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Calcitonin gene-related peptide facilitates serotonin release from guinea-pig colonic mucosa *via* myenteric neurons and tachykinin NK_2/NK_3 receptors

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1 The ability of calcitonin gene-related peptide (CGRP), to alter the outflow of 5-hydroxytryptamine (5-HT) from the guinea-pig proximal colon, was evaluated using three different isolated preparations: whole colon, mucosa-free muscle layer and submucosa/mucosa preparations.

2 In the presence of the monoamine oxidase A inhibitor, clorgyline, CGRP elicited a concentration-dependent increase in 5-HT outflow from the whole colon, but not from mucosa-free muscle layer preparations. The CGRP-evoked 5-HT outflow was sensitive to tetrodotoxin (TTX) or hexamethonium, but was not detectable in submucosa/mucosa preparations. $HCGRP_{8-37}$ (3 μM) inhibited the submaximal effect of CGRP on the 5-HT outflow. [Cys(ACM)^{2,7}]hCGRP had a slight stimulant influence on the 5-HT outflow.

3 The selective NK_2 and NK_3 receptor antagonists, SR48968 or SR142801, respectively, prevented the enhancing effect of CGRP. By contrast, a selective NK_1 receptor antagonist L703606, failed to block the effect of CGRP.

4 The enhancing effect of CGRP was mimicked by the NK_2 receptor agonist [β-Ala^8]-neurokinin A $(NKA)_{4-10}$ and the NK_3 receptor agonist senktide. The effect of [β-Ala^8]-NKA_{4-10} on the 5-HT outflow was unaffected by TTX, while the effect of senktide was prevented by TTX, hexamethonium or SR48968. The present data also demonstrated a synergistic action of the NK_2 and NK_3 receptor agonists on the CGRP-evoked 5-HT outflow.

5 We concluded that CGRP facilitates 5-HT release from the guinea-pig colonic mucosa through an action on myenteric neurons and that this effect is mediated by endogenously released tachykinins, acting *via* tachykinin NK_2/NK_3 receptors in cascade.
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Keywords: Calcitonin gene-related peptide; colon; enterochromaffin cells; 5-HT (5-hydroxytryptamine, serotonin); tachykinins; NK_2 and NK_3 receptors

Abbreviations: EC cells, enterochromaffin cells; NKA, neurokinin A; TTX, tetrodotoxin

Introduction

5-Hydroxytryptamine (serotonin, 5-HT) has long been recognized as an important messenger substance, which is involved in a wide variety of colonic functions through its interaction with multiple receptor subtypes (Kojima & Shimo, 1995a; Fox-x-orenstein *et al*., 1996; Cooke *et al*., 1997). Most of the intestinal 5-HT is produced and stored in the mucosal enterochromaffin (EC) cells from which this amine is released into both the intestinal lumen and portal circulation (Bülbring & Lin, 1958; Schwörer *et al*., 1987). The regulatory mechanism of 5-HT release from the EC cells has been studied extensively, using a variety of experimental models in rabbit, guinea-pig and porcine small intestine (Forsberg & Miller, 1983; Schwörer *et al*., 1989, 1992). Although it has long been known that the colonic mucosa also contains high levels of 5-HT (Sjolund *et al*., 1983), the precise mechanism controlling the release of 5-HT from the colonic mucosa remains poorly understood. As 5-HT release from the EC cells has been linked to functional bowel disorders such as IBS (Sanger, 1996), a comprehensive understanding of the regulatory mechanism(s) of 5-HT release may lead to new ways to control defecation or diarrhea in diseases such as IBS.

The main functions of the colon of mammals are fluid absorption and secretion, and the coordinated propulsion of luminal content towards the anus. The enteric nervous system including myenteric plexus plays a key role in the regulation of these functions. In the colon, however, relatively little information is available regarding the role of the myenteric neurons in the regulation of 5-HT release from the mucosal EC cells. Recently, we have shown that the colonic mucosa of guinea-pigs is capable of releasing 5-HT into the lumen (Kojima & Ikeda, 1998; Kojima, 1999). The guinea-pig colonic mucosa is not innervated by intrinsic 5-HT-ergic neurons (Wardell *et al*., 1994). Thus, the mucosal release of 5-HT is not contaminated by neuronal 5-HT, and can be used as a model for studying the release mechanism(s) of 5-HT from the mucosal EC cells.

