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Regional difference in correlation of 5-HT_4 receptor distribution with cholinergic transmission in the guinea pig stomach

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Abstract

Localization and function of 5-HT_4 receptors in the stomach were examined in mucosa-free preparations of antrum, corpus and fundus from guinea pig stomach by determination of acetylcholine release and in vitro receptor autoradiography. Specific [^{125}I]SB207710, (1-*n*-butyl-4-piperidinyl) methyl-8-amino-7-iodo-1,4-benzodioxane-5-carboxylate, binding sites were detected in 3 regions of the stomach. High densities of binding were observed in the myenteric plexus of antrum and corpus, but not fundus. In mucosa-free preparations treated with 5-HT_1, 5-HT_2 and 5-HT_3 receptor antagonists, 5-HT (10^{-8}–10^{-6} M) potentiated the electrically stimulated (0.5 Hz, 1 ms) outflow of [^{3}H]acetylcholine from antrum and corpus strips preloaded with [^{3}H]choline, but not from fundus strips, and the potentiation was antagonized by SB204070, (1-*n*-butyl-4-piperidinyl) methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate. Thus, 5-HT_4 receptors are located on myenteric cholinergic neurons in the antrum and corpus of guinea pig stomach and their activation evokes the release of acetylcholine.

Keywords: 5-HT_4 receptor; Stomach, guinea-pig; Myenteric plexus; Receptor autoradiography; Acetylcholine release

1. Introduction

Stimulation of the 5-HT_4 receptor modulates motility of the gastrointestinal tract, in an either excitatory or inhibitory manner, depending on the species and anatomical region (Ford and Clarke, 1993). In isolated preparations from rats, stimulation of the 5-HT_4 receptor causes relaxation of oesophagus (Baxter et al., 1991; Reeves et al., 1991; Mclean et al., 1995) and ileum (Mclean et al., 1995). In isolated preparations from guinea pigs, the 5-HT_4 receptor participates in the excitatory response, contraction of stomach (Buchheit and Buhl, 1994; Matsuyama et al., 1996), ileum (Craig and Clarke, 1990; Taniyama et al., 1991; Kilbinger and Wolf, 1992) and colon (Elswood et al., 1991; Briejer and Schuurkes, 1996). In isolated human colon preparations, stimulation of the 5-HT_4 receptor causes relaxation of circular muscle (Tam et al., 1994; Mclean et al., 1995; Mclean and Coupar, 1996), as is the case in rats. The inhibitory response appears to be induced by stimulation of 5-HT_4 receptors mainly located on smooth muscle cells (Mclean and Coupar, 1996), while excitatory responses are mediated by stimulation of the receptor mainly located on the excitatory neurons, such as cholinergic neurons and tachykinin-containing neurons (Craig and Clarke, 1990; Elswood et al., 1991; Taniyama et al., 1991; Kilbinger and Wolf, 1992; Briejer and Schuurkes, 1996; Matsuyama et al., 1996). The literature on functional studies on the gastrointestinal tract is extensive, but the localization of 5-HT_4 receptors has remained obscure. The stomach has three functions, storage, mixing and emptying, therefore the distribution of 5-HT_4 receptors is expected to be different among regions. Thus, in order to

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elucidate the relation between distribution and function of 5-HT_4 receptors in the stomach, we determined the localization of 5-HT_4 receptors involved in gastric motility of the guinea pig, using in vitro receptor autoradiography, and their function, by determination of acetylcholine release.

2. Materials and methods

Adult guinea pigs of either sex, weighing between 250 and 400 g, were killed by cervical dislocation, the stomach was immediately excised, and cut into strips of antrum, corpus and fundus. The mucosa was rapidly removed from the tissue.

