect non-cholinergic excitatory neurotransmission as indicated by a significant reduction in the atropine-resistant ascending contraction in the guinea-pig small intestine (Holzer *et al.*, 1989). The aim of the present study was to investigate the action of CGRP on non-adrenergic, non-cholinergic (NANC) excitatory neurones by elucidating the effect of CGRP on electrically-evoked, NANC contractions of guinea-pig proximal colon.

Methods

Experimental set-up

Male Dunkin Hartley guinea-pigs, weighing 250–600 g, were anaesthetized with halothane and bled. A segment of the proximal colon, 2–12 cm apart from the caecum was removed. Strips of mucosa-free longitudinal muscle from the proximal colon were prepared as described in a previous study (Kojima, 1991). The strips were suspended in a longitudinal direction under a 0.5 g load in 15 ml tissue baths filled with a modified Tyrode solution (mM: NaCl 136.8, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.05, NaH₂PO₄ 0.42, NaHCO₃ 11.9, glucose 5.56, ascorbic acid 0.12) (pH 7.4) at 37°C and bubbled with 5% CO₂: 95% O₂. The modified Tyrode solution always contained atropine (0.3 μM) and guanethidine (5 μM) for the elimination of cholinergic and adrenergic responses and indomethacin (3 μM), to minimize endogenous prostanoid biosynthesis in response to electrical field stimulation (EFS). Hexamethonium (100 μM) was present during EFS to ensure that the responses recorded were the result of stimulation of postganglionic nerves. After setup, the strips were allowed to equilibrate for at least 60 min with renewal of the bathing solution every 15 min. Changes in mechanical activity of the tissue were recorded isotonicly (isotonic transducer, Nihon Kohden, TD-112S; Nihon Kohden recticoder, RJG-3006). To obtain nerve-mediated contractions of guinea-pig colon, the tissue was placed between bipolar platinum ring electrodes (5 mm in-

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ternal diameter, 6 mm apart) and connected to a Nihon Kohden stimulator (SEN-7203). Optimal stimulus parameters (rectangular pulse with 1 Hz, 0.3 ms duration for 10 s every 6 min) to obtain tetrodotoxin-sensitive contractions with stable amplitude were determined in a preliminary study. Voltage was adjusted to give contractions which were approximately 37% of the substance P (30 nM)-induced contraction (6.5–18.5 V). When control responses to EFS were stable for at least 30 min, the colonic strips were incubated with a single concentration of CGRP (0.1–30 nM), calcitonin (10–100 nM) or 5-hydroxytryptamine (5-HT; 1–100 nM) for 5 min and further 4–6 stimulations performed. In each experiment, substance P, 30 nM, was added at the first stage of the experimental protocol. When the effects of tachykinin antagonists, DAU 6285 or human CGRP_{8–37} were evaluated, the colonic strips were incubated for 20–60 min with the antagonists before addition of CGRP. In most cases, one of four preparations served as control and the three others were used for the study of CGRP in the presence of a set concentration of antagonist. The effect of CGRP (30 nM) on exogenous substance P (0.3–1 nM)-induced contractions was evaluated by comparison of the response before and after the addition of CGRP in the same preparation at 40 min intervals.

Analysis of data

The effects of agonists and antagonists on the EFS-induced contractions were expressed as a percentage of the mean of the 3 consecutive control responses prior to drug addition. The percentage effects of agonists were plotted as mean values to obtain log concentration-response curves. pEC₅₀ (–log molar concentration of the agonist that enhances the response to EFS by 50% of the maximal effect that can be reached with that agonist) of agonists was determined from each curve, according to the method of Van Rossum (1963). Means ± s.e. mean of *n* experiments are given throughout the paper. Antagonist ef-

fects were compared with control experiments and significance assessed by Student's paired *t* test. Results were considered significant if *P* < 0.05.