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Calcitonin gene-related peptide (CGRP) is a 37 amino-acid neuropeptide that is widely distributed in the peripheral and central nervous system, and is also found in high concentrations in the enteric nervous system of the gastrointestinal tract (Yamamoto & Tohyama, 1989; Sternini, 1992). Although CGRP has a number of effects in the enteric nervous system, its most pronounced action is through its neuromodulatory effects. Our previous *in vitro* studies in the guinea-pig colon have demonstrated that CGRP excites myenteric neurons (Kojima & Shimo, 1995b). In this study, we tested whether CGRP affects the release of 5-HT from the guinea-pig colonic mucosa *via* myenteric neurons.

Methods

Tissue preparation

Male Dunkin–Hartley guinea-pigs (250–500 g body weight) were purchased from Shizuoka Laboratory Animal Center, Inc. (Shizuoka, Japan). Guinea-pigs were anesthetized with enflurane and bled *via* the femoral artery. A segment of the proximal colon, 3–6 cm distal from the caecum was removed, and the luminal contents were washed out with a modified Tyrode's solution (composition mM: NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.56, $EDTANa_2$ 0.06). Three preparations were used in this study. The first preparation was the whole intact colon (1.0 cm in length), which contained all layers of the intestinal wall. The second preparation consisted of a sheet of submucosa/mucosa, which was obtained by removal of the muscularis external by blunt dissection, as described in a previous study (Kojima *et al*., 2002). For the third preparation, a mucosa-free longitudinal/circular muscle preparation with an intact adherent myenteric plexus was prepared as previously described (Kojima & Shimo, 1995a). These three distinct isolated preparations were suspended in a longitudinal direction under a 0.5 g load in 2-ml tissue baths filled with modified Tyrode's solution at 37 °C and were aerated with 95% O_2/5% CO_2. To minimize endogenous monoamine oxidase A activity, Tyrode's solution contained clorgyline (1 μM). The tissue preparations were allowed to equilibrate for 60 min with fresh replacement of the bathing medium every 5 min. Following the equilibration period, the experiments were conducted by collecting the bathing medium every 10 min. The medium obtained during the first 60–70 min was discarded. CGRP, [β-Ala^8]-neurokinin A_{4-10} or senktide were added to the incubation medium from 90 to 110 min. Antagonists were added to the incubation medium 30–60 min before the start of the collection period. At the end of the collection period, the tissue preparations were blotted and weighed.

Measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

The collected medium was lyophilized, dissolved in 0.4 M perchloric acid (200 μl) and passed through a 0.45-μm filter (Dismic-13CP, Advantec, Japan). 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels in the filtrate were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD-300, Eicom, Japan) as described previously (Kojima & Ikeda, 1998). Known concentrations of 5-HT and 5-HIAA (Sigma, St Louis, MO, U.S.A.) were used as standards. The separation of 5-HT and 5-HIAA was achieved by a reverse-phase column (length 110 mm, inner diameter 4 mm, C-18, 3 μm, BAS), using a mobile phase consisting of 0.1 M monochloroacetic acid, 1 mM EDTA, 60 mg l^{-1} sodium octylsulfate and 8% acetonitrile (pH 3.2) at a flow rate of 0.5 ml min^{-1}. Aliquots (20 μl) of the filtrate were injected directly into the HPLC column. The levels of 5-HT and 5-HIAA in the incubation media are expressed in units of pmol g^{-1} 10 min^{-1}. The results are expressed as a percentage of the mean outflow observed during the first two collection samples (70–90 min of incubation) of the individual experiments.

Drugs

[β-Ala^8]-neurokinin A_{4-10}, [Cys(ACM)^{2,7}]hCGRP, L703606 oxalate salt and senktide were purchased from Sigma Chemicals (St Louis, MO, U.S.A.). Atropine sulfate, hexamethonium chloride dihydrate and tetrodotoxin (TTX) were purchased from Wako (Osaka, Japan). Human CGRPα and Human $CGRP_{8-37}$ were purchased from Peptide Institute Inc. (Osaka, Japan). SR48968 and SR142801 were gifts from Sanofi Recherche (Montpellier, France).

