J Pharmacol Sci **110**, 122 – 126 (2009)

Journal of Pharmacological Sciences

Short Communication

Further Investigation Into the Mechanism of Tachykinin NK_2 Receptor–Triggered Serotonin Release From Guinea-Pig Proximal Colon

Shu-ichi Kojima[1,*], Masashi Ikeda[2], and Yuichiro Kamikawa[1]

[1]*Department of Pharmacology,* [2]*Institute of International Education and Research, Dokkyo Medical University School of Medicine, Mibu, Tochigi 321-0293, Japan*

Received February 2, 2009; Accepted March 25, 2009

Abstract. The effects of the monoamine oxidase A (MAO-A) inhibitor clorgyline, the L-type calcium-channel blocker nicardipine, the syntaxin inhibitor botulinum toxin type C, and the potent thiol-oxidant phenylarsine oxide (PAO) on the selective tachykinin NK_2–receptor agonist [β-Ala^8]-neurokinin $A_{4\text{-}10}$ [βAla-NKA-(4-10)]–evoked 5-hydroxytryptamine (5-HT) outflow from colonic enterochromaffin (EC) cells was investigated in vitro using isolated guinea-pig proximal colon. The βAla-NKA-(4-10)–evoked outflow of 5-HT from clorgyline-treated colonic strips was markedly higher than that from clorgyline-untreated colonic strips. The βAla-NKA-(4-10)–evoked 5-HT outflow from the clorgyline-treated colonic strips was sensitive to nicardipine or botulinum toxin type C. Moreover, PAO concentration-dependently suppressed the βAla-NKA-(4-10)–evoked 5-HT outflow from the clorgyline-treated colonic strips. The suppressant action of PAO was reversed by the reducing agent dithiothrietol, but was not blocked by the protein tyrosine kinase inhibitor genistein. These results suggest that the tachykinin NK_2 receptor–triggered 5-HT release from guinea-pig colonic EC cells is mediated by syntaxin-related exocytosis mechanisms and that colonic mucosa MAO-A activity has the important function of modulating the tachykinin NK_2 receptor–triggered 5-HT release. It also appears that PAO-mediated sulfhydryl oxidation plays a role in modulating the tachykinin NK_2 receptor–triggered 5-HT release through a mechanism independent of inhibition of protein tyrosine phosphatase activity.

Keywords: colon, serotonin, tachykinin

5-Hydroxytryptamine (serotonin, 5-HT) has long been recognized as an important messenger substance that which regulates colonic motility, secretion, or sensation by acting via multiple receptor subtypes (1). 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 (2); therefore, alterations in the release of 5-HT from colonic EC cells affect the colonic function. The precise mechanism controlling the release of 5-HT from the colonic EC cells remains poorly understood; however, by using the clorgyline-treated preparations of guinea-pig isolated proximal colon, we have recently shown that the selective tachykinin NK_3–receptor agonist senktide is capable of inducing tetrodotoxin- or loperamide-sensitive 5-HT release from the colonic EC cells, whereas the selective tachykinin NK_2–receptor agonist [β-Ala^8]-neurokinin $A_{4\text{-}10}$ [βAla-NKA-(4-10)]–evoked 5-HT release is tetrodotoxin- or loperamide-insensitive (3, 4). Furthermore, the 5-HT–releasing effect of senktide was inhibited by the selective NK_2-receptor antagonist SR48968, thus suggesting that senktide releases neuronal tachykinins, which in turn enhances 5-HT release via NK_2 receptors on the colonic EC cells (3). Nevertheless, the underlying mechanism of the NK_2 receptor–triggered 5-HT release is still not completely understood.

In this study, we have examined the effects of the L-type calcium channel blocker nicardipine, the syntaxin inhibitor botulinum toxin type C, and the potent thiol oxidant phenylarsine oxide to obtain a better understanding of the underlying mechanism of the tachykinin NK_2

*Corresponding author. s-kojima@dokkyomed.ac.jp
Published online in J-STAGE on May 8, 2009 (in advance)
doi: 10.1254/jphs.09032SC

receptor–triggered 5-HT release from colonic EC cells. Additionally, we have determined if monoamine oxidase (MAO-A) plays a role in modulating the tachykinin NK_2 receptor–triggered 5-HT release by using the MAO-A inhibitor clorgyline.

