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A verification study of gastrointestinal motility-stimulating action of guinea-pig motilin using isolated gastrointestinal strips from rabbits and guinea-pigs

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ABSTRACT

Motilin (MLN), a 22-amino-acid peptide hormone, is generally present in the mucosa of the upper gastrointestinal (GI) tract, mainly the duodenum of mammals, and it regulates GI motility, especially that related to interdigestive migrating contraction. However, MLN and its receptor are absent in mice and rats, and MLN does not cause any mechanical responses in the rat and mouse GI tracts. The guinea-pig is also a rodent, but expression of the MLN gene in the guinea-pig has been reported. In the present study, two guinea-pig MLNs, FIPIFTYSELRRTQEREQNKGL found in the Ensemble Genome Database (gpMLN-1) and FVPIFTYSELRRTQEREQNKRL reported by Xu et al. (2001) (gpMLN-2), were synthesized, and their biological activities were evaluated in the rabbit duodenum and guinea-pig GI tract *in vitro*. Both gpMLNs showed contractile activity in longitudinal muscle strips of the rabbit duodenum. The EC_{50} values of gpMLN-1 and gpMLN-2 were slightly higher than that of human MLN (hMLN), but the maximum contractions were as same as that of hMLN. Treatment with GM109 and hMLN-induced receptor desensitization decreased the contractile activity of both gpMLNs, indicating that the two gpMLN candidates are able to activate the MLN receptor (MLN-R) of the rabbit duodenum. In guinea-pig GI preparations, hMLN and gpMLNs did not show any mechanical responses in circular muscle strips from the gastric antrum or in longitudinal strips of the duodenum, ileum and colon although acetylcholine and 1,1-dimethyl-4-phenylpiperazinium (DMPP) caused definite mechanical responses. The DMPP-induced neural responses in the gastric circular muscle and ileal longitudinal muscles were not modified by gpMLN-1. Even in the gastric and ileal strips with intact mucosa, no mechanical responses were seen with either of the gpMLNs. Furthermore, RT-PCR using various primer sets failed to amplify the gpMLN-2 mRNA. In conclusion, gpMLNs including one that was already reported and the other that was newly found in a database were effective to the rabbit MLN-R, whereas they did not cause any contractions or modification of neural responses in the guinea-pig GI tract, indicating that the MLN system is vestigial and not functional in regulation of GI motility in the guinea-pig as well as in other rodents such as rats and mice.

1. Introduction

Motilin (MLN), a 22-amino-acid-peptide, was first discovered from the porcine intestinal mucosa (Brown et al., 1971, 1973) and it has been shown to stimulate gastrointestinal (GI) motility in several mammals through activation of the MLN receptor (MLN-R) which was deorphanized as GPR 38 (Feighner et al., 1999). In humans, dogs and *Suncus*, MLN is considered to be an endogenous regulator of Phase-III activity of the interdigestive migrating contraction (IMC) in the stomach. The following findings support the involvement of MLN in gastric Phase-III activity of IMC: 1) peaks of endogenous MLN levels in plasma are highly associated with gastric Phase-III contractions, 2) exogenously applied MLN causes Phase-III-like gastric contraction, and 3) the Phase-III contraction is disrupted by administration of anti-MLN serum or MLN-R antagonists such as MA-2029 (Itoh et al., 1976, 1978; Peeters et al., 1980; Lee et al., 1983; Ozaki et al., 2009; Ogawa et al., 2012; Mondal et al., 2012).

The effects of MLN on GI motility have been investigated using dogs

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(*in vivo*) (Itoh, 1997; Ogawa et al., 2012) and rabbits (*in vivo, in vitro*) (Adachi et al., 1981; Kitazawa et al., 1994). However, since the body sizes of these animals are relatively large, they are unsuitable for animal models to investigate of MLN functions in detail. However, small experimental animals such as mice and rats lack genes for both MLN peptide and its receptor (He et al., 2010; Sanger et al., 2011), and hMLN does not affect the GI tract of either mice or rats *in vivo* and *in vitro* (Strunz et al., 1975; Depoortere et al., 2005).