Statistical analysis

Data are expressed as means ± standard error of the mean (s.e.m) from n experiments. The significance of the differences between two mean values was assessed using Student's t-test. For the comparison of one control with several experimental groups, the significance of differences was evaluated by the modified t-test according to Bonferroni. P-values <0.05 were considered significant.

Results

Effects of CGRP

The mean spontaneous outflow of 5-HT and 5-HIAA from the whole colonic strips incubated in modified Tyrode's solution (contained clorgyline 1 μM, a monoamine oxidase A inhibitor) in the absence of test compounds (determined between 70 and 90 min of incubation) amounted to 158.2 ± 11.3 and 39.2 ± 3.1 pmol g^{-1} 10 min^{-1}, respectively ($n=27$). In control experiments, the spontaneous outflow of 5-HT from the whole colonic strips did not change significantly during the period of observation up to 120 min (Figure 1a). Addition of CGRP to the incubation medium (1–100 nM, from 90 to 110 min of incubation) caused an increase in the outflow of 5-HT in a concentration-dependent manner, to a maximum of 269.9 ± 34.6 pmol g^{-1} 10 min^{-1} ($n=9$, 192.8 ± 17.3% compared to the initial outflow) at 100 nM CGRP (Figure 1a and b). The CGRP-evoked 5-HT outflow and basal 5-HT outflow were not detectable after removal of the underlying mucosa ($n=6$) (Figure 1a). CGRP (100 nM) had no effect on basal 5-HT outflow from the submucosa/mucosa preparations ($n=6$) (Figure 2). When TTX (1 μM) was added to the incubation medium 30 min before the start of the collection period, the enhancing effect of CGRP on the 5-HT outflow from the whole colonic strips was abolished ($n=6$) (Figure 2). The

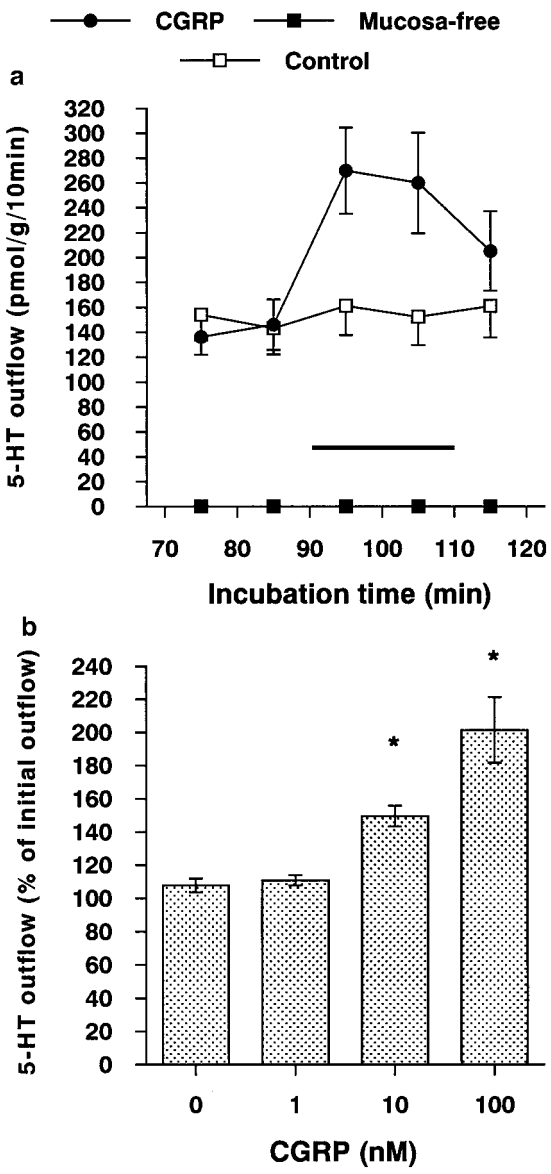

Figure 1 (a) Effects of CGRP (100 nM) on the outflow of 5-HT from the guinea-pig isolated whole colon and mucosa-free muscle preparations. CGRP was present from 90 to 110 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as pmolg tissue^{-1} 10 min^{-1}. (b) Effects of increasing concentrations of CGRP on the outflow of 5-HT from the whole colon. Height of columns: peak outflow of 5-HT, expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Mean values ± s.e.m (vertical bars) of six to nine experiments are shown. Significance of differences from the control: $*P<0.05$.

linear analogue [Cys(ACM)^{2,7}]hCGRP (100 and 300 nM) caused a slight but concentration-dependent increase in the 5-HT outflow from the whole colon: 109.0 ± 6.4% ($n=4$) and 135.0 ± 7.7% ($n=4$) at 100 and 300 nM, respectively.