All procedures were performed in accordance with the Dokkyo University School of Medicine animal care guidelines, which conform to the Guide for the Care and Use of Laboratory animal (NIH publication No. 85-23, revised 1985). Male Dunkin-Hartley guinea pigs (250 – 500 g body weight) were purchased from Shizuoka Laboratory Animal Center, Inc. (Shizuoka). 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 (136.8 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.56 mM glucose, and 0.06 mM $EDTANa_2$). Strips of whole colon (1.0 cm in length) or mucosa-free muscle layer only from the proximal colon were prepared as described in a previous study (3). The strips were suspended in a longitudinal direction under a 4.9-mN 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 type A activity (Fig. 1), the Tyrode's solution contained the monoamine-oxidase type A inhibitor clorgyline (1 μM). The tissue preparations were allowed to equilibrate for 90 min with fresh replacement of the bathing medium every 10 min. Following the equilibration period, the experiments were conducted by collecting the bathing medium every 10 min. The medium obtained during the first 90 – 100 min was discarded. βAla-NKA-(4-10) was added to the incubation medium from 120 to 140 min. At the end of the collection period, the tissue preparations were blotted and weighed.

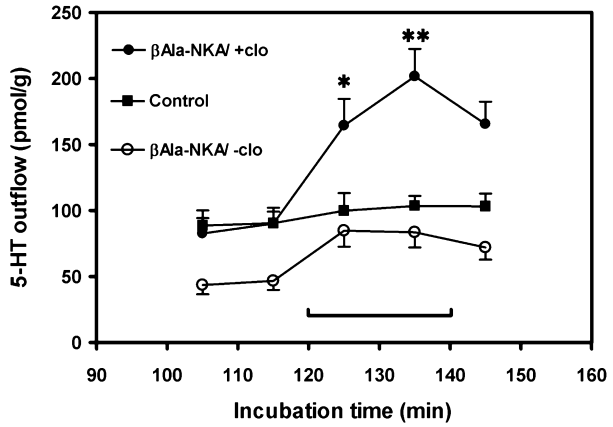

Fig. 1. Effects of βAla-NKA-(4-10) (1 μM) on the outflow of 5-HT (Control, closed square) from isolated guinea-pig colonic strips in the absence (open circle) or presence (closed circle) of clorgyline (1 μM). βAla-NKA-(4-10) was present from 120 to 140 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as pmol · g tissue^{-1} · 10 min^{-1}. Each point represents the mean ± S.E.M. (vertical bars) from six to nine experiments. *P<0.01, **P<0.001: βAla-NKA/ +clo vs. βAla-NKA/ –clo.

For the measurement of 5-HT and 5-hydroxy-indoleacetic acid (5-HIAA), 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, Tokyo). 5-HT and 5-HIAA levels in the filtrate were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD-300; Eicom, Tokyo) as described previously (3). Known concentrations of 5-HT and 5-HIAA (Sigma, St. Louis, MO, USA) were used as standards. The separation of 5-HT and 5-HIAA was achieved by a reverse-phase column [length of 100 mm, inner diameter of 4.6 mm, C-18 (3 μm); Shiseido, Tokyo], using a mobile phase consisting of 0.1 M monochloroacetic acid, 1 mM EDTA, 60 mg · l^{-1} sodium octylsulfate and 10% 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 medium 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 (100 – 120 min of incubation) of the individual experiments.

The following drugs were used: βAla-NKA-(4-10), genistein, phenylarsine oxide, and [sar^9,met(O$_2$)^{11}]-substance P (Sigma); clorgyline hydrochloride, nicardipine hydrochloride, and botulinus toxin C solution (Wako, Osaka). All drugs were dissolved in distilled water with the following exceptions: phenylarsine oxide (100 μM) and genistein (100 μM) were dissolved in 70% dimethylsulphoxide. All subsequent dilutions of the drugs were made with distilled water. The vehicles had no effects on βAla-NKA-(4-10)–evoked 5-HT outflow or basal 5-HT outflow.

Data are expressed as the mean ± S.E.M. from n experiments. Data were analyzed by ANOVA with the Newman-Keuls post hoc test. A value of P<0.05 was considered statistically significant.

The mean spontaneous outflow of 5-HT and 5-HIAA from the whole colonic strips incubated in modified Tyrode's solution (containing 1 μM clorgyline, a monoamine oxidase type A inhibitor) in the absence of test compounds (determined between 100 and 120 min of incubation) amounted to 89.5 ± 12.1 and 21.9 ± 6.4 pmol · g^{-1} · 10 min^{-1}, respectively (n = 7). Similar to previous observations (3, 4), the spontaneous outflow of 5-HT from the whole colonic strips did not change