Ghrelin (GHRL) is a MLN-related peptide that has some structural homology with MLN, and the growth hormone secretagogue receptor 1a (GHS-R1a, GHRL receptor) also has some structural similarity to the MLN-R (Asakawa et al., 2001; Peeters, 2005). GHRL stimulates gastric contraction in rats (Masuda et al., 2000; Fujino et al., 2003) and mice (Zheng et al., 2009), and a GHS-R1a antagonist attenuates the appearance of IMC in mice (Zheng et al., 2009), suggesting that GHRL serves as an alternative to MLN with regard to GI motility in MLN-lacking rodents. Recently, MLN and GHRL and their receptors have been identified in the house musk shrew (*Suncus murinus*), which is similar with humans and it has been shown that GHRL and MLN coordinate the IMC of the stomach (Sakahara et al., 2010; Suzuki et al., 2012; Mondal et al., 2012; Kuroda et al., 2015), proposing that *Suncus* is a suitable small laboratory animal for neurogastroenterological study of MLN and GHRL both *in vivo* and *in vitro*.

The guinea-pig (*Cavia porcellus*) belongs to rodentia as do rats and mice, and its GI tract responds to various bioactive substances and has abundant dense networks of enteric neurons. Therefore, the guinea-pig GI tract has been widely used for physiological and pharmacological studies on GI smooth muscle and enteric neurons. Several findings indicate the possible presence of the MLN system in the guinea-pig. In a previous molecular biological study, a MLN precursor was identified in the duodenal mucosa (GenBank accession number AF323752) (Xu et al., 2001). The results of an immunohistological study using a hMLN antibody and a hMLN-R antibody suggested the presence of a MLN-like peptide and MLN-R protein in the GI tract (Xu et al., 2005). In a functional study, superfusion of hMLN depolarized some of S and AH neurons in the myenteric plexus (Katayama et al., 2005). However, hMLN did not cause any contractions of the intestine either in a non-stimulated or electrically stimulated condition *in vitro* (Strunz et al., 1975; Minocha and Galligan, 1991), which is different from the results in the human gastric strips (Broad et al., 2012). Comparative functional studies using human, canine and chicken MLNs in isolated canine or chicken GI preparations indicated an obvious species-dependent difference in the contractile efficacy of MLNs (Poitras et al., 1987; Kitazawa et al., 1997). Therefore, the actions of MLN in the guinea-pig should be examined using homologous guinea-pig MLN instead of hMLN. In a search of a genome database (Ensemble, http://asia.ensembl.org/Cavia_porcellus/Info/Index), we found a 366-bp guinea-pig MLN cDNA encoding a 121 amino acid precursor (ENSCPOT00000008024), and a mature MLN peptide was deduced to be FIPIFTYSELRRTQEREQNKGL (gpMLN-1). As a result, unique substitutions were characterized in the second amino acid and in the middle part (from positions 11 to 13) and C-terminal part (from positions 16 to 22) of gpMLN-1: seven amino acids at positions 2, 8, 11, 13, 16, 18 and 22 were different when compared with hMLN (FVPIFTYGELQRMQE KERNKGQ). Actually, the primary structure of gpMLN-1 identified by us was similar to that of gpMLN reported by Xu et al. (2001) (gpMLN-2, FVPIFTYSELRRTQEREQNKRL): which has only two amino acid substitutions at position 2 (from I to V) and 21 (from G to R).

In the present study, the two gpMLNs, identified by us (gpMLN-1) and by Xu et al. (2001) (gpMLN-2), were synthesized, and their biological activities were evaluated both in the rabbit duodenum and the guinea-pig GI tract *in vitro*. Since it has been shown that MLN induces contractions of GI strips of mammals by actions on smooth muscles and on enteric neurons (Adachi et al., 1981; Kitazawa et al., 1994; Broad et al., 2012), the effects of MLN on smooth muscle tonus and on the neural responses were evaluated. Neural responses in the guinea-pig GI tract were evoked by 1,1-dimethyl-4-phenylpiperazinium (DMPP), an agonist for neural nicotinic receptor. Cloning of both gpMLN transcripts was also tried using various primer sets to examine the presence of gpMLN mRNA.