Effects of antagonists

Several antagonists were tested against the CGRP-evoked 5-HT outflow from the whole colonic strips. In experiments in which the interaction between antagonists and CGRP was tested, the respective antagonist was already present 30–60 min

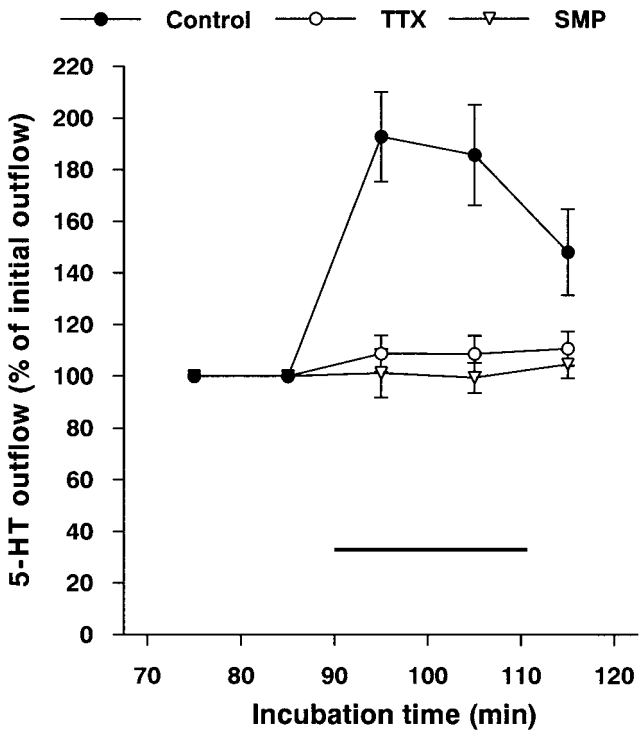

Figure 2 Effects of CGRP (100 nM) in the absence or presence of tetrodotoxin (TTX) (1 μM) on the outflow of 5-HT from the isolated whole colon of guinea-pig. CGRP had no effect on the outflow of 5-HT from submucosa/mucosa preparations (SMP). CGRP was present from 90 to 110 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Each point represents the mean ± s.e.m (vertical bars) from six to nine experiments.

before the collection period. In the whole colon preparations, the enhancing effect of CGRP (100 nM) was completely abolished by pretreatment with hexamethonium (100 μM, $n=6$), but was not significantly altered by atropine (0.2 μM, $n=6$) (Figure 3). The enhancing effect of CGRP (100 nM) was also prevented by the addition of SR48968 (1 μM, $n=6$) or SR142801 (1 μM, $n=6$), which are selective tachykinin NK_2 and NK_3 receptor antagonists, respectively (Figure 3). By contrast, L703606 (1 μM, $n=6$), a selective NK_1 receptor antagonist, did not affect the enhancing effect of CGRP (Figure 3). At 1 μM, $hCGRP_{8-37}$, a $CGRP_1$ receptor antagonist, failed to inhibit the submaximal effect of CGRP (10 nM), but this peptide prevented the submaximal effect of CGRP at a higher concentration of 3 μM (Figure 4). With the exception of atropine, which reduced the basal outflow of 5-HT by about 54%, none of the test drugs on its own significantly affected the basal outflow of 5-HT (data not shown).