significantly during the observation period in the control experiments (Fig. 1). As in previous studies (3, 4), the selective tachykinin NK_2–receptor agonist βAla-NKA-(4-10) (1 μM, from 120 to 140 min of incubation) caused a clear increase in the outflow of 5-HT; 5-HT outflow was enhanced to 201.5 ± 20.9 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1} (n = 7, 209.4 ± 9.6%, compared to the initial outflow), but not that of 5-HIAA (31.8 ± 5.3 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1}, n = 7) (Fig. 1). The mean spontaneous outflow of 5-HT and 5-HIAA from clorgyline-untreated preparations (determined between 100 and 120 min of incubation) amounted to 45.1 ± 6.8 and 111.1 ± 25.4 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1}, respectively (n = 6). The βAla-NKA-(4-10)–evoked maximal outflow of 5-HT from the clorgyline-treated preparations was markedly higher than that from the clorgyline-untreated preparations (5-HT: 84.7 ± 12.1 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1}, $P<0.001$; 5-HIAA: 166.0 ± 37.7 pmol $\cdot$ g^{-1} $\cdot$ 10 min^{-1}, n = 6; Fig. 1). The βAla-NKA-(4-10)–evoked 5-HT outflow and basal 5-HT outflow were not detectable after removal of the underlying mucosa (n = 4, data not shown). In contrast, the selective tachykinin NK_1–receptor agonist [sar^9,met(O$_2$)^{11}]-substance P (100 nM) did not have any significant effects on basal 5-HT outflow (n = 6, 122.4 ± 6.2% compared to the initial outflow, $P>0.05$).

When the L-type calcium channel blocker nicardipine (1 μM) was present from the start of incubation, nicardipine suppressed the βAla-NKA-(4-10) (1 μM)–evoked 5-HT outflow without affecting basal resting 5-HT outflow (n = 6) (Fig. 2). The syntaxin inhibitor botulinum toxin type C (3 μg/ml, from the start of incubation) also significantly reduced the βAla-NKA-(4-10)–evoked 5-HT outflow without affecting basal resting 5-HT outflow (n = 6) (Fig. 2).

We have tested the effect of the potent thiol oxidant phenylarsine oxide (PAO) on the βAla-NKA-(4-10)–evoked 5-HT outflow. PAO (1 – 100 μM, n = 6), when present from the start of incubation, suppressed the βAla-NKA-(4-10)–evoked maximal increase in 5-HT outflow in a concentration-dependent manner, by 112.6% at 10 μM, without affecting basal resting 5-HT outflow (Fig. 3). Since PAO is thought to act by an oxidative mechanism that is inhibited by reducing agents, we have also tested the effect of the reducing agent dithiothrietol (DTT) on the inhibitory effect of PAO. DTT (300 μM, n = 6, from the start of incubation) reversed the inhibitory effect of PAO (Fig. 3). Addition of DTT (300 μM, n = 4) alone to the incubation medium (from 120 to 140 min of incubation) caused a marginal increase in basal 5-HT outflow ($P>0.05$, data not shown). The inhibitory effect of PAO (1 μM) was not altered by the protein tyrosine kinase inhibitor genistein (GST, 100 μM, n = 4, from the start of incubation) (Fig. 3).

The results of the present study confirm our previous observations (3, 4): the selective tachykinin NK_2–receptor agonist βAla-NKA-(4-10) enhances 5-HT outflow from the mucosal 5-HT storage sites. Preliminary observations from our laboratory indicate that 5-HT–

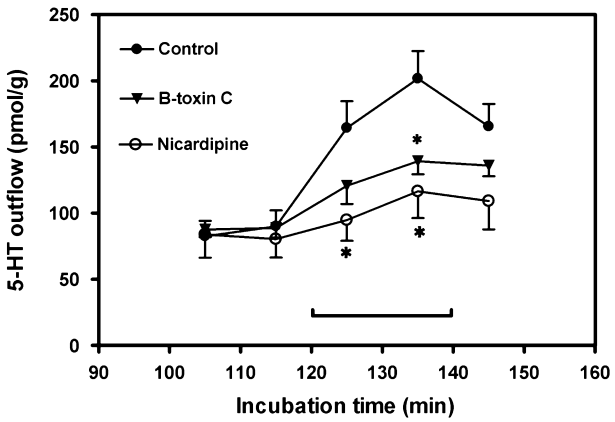

Fig. 2. Effects of βAla-NKA-(4-10) (1 μM) on the outflow of 5-HT from the clorgyline-treated colonic strips in the absence (closed circle, Control) or presence of botulinum toxin type C (3 μg/ml, closed triangle, B-toxin C) or nicardipine (1 μM, open circle). βAla-NKA-(4-10) was present from 120 to 140 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as pmol $\cdot$ g tissue^{-1} $\cdot$ 10 min^{-1}. Each point represents the mean ± S.E.M (vertical bars) from six to seven experiments. Significance of differences from the control: *$P<0.05$.