Drugs

The following drugs were used: atropine sulphate, hexamethonium chloride dihydrate, histamine dihydrochloride (Wako, Osaka, Japan); guanethidine sulphate (Ciba, Basel, Switzerland); tetrodotoxin (Sankyo, Tokyo, Japan); substance P, CGRP (rat, α -CGRP), human CGRP_{8–37}, calcitonin (human) (Peptide Institute, Osaka, Japan); 5-HT creatine sulphate (Merck, Darmstadt, Germany); indomethacin (Sigma, St. Louis, MO, U.S.A.); DAU 6285 hydrochloride (endo-6-methoxy-8-methyl-8-azabicyclo[3,2,1]oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride; gift from Dr. C.A. Rizzi, Boehringer Ingelheim, Milano, Italy); FK 888 (2-(N-Me)indolil)-CO-Hyp-Nal-NMe-Bzl; gift from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan); MEN 10,376 ([Tyr⁵,D-Trp^{6,8,9},Lys¹⁰]NKA(4-10) Research Biochemicals Inc, Natick, U.S.A.). All drugs were initially dissolved in saline with the following exceptions: indomethacin (100 μ M) was dissolved in distilled water containing equimolar concentrations of Na₂CO₃, and FK 888 (100 μ M) was dissolved in saline containing 50% dimethylsulphoxide. The vehicles had no effects on EFS-induced contractions. The reported concentrations are the calculated final concentrations in the bath solution.

Results

General

Isolated strips of guinea-pig proximal colon exhibited a low-amplitude spontaneous activity (Figure 1). In the presence of atropine (0.3 μ M), guanethidine (5 μ M), hexamethonium

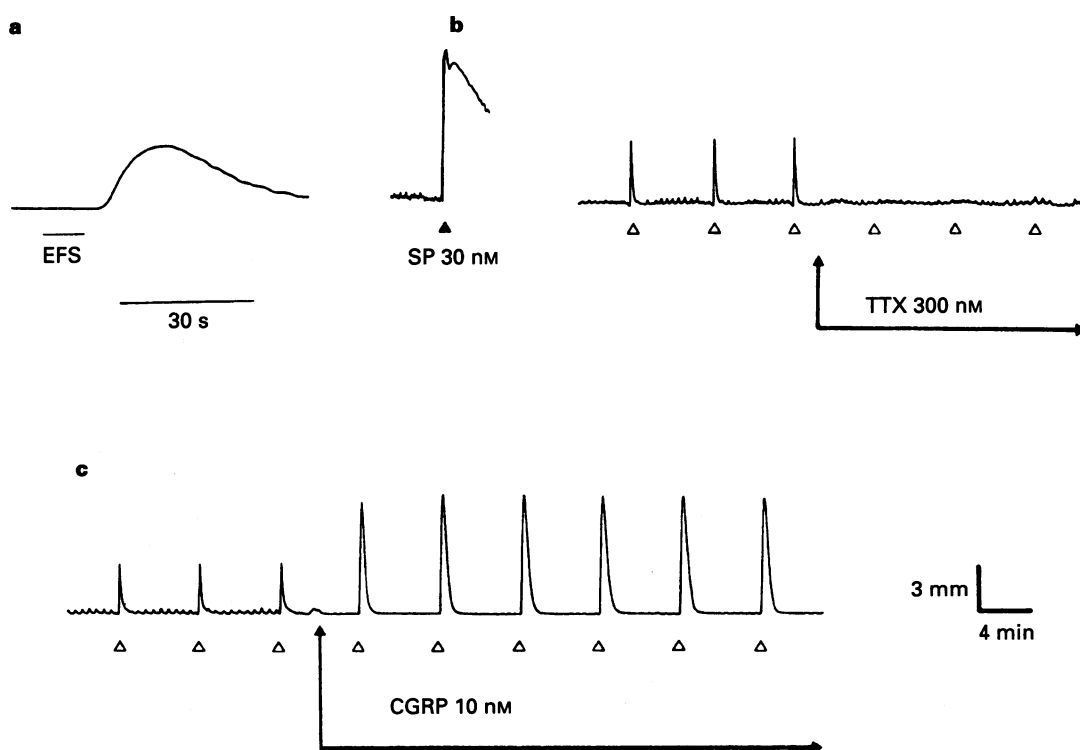


Figure 1 Typical tracings showing (a) Contractile response to electrical field stimulation (1 Hz, 0.3 ms for 10 s) in the mucosa-free longitudinal muscle of guinea-pig isolated proximal colon and the effects of (b) tetrodotoxin (TTX, 300 nM), (c) calcitonin gene-related peptide (CGRP, 10 nM) on electrically evoked (Δ , at 6 min intervals) contractions of guinea-pig isolated proximal colon. Vertical calibration indicates 3 mm shortening of the tissue, horizontal calibration indicates (a) 30 s and (b), (c) 4 min. Atropine (0.3 μ M), guanethidine (5 μ M), hexamethonium (100 μ M) and indomethacin (3 μ M) were present throughout the experiments.