Effects of tachykinin receptor agonists

[β-Ala^8]-neurokinin A $(NKA)_{4-10}$ (0.1. and 1 μM) and senktide (10 and 100 nM) are the selective NK_2 and NK_3 receptor agonists in the guinea-pig colon, respectively (Maggi *et al.*, 1994a, b). Herein, we examined whether the selective NK_2 receptor agonist, [β-Ala^8]-NKA_{4-10} and the selective NK_3 receptor agonist, senktide, mimic the enhancing effect of CGRP. In the whole colon, the addition of [β-Ala^8]-NKA_{4-10} (0.1 and 1 μM, from 90 to 110 min of incubation) to the

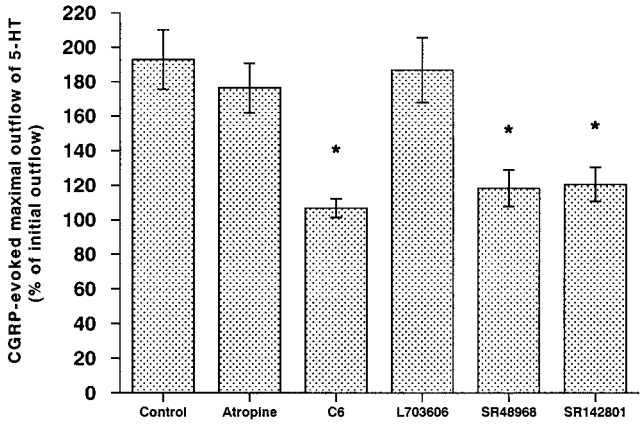

Figure 3 Effects of atropine (0.2 μM), hexamethonium (C6, 100 μM), L703606 (1 μM), SR48968 (1 μM) or SR142801 (1 μM) on the maximal outflow of 5-HT from the guinea-pig isolated whole colon evoked by CGRP (100 nM). Height of columns: maximal outflow of 5-HT, expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Mean values ± s.e.m (vertical bars) from six to nine experiments are shown. Significance of differences from the control: $^*P<0.001$.

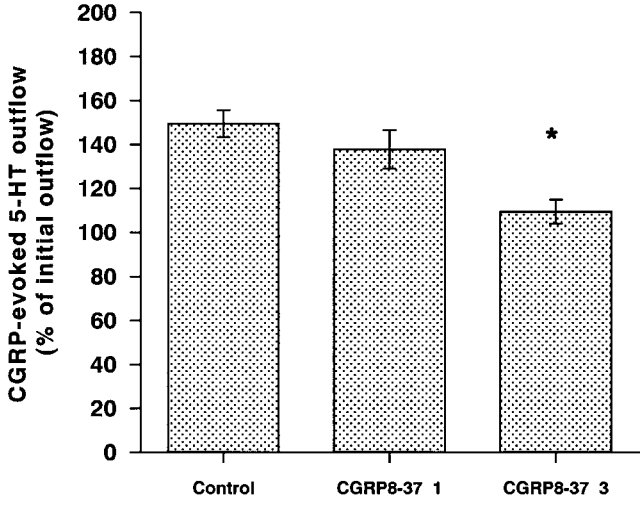

Figure 4 Effects of $hCGRP_{8-37}$ (1 and 3 μM) on the outflow of 5-HT from the guinea-pig isolated whole colon evoked by CGRP (10 nM). Height of columns: CGRP-evoked peak 5-HT outflow, expressed as % of the initial outflow (70–90 min of incubation) in the respective experiments. Mean values ± s.e.m (vertical bars) from four to six experiments are shown. Significance of differences from the control: $^*P<0.05$.

incubation medium caused an increase in the outflow of 5-HT in a concentration-dependent manner, to a maximum of 188.8 ± 14.5% at 1 μM ($n=7$) (Figure 5). When TTX (1 μM, $n=6$) was present, the enhancing effect of [β-Ala^8]-NKA_{4-10} was not significantly affected (Figure 5). [β-Ala^8]-NKA_{4-10} (1 μM) also increased basal 5-HT outflow from the submucosa/mucosa preparations: 157 ± 2.5% ($n=5$), as compared to the initial outflow. Likewise, senktide (10 and 100 nM) also caused a concentration-dependent increase in the outflow of 5-HT: 140.8 ± 4.3% ($n=6$) and 215.8 ± 14.6% ($n=6$), as compared to the initial outflow at 10 and 100 nM, respectively. TTX (1 μM, $n=4$), hexamethonium (100 μM, $n=4$) or SR48968 (1 μM, $n=4$) significantly inhibited the increase of 5-HT outflow evoked by senktide (100 nM) (Figure 6). Senktide (100 nM) had