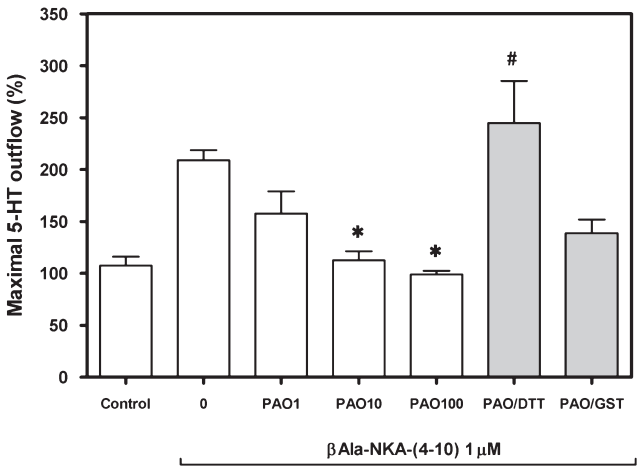

Fig. 3. Effects of PAO (1 – 100 μM) alone or PAO (10 μM) in the presence of DTT (300 μM, PAO/DTT) or genistein (100 μM, PAO/GST) on the maximal outflow of 5-HT from colonic strips evoked by 1 μM βAla-NKA-(4-10). Height of column: maximal outflow of 5-HT, expressed as % of the initial outflow (100 – 120 min of incubation) in the respective individual experiments. Mean values ± S.E.M (vertical bars) from six to seven experiments are shown. Significance of differences: *$P<0.01$, from the βAla-NKA-(4-10) alone; $^{\#}P<0.01$, from the βAla-NKA-(4-10) in the presence of PAO (10 μM).

immunoreactve 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 (5). Thus, the mucosal 5-HT storage sites are most likely to be within EC cells. The present results are also compatible with previous work indicating that strong NK_2-receptor immunoreactivity on the surfaces of enterocytes at the bases of crypts was found in the guinea-pig proximal colon, whereas the ileum and duodenum were devoid of immunoreactve epithelial cells (6). In the present study, clorgyline, which is a selective MAO-A inhibitor, markedly facilitated the βAla-NKA-(4-10)–evoked 5-HT outflow from the colonic EC cells. MAO-A has been shown to preferentially metabolize 5-HT and the non-selective MAO inhibitor pargyline is capable of inhibiting the level of mucosal 5-HT oxidation in rabbit small intestine (7). Thus, these findings favor the view that MAO-A is an important regulator of the tachykinin NK_2 receptor–triggered 5-HT release from colonic EC cells.

In the present study, we also found that the βAla-NKA-(4-10)–evoked 5-HT outflow was sensitive to the L-type calcium-channel blocker nicardipine or the syntaxin inhibitor botulinum toxin type C, indicating that the tachykinin NK_2 receptor–triggered 5-HT release is mediated by syntaxin-related exocytosis mechanisms. This result is also compatible with previous work indicating a major role for L-type calcium channel in mediating 5-HT release from EC cells caused by bile salt, cholera toxin, and 5-hydroxytryptophan (8, 9). However, the L-type calcium channel blocker did not completely abolish the βAla-NKA-(4-10)–evoked 5-HT outflow. Hence, a mechanism unrelated to the activation of L-type calcium channels might be partially involved in the βAla-NKA-(4-10)–evoked 5-HT release.

We have also examined the effect of the membrane-permeable thiol oxidant PAO in order to elucidate the role of sulfhydryl oxidation since PAO has been demonstrated to affect the guinea-pig L-type calcium channel activity by modifying the channel protein through sulfhydryl oxidation (10). In the present study, PAO markedly attenuated the βAla-NKA-(4-10)–evoked 5-HT outflow with a low micromolar range threshold concentration. PAO acts as a selective antagonist of protein tyrosine phosphatase (PTP) when used in this range of concentrations (11, 12). Therefore, an important question is whether the results observed in the present study can be attributed to the inhibition of PTP activity. However, genistein, which is a broad spectrum protein tyrosine kinase inhibitor, did not block the inhibitory effect of PAO. This suggests that the inhibitory action of PAO observed in the present study does not involve the inhibition of PTP activity. It is also thought that PAO acts by a mechanism involving sulfhydryl oxidation of cysteine residues and the membrane-permeable reducing agent DTT is known to reverse the action of PAO (10, 11). In the present study, the inhibitory action of PAO on βAla-NKA-(4-10)–evoked 5-HT outflow was reversed by DTT, consistent with its mechanism of action involving sulfhydryl oxidation of cysteine residues. This suggests that PAO-mediated sulfhdryl oxidation can attenuate the tachykinin NK_2 receptor–triggered 5-HT release. PAO has been demonstrated to affect the guinea-pig L-type calcium channel function by redox mechanisms that do not involve the inhibition of PTP activity (10). Therefore, the PAO-mediated sulfhydryl oxidation might play a role in tachykinin NK_2 receptor–triggered modulation of L-type calcium-channel function in colonic EC cells. It is also reported that PAO is capable of generating reactive oxygen species (ROS) (13). We previously showed that 5-HT levels in guinea-pig colonic mucosa were increased by catalase and decreased by hydrogen peroxide (14). Thus, we cannot exclude the possibility that ROS generation may contribute to the suppressant effect of PAO.