(100 μM) and indomethacin (3 μM), electrical field stimulation (EFS, 1 Hz, 0.3 ms for 10 s every 6 min) of the colonic strips produced a phasic contraction, the amplitude of which averaged to $36.5 \pm 1.4\%$ of the substance P (30 nM, $n=18$)-induced contraction within a few seconds after the 10 s stimulation period (Figure 1a). The submaximal contractions evoked by EFS were completely abolished by tetrodotoxin (300 nM, $n=4$, Figure 1b). Moreover, the contractions evoked by EFS were significantly reduced ($P < 0.001$) by the combined administration of a highly selective tachykinin NK₁ receptor antagonist, FK 888 (10 μM) plus, a highly selective NK₂ receptor antagonist, MEN 10,376 (0.3 μM) ($43.0 \pm 6.6\%$, $n=6$, compared to pre-drug control).

Effects of CGRP

CGRP (0.1–30 nM) significantly enhanced neurogenic contractile responses evoked by EFS ($P < 0.001$) in a concentration-dependent manner, with a maximal effect of $204.3 \pm 10.2\%$ at 10 nM, resulting in pEC₅₀ value of 9.20 ± 0.17 ($n=6$, Figure 2). In this concentration-range, CGRP did not produce any contraction of the colonic strips, but caused suppression of spontaneous activity at concentrations higher than 1 nM. Removal of CGRP by repeated washing resulted in a prompt recovery in the amplitude of the spontaneous activity. Tracings showing the two effects of CGRP (10 nM) on the colonic strips are presented in Figure 1c. As shown in Figure 1c, the enhancing action of CGRP was rapid in onset and in all strips the maximal effect was reached within 5 min after the peptide administration. 5-Hydroxytryptamine (5-HT, 1–100 nM, pEC₅₀ = 8.06 ± 0.09 , $n=4$) also enhanced the EFS-evoked contractions, but calcitonin (10–100 nM, $n=4$) did not affect the EFS-evoked contractions (Figure 2). The maximal enhancement by 5-HT ($200.2 \pm 11.2\%$ at 30 nM) was close to that produced by CGRP. DAU 6285 (3 μM), a 5-HT₄ receptor antagonist, abolished the enhancement of EFS-induced responses by 5-HT (30 nM, $n=4$, $99.6 \pm 1.6\%$ compared to pre-drug control).

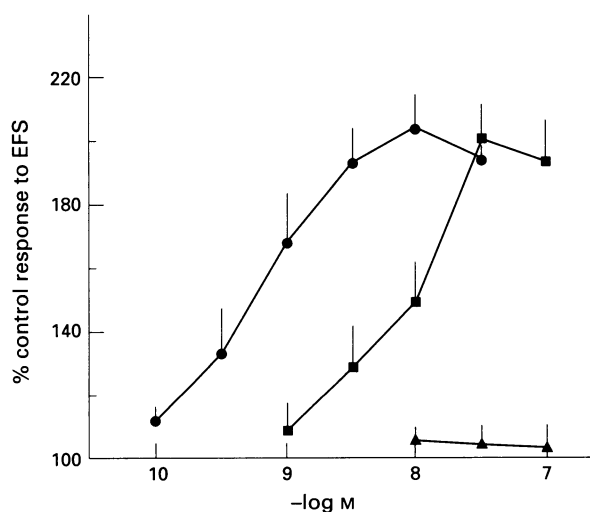


Figure 2 Concentration-response curves for the enhancing effect of calcitonin gene-related peptide (CGRP, ●), 5-hydroxytryptamine (5-HT, ■) and calcitonin (▲) on electrically-evoked contractions of the guinea-pig isolated proximal colon. The enhancement of neurogenic contractions was expressed as a percentage of the control response to electrical stimulation prior to the drug addition. Each point represents the mean \pm s.e. mean of 4–6 experiments. Atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM) and indomethacin (3 μM) were present throughout the experiments.

Effects of antagonist

To evaluate the participation of endogenous tachykinins or 5-HT in the enhancing action of CGRP, the combined administration of FK 888 plus MEN 10,376 or the effect of DAU 6285 were studied. When the tissue was preincubated with FK 888 (10 μM) plus MEN 10,376 (0.3 μM) for 30 min, the enhancement of the electrically evoked contractions induced by CGRP (10 nM) was completely prevented (Figure 3).