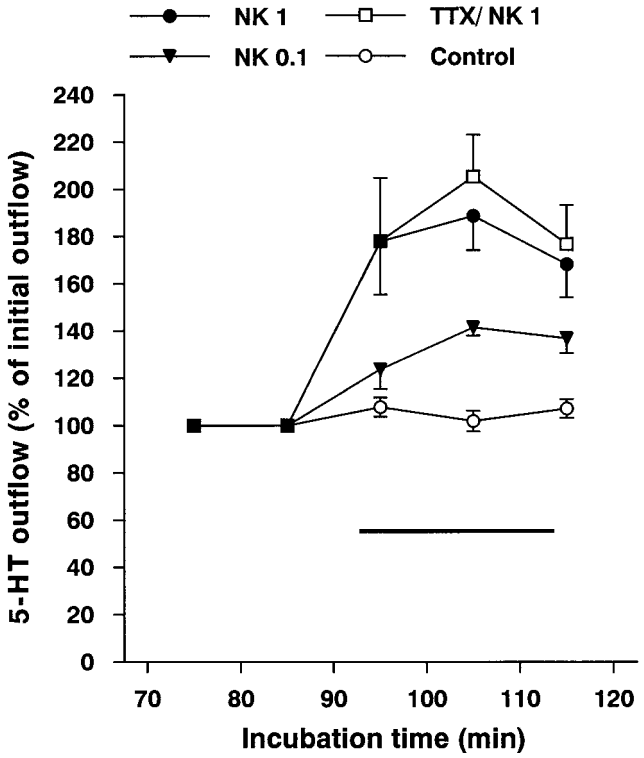

Figure 5 Effects of [β-Ala^8]-NKA_{4-10} (NK, 0.1 and 1 μM), in the absence or presence of tetrodotoxin (TTX, 1 μM) on the outflow of 5-HT from the guinea-pig isolated whole colon. [β-Ala^8]-NKA_{4-10} was present from 90 to 110 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT , expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Each point represents the mean ± s.e.m (vertical bars) from six to seven experiments.

no effect on basal 5-HT outflow from the submucosa/mucosa preparations ($n=4$) (Figure 6).

The combined effects of CGRP and the tachykinin NK_2/NK_3 receptor agonists were also examined. When [β-Ala^8]-NKA_{4-10} (100 nM) was coincubated with CGRP (10 nM), a synergistic increase in the 5-HT outflow was observed (Figure 7). Likewise, the synergistic rise of the 5-HT outflow was seen when senktide (10 nM) was coincubated with CGRP (10 nM) (Figure 7).

Discussion

The ability of CGRP to alter the outflow of 5-HT from the guinea-pig proximal colon was evaluated using three different isolated preparations: whole colon, mucosa-free muscle layer preparations and submucosa/mucosa preparations. As the first main finding of the present study, the outflow of 5-HT from the whole colon, but not from mucosa-free muscle layer preparations, was markedly increased in the presence of CGRP, suggesting that CGRP facilitates 5-HT release from mucosal store sites. The EC cells have commonly been addressed as a candidate of the 5-HT storage sites in the intestinal mucosa. Preliminary observations from our laboratory indicate that 5-HT-immunoreactive cells are identified as eggplant-shaped cells within the guinea-pig colonic mucosa, and that these cells are detected in the neighborhood of the base of crypt mucosal cells (Kojima *et al.*, 2003). Thus, the