In conclusion, our results suggest that the tachykinin NK_2 receptor–triggered 5-HT release from guinea-pig colonic EC cells is mediated by syntaxin-related exocytosis mechanisms and that colonic mucosal MAO-A activity has the important function of modulating the tachykinin NK_2 receptor–triggered 5-HT release. It also appears that the PAO-mediated sulfhdryl oxidation plays a role in modulating the tachykinin NK_2 receptor–triggered 5-HT release through a mechanism independent of inhibition of PTP activity.

References

1 Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 2007;132:397–414.

2 Racke, K, Schworer H. Regulation of serotonin release from the intestinal mucosa. Pharmacol Res. 1991;23:13–25.

3 Kojima S, Ueda S, Ikeda M, Kamikawa Y. Calcitonin gene-related peptide facilitates serotonin release from guinea-pig colonic mucosa via myenteric neurons and tachykinin NK_2/NK_3 receptors. Br J Pharmacol. 2004;141:385–390.

4 Kojima S, Ikeda M, Kamikawa Y. Loperamide inhibits tachykinin NK_3-receptor-triggered serotonin release without affecting NK_2-receptor-triggered serotonin release from guinea-pig colonic mucosa. J Pharamcol Sci. 2005;98:175–180.

5 Kojima S, Ueda S, Ikeda M, Miyoshi H, Kamikawa Y. Calcitonin gene-related peptide facilitates serotonin release from guinea-pig colonic enterochromaffin cells. J Pharmacol Sci. 2003;91:173.

6 Portbury AL, Furness JB, Southwell BR, Wong H, Walsh JH, Bunnett NW. Distribution of neurokinin-2 receptors in the guinea-pig gastrointestinal tract. Cell Tissue Res. 1996;286:281–292.

7 Forsberg EJ, Miller RJ. Regulation of serotonin release from rabbit intestinal enterochromaffin cells. J Pharmacol Exp Ther. 1983;227:755–766.
8 Timar-Peregrin A, Ahlman H, Jodal M, Lundgren O. Involvement of serotonin and calcium channels in the intestinal fluid secretion evoked by bile salt and cholera toxin. Br J Pharmacol. 1999;127:887–894.
9 Kojima S, Ikeda M, Kamikawa Y. Investigation into the 5-hydroxytryptophan-evoked luminal 5-hydroxytryptamine release from the guinea-pig colon. Jpn J Pharmacol. 2000;84:174–178.
10 Sims C, Harvey RD. Redox modulation of basal and β-adrenergically stimulated cardiac L-type Ca^{2+} channel activity by phenylarsine oxide. Br J Pharmacol. 2004;142:797–807.
11 Oetken C, Von Willebrand M, Autero M, Ruutu T, Andersson LC, Mustelin T. Phenylarsine oxide augments tyrosine phosporylation in hematopoietic cells. Eur J Haematol. 1992;49:208–214.
12 Wijetunge S, Lymn JS, Hughes AD. Effect of inhibition of tyrosine phosphatases on voltage-operated calcium channel currents in rabbit isolated ear artery cells. Br J Pharmacol. 1998;124:307–316.
13 Fanelus I, Desrosiers RR. Reactive oxygen species generated by thiol-modifying phenylarsine oxide stimulate the expression of protein L-isoaspartyl methyltransferase. Biochem Biophys Res Commun. 2008;371:203–208.
14 Kojima S, Ikeda M, Shibukawa A, Kamikawa Y. Modification of 5-hydroxytryptophan-evoked 5-hydroxytryptamine formation of guinea-pig colonic mucosa by reactive oxygen species. Jpn J Pharmacol. 2002;88:114–118.