The substance P (30 nM)-induced contraction (the amplitude of which was $85.2 \pm 5.8\%$ of 30 μM histamine ($n=6$)-induced maximal contraction) was also blocked by preincubation of the tissue with a combination of FK 888 (10 μM) plus MEN 10,376 (0.3 μM) ($2.5 \pm 2.2\%$ of 30 μM histamine, $n=6$). However, 60 min preincubation of the tissue with DAU 6285 (3 μM) did not affect the CGRP-induced enhancement of the electrically evoked contractions (Figure 4). Moreover, a CGRP₁ receptor antagonist, human CGRP_{8–37} was tested against the enhancing effects of CGRP. Human CGRP_{8–37} (0.3–1 μM) displayed no significant agonist activity. Human CGRP_{8–37} (1 μM) was ineffective against the submaximal enhancement of electrically evoked contractions

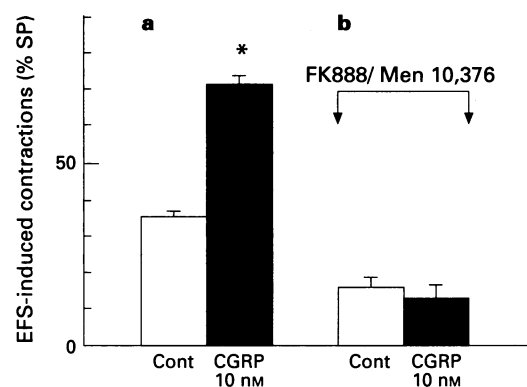


Figure 3 Histogram showing the effect of calcitonin gene-related peptide (CGRP, 10 nM, solid columns) on EFS-induced contractions (% of substance P, 30 nM) in the absence (a) and presence (b) of both FK 888 (10 μM) and MEN 10,376 (0.3 μM). Columns represent mean values \pm s.e. mean of 6 experiments. Significantly different from control response (Cont, open columns), * $P < 0.001$.

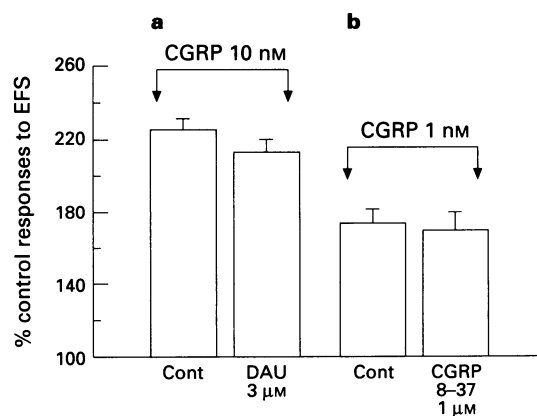


Figure 4 Histogram showing the effects of DAU 6285 (3 μM , a) and human CGRP_{8–37} (1 μM , b) on the enhancement of EFS-induced contractions by calcitonin gene-related peptide (CGRP, 1 or 10 nM). Columns represent mean values \pm s.e. mean of 6 experiments. Not significantly different from control (Cont).

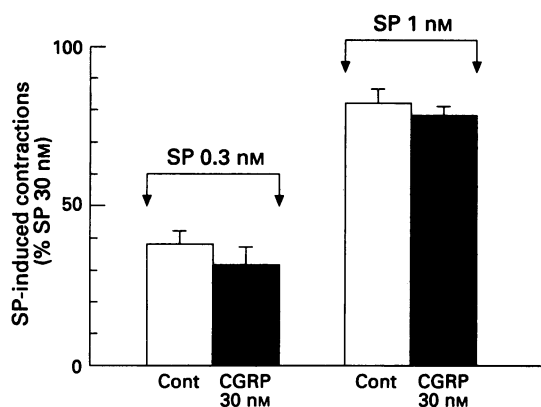


Figure 5 Histogram showing the effect of calcitonin gene-related peptide (CGRP, 30 nM, solid columns) on exogenous substance P (SP, 0.3 or 1 nM)-induced contractions. Columns represent mean values \pm s.e. mean of 6 experiments. Not significantly different from control (Cont, open columns).

induced by CGRP (1 nM, Figure 4). The depressant effect of CGRP (10 nM) on the spontaneous phasic contractile activity was also unaffected by human CGRP₈₋₃₇ (1 μ M).