2.1. Receptor autoradiography

Five animals were used for receptor autoradiographic experiments. The antrum, corpus and fundus were immediately immersed in isopentane at −30°C. Frozen tissues were cut into 20-μm-thick sections on a cryostat, thaw-mounted onto gelatin-coated glass slides and stored overnight under vacuum at 4°C. After preincubation in the incubation buffer of the following composition: 50 mM Tris–HCl buffer (pH 7.4) containing 4 mM $MgCl_2$, 0.3% bovine serum albumin, 0.2 mM ascorbic acid and 10 μM pargyline, at 23°C for 30 min, tissue sections were incubated in 2 ml of incubation buffer containing [^{125}I]SB207710 at a concentration of 10 pM at 23°C for 2 h. Consecutive tissue sections were labelled to characterize [^{125}I]SB207710 binding in the presence of 1 μM unlabelled SB204070 (non-specific binding). Then, the labelled sections were washed three times (for 1 min each) at 4°C in 50 mM Tris–HCl buffer (pH 7.2) and rinsed in ice-cold distilled water, then dried under a stream of cold air. To obtain autoradiograms with a higher resolution, the dry-labelled sections were apposed against Hyperfilm-[^{3}H] (Amersham, UK) for 1 week and the films were developed with a D19 developer (Eastman Kodak, USA) for 7 min at 4°C. Cholinesterase in consecutive tissue sections was stained according to a modified Karnovsky's method (Karnovsky and Roots, 1964) to verify the anatomical location of the myenteric plexus.

2.2. Measurement of [^{3}H]acetylcholine outflow

The methods of incubation and superfusion were as described by Kusunoki et al. (1985) and Takeda et al. (1991). Preparations from the 3 regions, the antrum, corpus and fundus were incubated at 35°C for 60 min with [^{3}H]choline at a final concentration of 200 nM in Krebs solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and glucose 11. After a wash in fresh Krebs solution for 30 min, the preparations were mounted in the superfusion apparatus and superfused at 1.2 ml/min with Krebs solution gassed with 95% O_2–5% CO_2, maintained at 35–37°C. Krebs solution containing 10 μM hemicholinium-3 to prevent the uptake of choline formed from acetylcholine was the superfusion medium. The superfusate was collected every 5 min. Radioactivity of the superfusates and of the tissue dissolved in Soluene at the end of the release experiment was counted in a liquid scintillation spectrometer (Packard Instrument, Ill, USA). Experiments were begun 60 min after the spontaneous [^{3}H] outflow had reached a plateau.

The validity of assuming total tritium as a measure of [^{3}H]acetylcholine release under the present experimental conditions has been documented in our previous studies (Kusunoki et al., 1985; Takeda et al., 1991). The outflow of [^{3}H] was represented as the fractional rate obtained by dividing the amount of [^{3}H] in the superfusate by the respective amount of [^{3}H] in the tissue. The [^{3}H] content of the tissue at each period was calculated by adding cumulatively the amount of each fractional [^{3}H] outflow to the [^{3}H] content of the tissue at the end of the experiment. From each of the outflow curves obtained by plotting the fractional outflow of [^{3}H] against time, the peak outflow of [^{3}H] evoked by stimulation in each case was calculated as the percentage increase over the basal outflow. When electrical transmural stimulation was applied successively three times to the preparation at 30 min intervals, there were no significant differences between the outflow of [^{3}H] evoked by the first (S1), the second (S2) and the third (S3) applications. Therefore, the ratio of S2/S1 calculated from the S1 and S2 without 5-HT was used as control, and the effect of 5-HT on the electrically evoked outflow was evaluated from the ratio of S2/S1 calculated from the S2 in the presence of 5-HT. The data were analyzed using Dunnett's *t*-test and a P value of 0.05 or less was considered statistically significant.

2.3. Chemicals

Substances used were as follows: [^{125}I]SB207710: (1-*n*-butyl-4-piperidinyl) methyl-8-amino-7-iodo-1,4-benzodioxane-5-carboxylate (74 TBq/mmol) (Amersham, UK), [^{3}H]choline (3.33 TBq/mmol) (New England Nuclear, Boston, Mass, USA), hemicholinium-3 and EGTA (Sigma, St. Louis, MO, USA), Soluene (Packard, Downers Groves, IL, USA), 5-hydroxytryptamine (5-HT) creatinine sulfate (Sigma), methysergide maleate and ketanserine tartrate (Research Biochemicals Int., Natick, MA, USA) and tetrodotoxin (Wako, Osaka, Japan). Other chemicals used were of reagent grade. SB204070: (1-*n*-butyl-4-piperidinyl) methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate and granisetron (BRL43694) were generously provided by Smith Kline Beecham, UK.