2. Materials and methods

All experiments were performed in accordance with institutional guidelines for animal care at Rakuno Gakuen University (VH25A18).

2.1. Animals and tissue preparations

Hartley guinea-pigs (*Cavia porcellus*) of both sexes (weighing 200–250 g) were obtained from Sankyo Lab Service (Sapporo, Japan). Since the rabbit duodenum is highly sensitive to MLN and has been used to investigate the mechanical responses to MLNs (Adachi et al., 1981; Kitazawa et al., 1994), Japanese white rabbits of both sexes (weighing 3–4 kg) were also obtained from Sankyo Lab Service. Both guinea-pigs and rabbits were in stainless steel cages at a regulated temperature (22 ± 2 °C) and at 60%–65% humidity with a normal 12–12-h light/dark cycle.

2.2. In vitro contraction study of gastrointestinal strips

Rabbits were deeply anesthetized by intravenous injection of pentobarbital sodium (50 mg/kg) and then killed by bleeding from the carotid vein. After a midline incision, the duodenum (next part of the gastric antrum, approx. 10 cm) was dissected out and longitudinal muscle strips were peeled out using fine forceps as previously described (Kitazawa et al., 1994). Guinea-pigs were also anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and killed by the same method as that for rabbits. After a midline incision, smooth muscle strips from different parts of the GI tract (stomach, duodenum, jejunum, ileum, proximal colon and distal colon) were prepared. We used two kinds of GI preparations for the guinea-pigs: muscle strips with the mucosa (whole tubular preparation of the intestine) and muscle strips without the mucosa (longitudinal muscle layers were peeled out as previously described, Kitazawa et al., 2011).

The GI strips of both rabbits and guinea-pigs were suspended vertically in an organ bath (5 mL) containing warmed (37 °C) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.5, bubbled with 95%O_2 + 5%CO_2 (pH 7.4). Mechanical activity of longitudinal muscles was measured with an isometric force transducer (SB-612T, Nihon Kohden), recorded on a computer, and analyzed using a computer-aided analysis system (Power Lab 2/25, Japan Bioresearch center, Nagoya, Japan). The initial load was set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to MLN application, each muscle strip was subjected to 3 or 4 continuous stimulations with 10^{-4} M acetylcholine (ACh) (15-min intervals) until a reproducible contraction was obtained.

For examining the response to MLN in the rabbit duodenum, MLN was applied cumulatively at concentrations from 10^{-10} M to 10^{-6} M. Since MLN caused distinct contractions, MLN was applied immediately after observing the peak amplitude of each concentration. In the guinea-pig, the responses to ACh (a muscarinic and nicotinic receptors agonist) and DMPP (a neural nicotinic receptor agonist) were observed to confirm the responsiveness of smooth muscles and enteric neurons in the GI preparations. MLN was applied cumulatively at 2–3-min intervals, and the mechanical response was observed. Changes in smooth muscle tonus caused by MLN peptides were normalized using 10^{-4} M ACh-induced contraction and indicated as relative change (% response) in smooth muscle tonus. The effects of pretreatment with gpMLN-1(3 min) on the neural responses to DMPP were also examined in the gastric circular muscle and ileal longitudinal muscle.

Table 1
Six primer sets for RT-PCR detection of gpMLN-2 in the present experiments.

Primer sets for RT-PCR		Direction	Sequence
Set A	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	GAGGAGTCTGCCTTGGAGAG
Set B	guinea pig motilin	FWD	GCGTACATCCAGAATGCTGTC
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set C	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	GAGGAGTCTGCCTTGGAGAG
Set D	guinea pig motilin	FWD	AGAATGCTGTCCCGAAAGG
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set E	guinea pig motilin	FWD	TTCCAATCTTCACTTACAGCGAG
	guinea pig motilin	BWD	CCAATTTCCACTGGAGCAG
Set F	guinea pig motilin	FWD	GTCCCTGAGGGTACAGCAGA
	guinea pig motilin	BWD	CCTCACTGAGCAGAGCTTCC