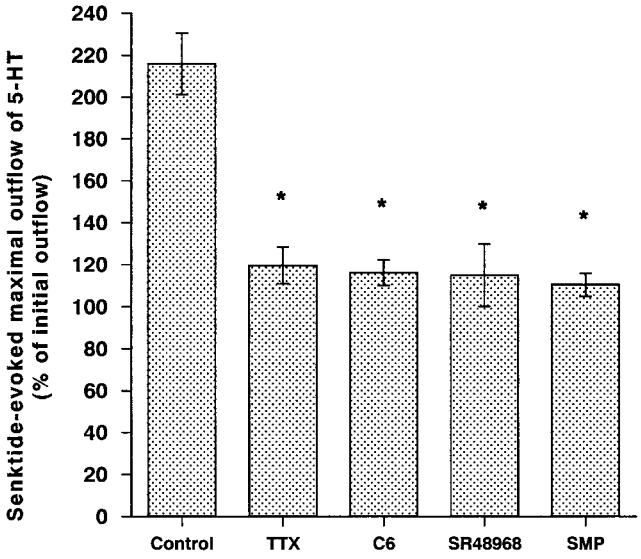

Figure 6 Effects of tetrodotoxin (TTX, 1 μM), hexamethonium (C6, 100 μM) or SR48968 (1 μM) on the maximal outflow of 5-HT from the whole colon evoked by senktide (100 nM). Senktide had no effect on the outflow of 5-HT from submucosa/mucosa preparations (SMP). Height of columns: maximal outflow of 5-HT, expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Mean values ± s.e.m (vertical bars) from four to six experiments are shown. Significance of differences from the control: $^*P<0.001$.

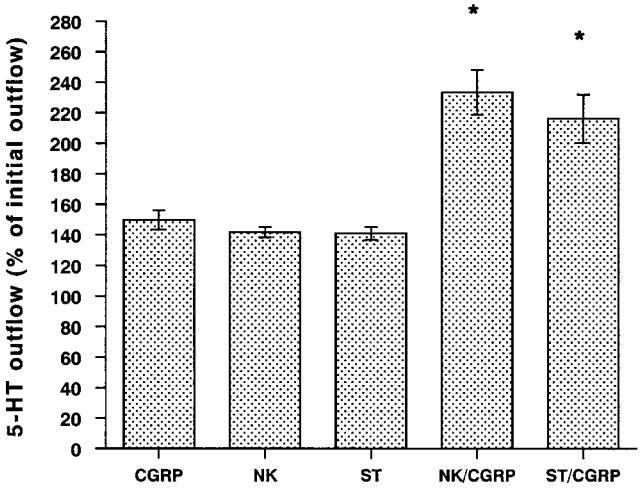

Figure 7 Effects of [β-Ala^8]-NKA$_{4-10}$ (NK, 100 nM) and senktide (ST, 10 nM) on the outflow of 5-HT from the whole colon in the absence or presence of CGRP (10 nM). Height of columns: peak outflow of 5-HT, expressed as % of the initial outflow (70–90 min of incubation) in the respective individual experiments. Mean values ± s.e.m (vertical bars) from four to six experiments are shown. Significance of differences from the CGRP alone: $^*P<0.001$.

mucosal 5-HT storage sites are most likely to be within the EC cells. The CGRP-evoked 5-HT outflow from the whole colon was sensitive to neuronal blockade with TTX, suggesting an indirect, neuronally mediated action. Furthermore, the CGRP-evoked 5-HT outflow was not detectable in the submucosa/mucosa preparations, suggesting that the CGRP-evoked 5-HT outflow requires the presence of intact myenteric plexuse neurons rather than submucosal plexuse neurons. Taken together, these data strongly support the view that CGRP facilitates 5-HT release from the EC cells through its actions on the myenteric neurons. In the present study, the CGRP-evoked 5-HT outflow was also sensitive to nicotinic cholinergic blockade with hexamethonium, suggesting that the CGRP-evoked 5-HT outflow is mediated by the release of acetylcholine from myenteric cholinergic interneurons. However, the role of the muscarinic cholinergic receptors in the enhancing effect of CGRP is questionable, because the CGRP-evoked 5-HT outflow was not affected by atropine.

CGRP receptors have been subdivided into $CGRP_1$ and $CGRP_2$, based on studies using CGRP peptide analogues. The $CGRP_1$ receptor is sensitive to the antagonism of $hCGRP_{8-37}$ (pA_2 7.2–7.7), but mostly insensitive to the weak agonistic effects of the linear analogue [Cys(ACM)^{2,7}]hCGRP (Juaneda *et al*., 2000). By contrast, the $CGRP_2$ receptor is less sensitive to $hCGRP_{8-37}$ (pA_2 6.2–6.6), whereas [Cys(ACM)^{2,7}]hCGRP demonstrated a weak agonistic potency for this receptor (Juaneda *et al*., 2000). In the present study, $hCGRP_{8-37}$ had less potency in antagonism of the submaximal effect of CGRP (10 nM), whereas [Cys(ACM)^{2,7}]hCGRP showed an agonistic effect, but the effect was much less than that of CGRP. Thus, these results rule out an action of CGRP at $CGRP_1$ receptors. The lesser potency of $hCGRP_{8-37}$ may reflect differences in CRLR (calcitonin receptor-like receptor) and the accessory protein RAMP (receptor activity-modifying proteins), but more detailed molecular pharmacological studies will be necessary for a clear characterization of the present CGRP receptor.