3. Results

3.1. In vitro receptor autoradiography

Fig. 1A–I shows typical autoradiograms of the [^{125}I]SB207710 binding sites in mucosa-free tissues of antrum, corpus and fundus of the guinea pig stomach. [^{125}I]SB207710 binding was visible in 3 regions (Fig. 1A–C), and was abolished by the addition of unlabelled 1 μM SB204070 (Fig. 1D–F). The distribution of [^{125}I]SB207710 binding sites in antrum and corpus was not even; high densities of binding sites were detected in the outer muscle layer including the myenteric plexus (indicated by arrows in Fig. 1A and B). When the distribution of [^{125}I]SB207710 binding sites (Fig. 1A–C) was compared with the consecutive sections stained with hematoxylin (Fig. 1G–I), the dense binding sites of [^{125}I]SB207710 indicated by arrows (Fig. 1A and B) corresponded to the myenteric plexus stained with hematoxylin (indicated by arrows in Fig. 1G and H). The binding sites of [^{125}I]SB207710 in the fundus were distributed moderately in the inner and outer muscle layers, including myenteric plexus (Fig. 1C). The magnified photographs of Fig. 1A and B (Fig. 2A and B) were compared with the consecutive cholinesterase-stained sections (Fig. 2C and D). The distribution of the highest binding sites of [^{125}I]SB207710 (indicated by arrows in Fig. 2A and B) correlated with that of cholinesterase staining in the antrum and corpus (Fig. 2C and D), thereby indicating that the densities of [^{125}I]SB207710 binding in the antrum and corpus were higher in the myenteric plexus than in the muscle layers.

3.2. Effect of 5-HT on the outflow of [^{3}H]acetylcholine from the antrum, corpus and fundus

The mucosa-free preparations of the antrum, corpus and fundus were superfused with the medium containing 10^{-7} M methysergide, 10^{-7} M ketanserin and 10^{-7} M granisetron to prevent the effect of 5-HT on the 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ receptors. The spontaneous outflow of [^{3}H]acetylcholine from preparations of the antrum, corpus and fundus preloaded with [^{3}H]choline reached a steady level to a single exponential curve, 60 min after the start of