2.3. PCR cloning of guinea-pig MLN

Based on the guinea-pig MLN sequence reported by Xu et al. (2001), we designed several primer sets for PCR cloning of gpMLN-2 (Table 1). Total RNA was extracted from the guinea-pig small intestine using ISOGEN (Nippon Gene) or an RNeasy mini kit (QIAGEN). After DNase I (Promega) treatment of total RNA to remove genome DNA contamination, 1 μg total RNA was reverse-transcribed to cDNA by using Superscript III reverse transcriptase (Invitrogen) at several temperatures (42℃ to 50℃) with a random or oligo-dT primer or gene-specific primers. RT-PCR was performed by using Ex taq polymerase (Takara) or AmpliTaq Gold (Thermo Fisher Scientific) with annealing temperature of 50-70℃ in each cDNA and primer sets. In addition, one-step RT-PCR kits (QIAGEN) were also used with each of the primer sets following the manufacture's instructions.

2.4. Chemicals

Two guinea-pig MLNs were custom-synthesized by Scrum Co. Ltd. (gpMLN-1, Tokyo, Japan) and Peptide Institute Inc. (gpMLN-2, Osaka, Japan), and their purity was commercially confirmed by a single peak of high-performance liquid chromatography. Human MLN (hMLN) was purchased from Peptide Institute Inc. Acetylcholine chloride and tetrodotoxin (TTX) were obtained from Wako Co. Ltd. (Tokyo, Japan). Atropine sulfate, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and L-nitroarginine methylester hydrochloride (L-NAME) were obtained from Sigma-Aldrich (MO, USA). GM109, a MLN-R antagonist was kindly donated by Chugai Co. Ltd. (Tokyo, Japan). All chemicals were dissolved in distilled water and the desired concentration was directly applied to an organ bath using a micropipette. The applied volume was less than 0.5% of the bath volume (5 mL).

2.5. Statistical analysis

Data are expressed as means ± S.E.M of more than four experiments. The significance of differences between the values was determined at $P < 0.05$ using Student's *t*-test (paired and unpaired) for single comparison or ANOVA followed by Dunnett's test or Bonferroni test for multiple comparison of the mechanical responses.

3. Results

3.1. Effects of hMLN and gpMLNs in the rabbit duodenum

In the isolated longitudinal muscle strips from the rabbit duodenum, hMLN caused concentration-dependent contraction at concentrations from 10^{-10} M to 10^{-6} M. EC_{50} and relative maximum contraction (% to 10^{-4} M ACh-induced contraction) were 4.2 ± 0.7 × 10^{-9} M and 89.3 ± 3.0% (n = 15), respectively (Fig. 1, left panel and Fig. 2). Synthetized gpMLN-1 caused contraction of the rabbit duodenum (Fig. 1, middle panel). The contraction-response curve was located slightly right to that of hMLN, but EC_{50} (5.3 ± 1.0 × 10^{-9} M, n = 8) and maximum amplitude of contraction (81.1 ± 4.3%, n = 8) were comparable to those of hMLN (Fig. 2). Only the contraction at 3 × 10^{-8} M (71.6 ± 4.6%, n = 8) was significantly smaller ($P = 0.048$) than that of hMLN (83.1 ± 3.0%, n = 15). Synthetized gpMLN-2 also showed contractile activities in the isolated rabbit duodenum in a concentration-dependent manner (Fig. 1, right panel and Fig. 2). The EC_{50} value of gpMLN-2 was 10.7 ± 1.8 × 10^{-9} M (n = 9), which was significantly higher than those of hMLN ($P = 0.0005$) and gpMLN-1 ($P = 0.012$).

To confirm the involvement of the MLN-R in gpMLN-induced contractions, the effect of a MLN-R antagonist (GM109, Takanashi et al., 1995) was examined. GM109 alone at a dose of 10^{-6} M did not affect the concentration-response curve of ACh as previously reported (Kitazawa et al., 2017), but it inhibited contractile responses to hMLN, gpMLN-1 and gpMLN-2, and the concentration-response curves shifted rightward by the same degrees (Fig. 2).