We have also examined the effects of selective NK_1 and NK_2 receptor antagonists in order to elucidate the role of tachykinins in the CGRP-evoked 5-HT outflow, since we have previously demonstrated interactions between CGRP and intrinsic tachykinergic neurons in the guinea-pig colon (Kojima & Shimo, 1995b). As the second main result in the present study, the CGRP-evoked 5-HT outflow was markedly attenuated by the NK_2 receptor antagonist SR48968, but not by the NK_1 receptor antagonist L703606, suggesting that the CGRP-evoked 5-HT outflow is mediated by endogenously released tachykinins, acting preferentially *via* NK_2 receptors. As would be expected from the result obtained with the NK_2 receptor antagonist, the NK_2 receptor agonist [β-Ala^8]-NKA$_{4-10}$ effectively enhanced the outflow of 5-HT from the whole colon. However, it is unlikely that [β-Ala^8]-NKA$_{4-10}$ facilitates the 5-HT outflow from the colon *via* an action on myenteric neurons, because the enhancing action of the NK_2 agonist was also seen in submucosa/mucosa preparations. Moreover, the enhancing effect of [β-Ala^8]-NKA$_{4-10}$ remained unaffected by the presence of TTX, excluding an indirect, neuronally mediated mechanism and supporting the hypothesis that stimulant tachykinergic NK_2 receptors may be localized directly at the EC cells. This concept is further corroborated by the observation that in the rat colonic mucosa, NK_2 receptors exist predominantly on epithelial cells but not on submucosal cell bodies (Cox *et al*., 1993). However, TTX is an axonal blocker; it does not block neurotransmitter release from mucosal nerve terminals. It is therefore possible that transmitters are released by a direct action of NK_2 agonist on the mucosal nerve terminals, and are thus responsible for 5-HT release.

Furthermore, we have made use of the NK_3 receptor antagonist, SR142801, to define a role for NK_3 receptors, as tachykinergic neuronal transmission *via* NK_3 receptors has been documented in the enteric nervous system in the guinea-

pig small intestine (Yau *et al*., 1992). As the third important finding, NK_3 receptors also played a role in the CGRP-evoked 5-HT outflow, as SR142801 markedly attenuated the 5-HT outflow evoked by CGRP. In the present study, the enhancing effect of CGRP was mimicked by the NK_3 receptor agonist senktide with a similar intrinsic activity. Since the effect of senktide was prevented by SR48968 and by TTX, we suggest that senktide releases neuronal tachykinins, which in turn enhances 5-HT release *via* NK_2 receptors on the EC cells or the mucosal nerve terminals. Moreover, the senktide-evoked 5-HT outflow was sensitive to hexamethonium, and was not detectable in sheets of submucosa/mucosa, suggesting that the NK_3 receptor-mediated 5-HT outflow is also mediated by the release of acetylcholine from myenteric cholinergic interneurons. We also found a synergistic action of the NK_2 and/or NK_3 receptor agonists on the CGRP-evoked 5-HT outflow. Taken together, these results indicate that under the conditions used in the present study, the CGRP-evoked 5-HT outflow is mediated by the activation in the cascade of NK_2 and NK_3 receptors.

In conclusion, our findings support the view that CGRP facilitates 5-HT release from the guinea-pig colonic EC cells through an action on myenteric neurons and that this effect is mediated by endogenously released tachykinins, acting *via* NK_2 and NK_3 receptors in cascade. Thus, CGRP and tachykinins appear to play a messenger role at the interface between the enteric nervous system and the mucosal EC cells. There is an abundance of evidence, which suggests that CGRP and tachykinins contribute to motor, secretory, vascular and immunological disturbances in intestinal anaphylaxis, infection and inflammation (Holzer, 1998). Therefore, in the pathophysiological states, excessive 5-HT secretion caused by the synergistic action between CGRP and tachykinins may participate in a variety of hypersecretory and inflammatory reactions of the colon.

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