Effects of CGRP on substance P-induced contractions

Substance P (0.3–1 nM) produced transient contraction of the colonic strip in a concentration-dependent manner. The substance P-induced contraction was unaffected by preincubation of the tissue with tetrodotoxin (300 nM, $n=4$). As shown in Figure 5, CGRP (30 nM) was without effect on contractions evoked by exogenous substance P (0.3 or 1 nM).

Discussion

In the presence of atropine, guanethidine, hexamethonium and indomethacin, the electrical field stimulation (EFS)-induced contraction of the longitudinal muscle of guinea-pig proximal colon is probably mediated via non-adrenergic, non-cholinergic (NANC) excitatory neurones, because tetrodotoxin abolished the contraction. Moreover, the EFS-induced NANC contraction was significantly reduced by the combined administration of a highly selective NK₁ receptor antagonist, FK 888 and a highly selective NK₂ receptor antagonist, MEN 10,376 (Maggi *et al.*, 1994). The results are consistent with previous work indicating that endogenous tachykinins are involved in non-cholinergic excitatory responses of the guinea-pig proximal colon *in vivo* (Giuliani *et al.*, 1993). In the present study, we found that the NANC contraction of the colonic strip evoked by EFS was enhanced in the presence of CGRP. The combined administration of FK 888 plus MEN 10,376 abolished the enhancement of EFS-induced NANC contraction by CGRP, suggesting that an increase in tachykinergic neurotransmission is involved in this enhancement. The in-

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ability of CGRP to affect the contractile response to exogenous substance P suggests that the enhancement of EFS-induced NANC contractions by CGRP was not related to a post-junctional change in colonic smooth muscle function, such as enhanced tachykinin receptor binding or potentiation of intrinsic contractile processes. Therefore, this suggests that the enhancement of NANC contractions by CGRP may involve a pre-junctional rather than a postjunctional mechanism, such as facilitation of tachykinin release from NANC neurones. This conclusion is consistent with the notion that CGRP is an endogenous neuromodulator in the enteric nervous system (Palmer *et al.*, 1986). CGRP is known to interact with calcitonin binding sites in kidney and brain (Goltzman & Mitchell, 1985). However, unlike CGRP, the lack of enhancing effect of calcitonin on EFS-induced NANC contractions in the present study rules out an action of CGRP at calcitonin receptors. The submaximal effect of CGRP (1 nM) was unaffected by a CGRP₁ receptor antagonist, human CGRP₈₋₃₇ (Dennis *et al.*, 1990). This compound has been reported to have a pA₂ value of 7.2 against CGRP-induced relaxations of the guinea-pig ileum and a value of 7.61 against the chronotropic action of CGRP on the guinea-pig right atrium, both responses reported to be mediated via CGRP₁ receptor activation (Poyner, 1992). However, at the concentration used in the present study (1 μ M) the lack of effect of this antagonist rules out an action of CGRP at the CGRP₁ receptor. Thus, these results support the hypothesis that the activation of non-CGRP₁ receptor can augment the NANC contractions evoked by EFS through the facilitation of intramural tachykinergic neurotransmission of the guinea-pig proximal colon.

We recently showed that 5-HT enhances the non-cholinergically mediated contraction of guinea-pig proximal colon by stimulation of 5-HT₄ receptors located on intramural preganglionic cholinergic neurones and tachykinergic neurones (Kojima & Shimo, 1995). Therefore, we were interested to determine whether 5-HT was implicated in this enhancing action of CGRP. However, it is unlikely that 5-HT plays a role in the enhancement of electrically-evoked NANC contractions induced by CGRP, because pretreatment with a 5-HT₄ receptor antagonist, DAU 6285 (Dumuis *et al.*, 1991), applied in a concentration sufficient to block the effect of 5-HT, did not modify the enhancing action of CGRP.

In conclusion, our data indicate that in the guinea-pig proximal colon, CGRP produces an enhancement of NANC contractions induced by EFS through prejunctional mechanisms and that the enhancement is mediated by the stimulation of non-CGRP₁ receptors located on intramural tachykinergic neurones. The exact role of CGRP in colonic motility is not well understood, but these studies are consistent with a neuromodulatory role for CGRP in tachykinergic neurotransmission and support the view that it might also participate in the regulation of colonic motility through a neuromodulatory function.

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