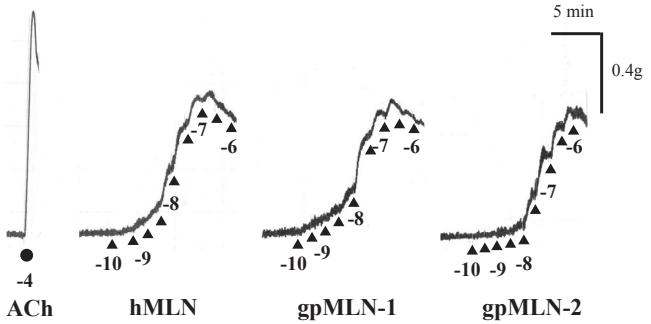

Fig. 1. Representative contractile responses to human MLN (hMLN), guinea-pig MLN-1 (gpMLN-1) and guinea-pig MLN-2 (gpMLN-2) in the rabbit duodenal longitudinal muscles. hMLN, gpMLN-1 and gpMLN-2 were applied cumulatively (10^{-10} M–10^{-6} M) and evoked contractions were observed. The amplitude of MLN-induced contraction was normalized by acetylcholine (ACh, 10^{-4} M, ●)-induced contraction. The number under each triangle indicates the concentration of MLNs (log M).

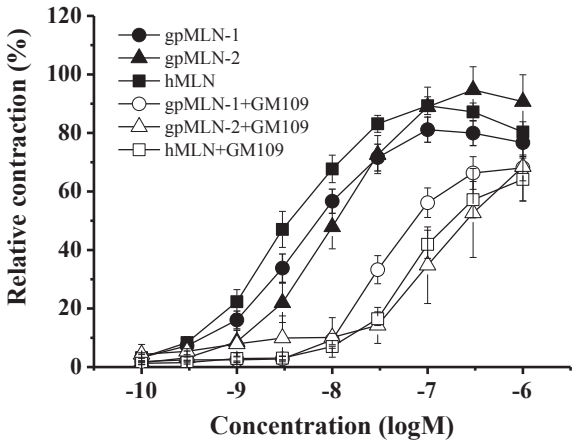

Fig. 2. Concentration-contraction curves for hMLN and gpMLNs and effects of GM109 on MLN-induced contractions in isolated rabbit duodenal strips. The symbols indicate the concentration-response curves for hMLN (■), gpMLN-1 (●) and gpMLN-2 (▲) in the normal condition and in the presence of GM109 (10^{-6} M). (hMLN: □, gpMLN-1: ○, gpMLN-2: △). The amplitude of MLN-induced contractions (y-axis) was normalized by a standard contraction by ACh (10^{-4} M) in the absence of GM109. GM109 did not change the responses to ACh (10^{-4} M). The x-axis is the concentration of MLN (log M). Values are means ± S.E.M. (4 experiments or more).

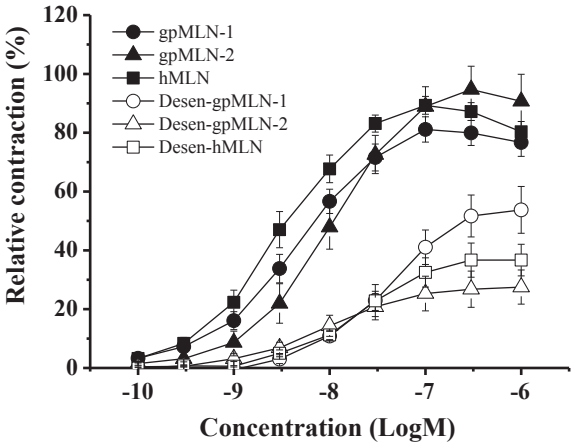

Fig. 3. Effects of desensitizing treatment of the MLN receptor on the concentration-response curves of hMLN and gpMLNs in isolated rabbit duodenal strips. The symbols indicate the concentration-response curves for hMLN (■), gpMLN-1 (●) and gpMLN-2 (▲) in the normal condition and in the condition of hMLN-induced desensitization (see text, hMLN: □, gpMLN-1: ○, gpMLN-2: △). The desensitization treatment by hMLN (10^{-6} M for 20 min) decreased the responses to both gpMLNs. The amplitude of MLN-induced contractions (y-axis) was normalized by a standard contraction by ACh (10^{-4} M). The x-axis is the concentration of MLN (log M). Values are means ± S.E.M. (4 experiments or more).