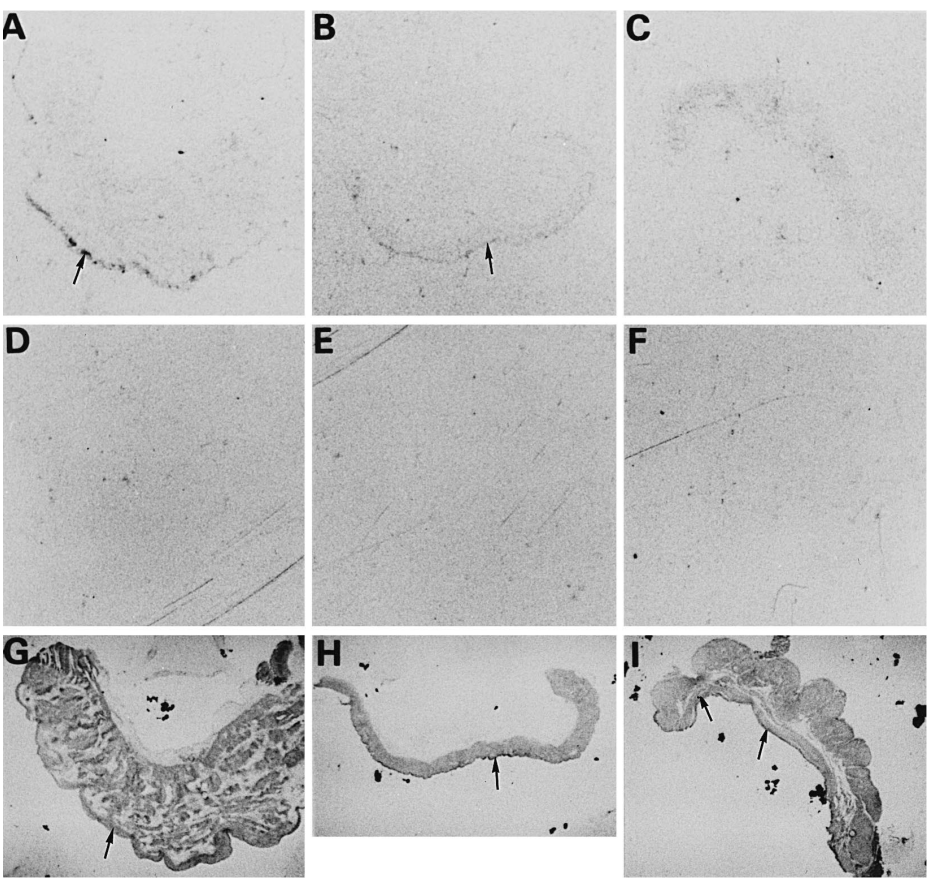

Fig. 1. Typical receptor autoradiographic localization of [^{125}I]SB207710 binding (A–F) sites and hematoxylin staining (G–I) in mucosa-free preparations from the antrum (A, D and G), corpus (B, E and H) and fundus (C, F and I) of guinea pig stomach. Consecutive, 20-μm-thick sections were labeled with 10 pM [^{125}I]SB207710 in the absence (total binding) (A–C) and presence of 1 μM SB204070 (non-specific binding) (D–F), in vitro. Arrows in G, H and I indicate the myenteric plexus of antrum, corpus and fundus. The high densities of binding sites indicated by arrows in A and B corresponded to the myenteric plexus in G and H.

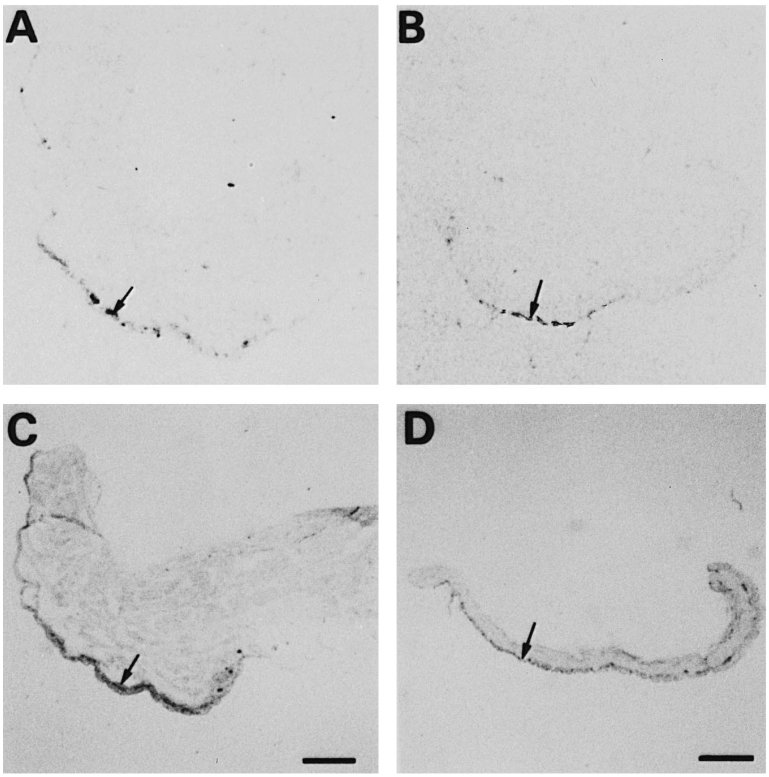

Fig. 2. Typical high magnification of receptor autoradiograms of [^{125}I]SB207710 binding sites (A and B) and cholinesterase staining (C and D) in mucosa-free preparations from the antrum (A and C) and corpus (B and D) of guinea pig stomach. Consecutive, 20-μm-thick sections were labeled with 10 pM [^{125}I]SB207710 in vitro and stained by a modified Karnovsky's method. Arrows in C and D indicate the highest distribution of cholinesterase staining in myenteric plexus, and correspond to the high densities of binding sites indicated by arrows in A and B. Scale bar: 0.5 mm.

the superfusion. Electrical transmural stimulation (0.5 Hz, 15 V, 1 ms, for 5 min) induced an increase in the outflow of [^{3}H]acetylcholine from preparations of the 3 regions. Superfusion with the Ca^{2+}-free medium or with the medium containing 3×10^{-7} M tetrodotoxin prevented the stimulation-evoked outflow of [^{3}H]acetylcholine (data not shown).