MLN-R desensitization by exposure for a long time to a high concentration of hMLN has been used to examine the possible involvement of the MLN-R in the response (Kitazawa et al., 1994). Duodenum longitudinal strips were treated with 10^{-6} M hMLN for 20 min and were then washed for 20–30 min. During washing, tonus of the preparations decreased but did not completely recover to the level before application of hMLN. In this condition, 10^{-4} M ACh-induced contraction was slightly reduced (74.6 ± 4.8% of the control, n = 7). The contractions induced by hMLN, gpMLN-1 and gpMLN-2 were markedly decreased by the desensitization treatment (Fig. 3). The relative changes in smooth muscle tonus caused by 10^{-9} M, 3×10^{-9} M and 10^{-8} M MLN were 0.6 ± 0.2%, 5.0 ± 1.2% and 11.2 ± 1.6% (n = 7), respectively, for hMLN, and 0 ± 1.4%, 3.0 ± 1.4% and 10.9 ± 1.6% (n = 4), respectively, for gpMLN-1 and 3.2 ± 1.0, 6.8 ± 1.9% and 14.3 ± 3.6% (n = 6), respectively, for gpMLN-2. The degree of inhibition of MLN-induced contraction by desensitization treatment was markedly high compared with that of ACh-induced contraction (25%).

3.2. Effects of gpMLNs in the guinea-pig GI tract

Gastric circular muscle strips were prepared from the distal part (antral region) of the guinea-pig stomach. The circular muscles showed small spontaneous contractions (Figs. 4a and 5a), and ACh (10^{-4} M) caused a marked contraction (data not shown). Neither gpMLN-1 (Fig. 4a) nor gpMLN-2 (Fig. 5a) applied cumulatively caused mechanical changes in muscle tonus of gastric strips without the mucosa. In the gastric circular muscle with intact mucosa, neither of the gpMLNs caused contraction of the muscle strips (n = 2 or 3) (data not shown). The effects of hMLN were also examined in the same gastric strips without mucosa and with mucosa, and hMLN (10^{-6} M) did not cause mechanical responses regardless of the presence of intact mucosa (without mucosa: 3.4 ± 1.0%, n = 3, with mucosa: 0.84%, n = 2). In this experimental condition, DMPP (10^{-5} M) caused a relaxation of gastric circular muscles without mucosa, which was changed into contraction by L-NAME (10^{-4} M). This DMPP-induced contraction was decreased by atropine (10^{-6} M, relative contraction 4.2 ± 3.5%, n = 4, $P = 0.046$) and was abolished by TTX (10^{-6} M, 1.6 ± 1.6%, n = 4), indicating that the DMPP-induced contractions are neurally