5-HT at 10^{-8} M to 10^{-6} M potentiated the electrical transmural stimulation-evoked outflow of [^{3}H]acetylcholine from the preparations of antrum and corpus, but not from the preparation of the fundus, in a concentration-dependent fashion (Fig. 3). The 5-HT (10^{-7} M)-induced potentiation was antagonized by SB204070 at concentra-

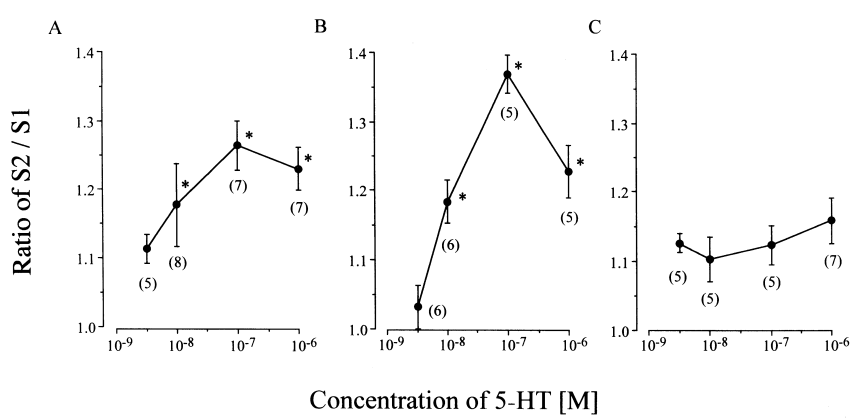

Fig. 3. Concentration–response curves of 5-HT$_4$ receptor-mediated potentiation of electrically stimulated [^{3}H] outflow from mucosa-free preparations of antrum (A), corpus (B) and fundus (C) preloaded with [^{3}H]choline. Electrical stimulation (0.5 Hz, 1 ms, 20 V) was applied for 5 min in the presence of 10^{-7} M methysergide, 10^{-7} M ketanserin and 10^{-7} M granisetron. The [^{3}H] outflow was represented as a ratio of the release evoked by the second stimulation (S2) to that by the first stimulation (S1). 5-HT was present during the second stimulation (S2). Each point represents the mean from the number of animals in parenthesis with S.E.M. of the mean shown by vertical lines. *Significance of difference from the value of S2/S1 obtained in the S2 without 5-HT was calculated by Dunnett's *t*-test, at the < 0.05 level of probability.

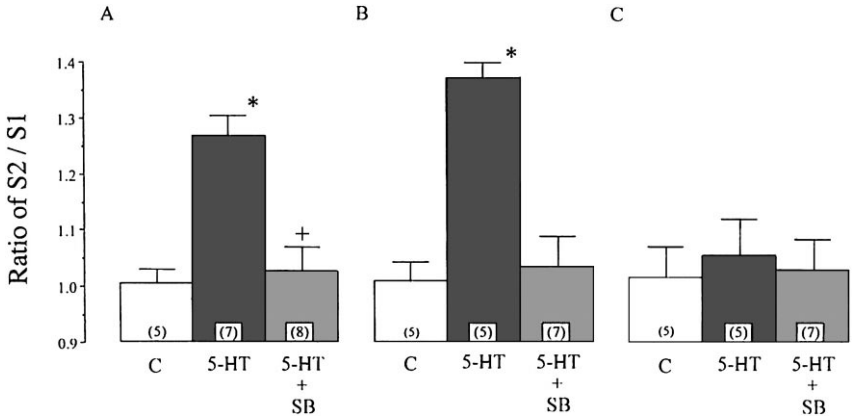

Fig. 4. Effect of SB204070 on the 5-HT_4 receptor-mediated potentiation of electrically-stimulated [^{3}H] outflow from mucosa-free preparations from antrum (A), corpus (B) and fundus (C) preloaded with [^{3}H]choline. The [^{3}H] outflow was represented as a ratio of the release evoked by the second stimulation (S2) to that by the first stimulation (S1). 5-HT (10^{-7} M) and SB204070 (SB, 10^{-8} M) were present during the second stimulation (S2). Each column represents the mean for the number of animals given in columns with S.E.M. of the mean shown by vertical lines. * Significance of difference from the control value, and $^+$*from* the value in the presence of 5-HT only was calculated by Dunnett's *t*-test, at the < 0.05 and 0.01 level of probability, respectively.

tions from 10^{-10} M and was inhibited completely by 3×10^{-8} M of SB 204070, a 5-HT_4 receptor antagonist (Wardle et al., 1994) (Fig. 4).

4. Discussion

We found a correlation between localization and function of 5-HT_4 receptors in the guinea pig stomach. Specific binding sites of [^{125}I]SB207710 were detected in the antrum, corpus and fundus. [^{125}I]SB207710 is a radioligand of high specific activity and selectivity for 5-HT_4 receptors (Brown et al., 1993) and [^{125}I]SB207710 binding was abolished by the addition of unlabelled SB 204070, a selective antagonist for the 5-HT_4 receptors (Wardle et al., 1994), therefore specific [^{125}I]SB207710 binding sites may indicate 5-HT_4 receptors. Photographs with a high magnification of specific [^{125}I]SB207710 binding sites showed the same pattern in the antrum and corpus. Comparison with cholinesterase-stained tissue demonstrated that the densities of [^{125}I]SB207710 binding were markedly higher in the myenteric plexus than in the muscle layers. In the fundus, the specific [^{125}I]SB207710 binding sites were distributed in the myenteric plexus and muscle layers, but were not clearly visible in the myenteric plexus. The receptors detected in the myenteric plexus indicate the receptors located on the nerve cell body/dendrite, while for the receptors detected in the muscle layer, it remains to be elucidated whether the receptors are located on the smooth muscle cells and/or on the nerve terminals innervating smooth muscle cells. Thus, the 5-HT_4 receptors may be predominantly located on the cell body/dendrite of enteric neurons in the antrum and corpus of guinea pig stomach.

Since our study was directed to 5-HT_4 receptors within the myenteric plexus, the function of these receptors was examined in relation to the activity of cholinergic neurons, in mucosa-free preparations with the 5-HT_1, 5-HT_2 and 5-HT_3 receptors blocked. 5-HT potentiated the electrically stimulated release of acetylcholine from preparations of the antrum and corpus, but not of the fundus. As the potentiation by 5-HT was inhibited by SB204070, a selective 5-HT_4 receptor antagonist (Wardle et al., 1994), 5-HT acted at the 5-HT_4 receptors located on cholinergic neurons and stimulation of the 5-HT_4 receptors increased the release of acetylcholine from the antrum and corpus, but not from the fundus. Functional 5-HT_4 receptors may correspond to the [^{125}I]SB207710 binding sites in the myenteric plexus demonstrated in the receptor autoradiographic study.

In the stomach, the 5-HT_4 receptors express contractile functions. Contractile 5-HT_4 receptors have been identified in the human gastric fundus, corpus and antrum (Schuurkes et al., 1991), rat gastric fundus (Amemiya et al., 1996), and guinea pig gastric corpus (Buchheit and Buhl, 1994; Matsuyama et al., 1996) and antrum (Tamura et al., 1996). These responses appear to be mediated via 5-HT_4 receptors located on the cholinergic neurons in the guinea-pig corpus (Matsuyama et al., 1996) and the non-cholinergic neurons in the rat fundus (Amemiya et al., 1996). Thus, stimulation of the 5-HT_4 receptor may enhance gastric emptying, as shown in in vivo studies (Gullikson et al., 1993; Hegde et al., 1995). Whether the 5-HT_4 receptors detected in the muscle layers are located on smooth muscle cells or on neurons innervating smooth muscle now remains to be elucidated. The non-neuronally located 5-HT_4 receptor has been implicated in secretory effects of 5-HT in the human small intestine (Borman and Burleigh, 1993) and colon (Borman and Burleigh, 1996) and ion transport in the rat colon (Budhoo et al., 1996), therefore examination of localization and function of the 5-HT_4 receptors in the muscle layers and mucosa is a topic of ongoing studies.

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