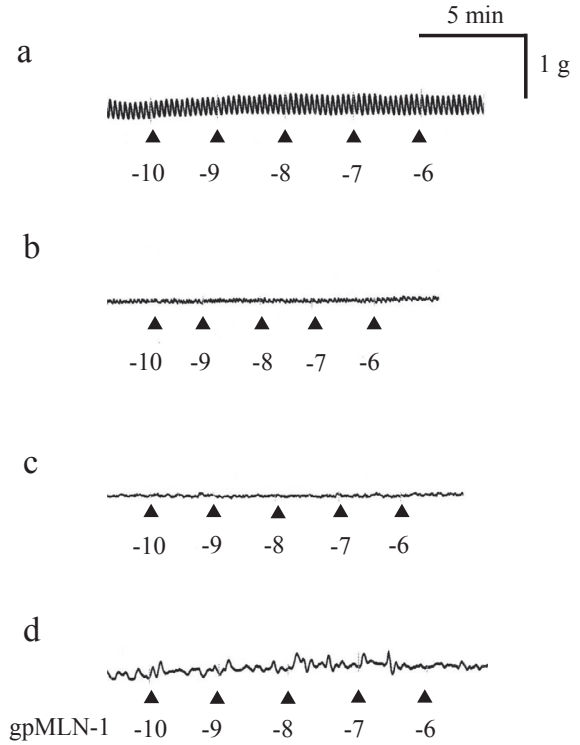

Fig. 4. Representative responses to gpMLN-1 in various regions of isolated gastrointestinal strips from the guinea-pig. gpMLN-1 applied cumulatively (10^{-10} M–10^{-6} M) did not cause any mechanical changes in gastric circular muscle strips (a), duodenal longitudinal muscle strips (b), ileal longitudinal muscle strips (c) and proximal colon longitudinal strips (d). The number under each triangle indicates the concentration of gpMLN-1 (log M).

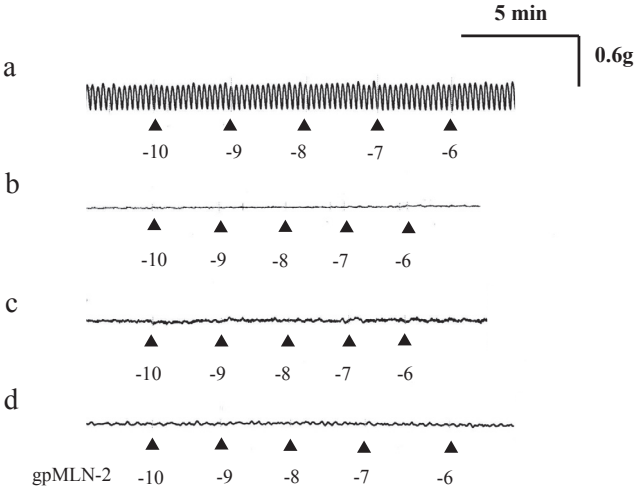

Fig. 5. Representative responses to gpMLN-2 in various regions of isolated gastrointestinal strips from the guinea-pig. gpMLN-2 applied cumulatively (10^{-10} M–10^{-6} M) did not cause any mechanical changes in gastric circular muscle strips (a), duodenal longitudinal muscle strips (b), ileal longitudinal muscle strips (c) and proximal colon longitudinal strips (d). The number under each triangle indicates the concentration of gpMLN-2 (log M).

mediated. The DMPP (10^{-5}M)-induced contraction was not affected by treatment of gpMLN-1 (10^{-9} – 10^{-6} M). Relative contractions of DMPP were 96 ± 8% for 10^{-9} M, 89 ± 12% for 10^{-8} M, 98 ± 13% for 10^{-7} M and 105 ± 24% for 10^{-6} M (n = 4), respectively.

Typical effects of gpMLNs on contractility of the duodenum longitudinal muscle preparations are shown in Fig. 4b (gpMLN-1) and 5b (gpMLN-2). Neither gpMLN-1 nor gpMLN-2 caused any mechanical

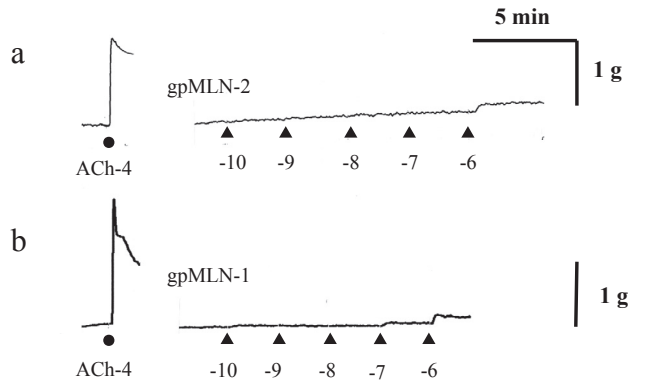

Fig. 6. Small contraction induced by gpMLN-1 and gpMLN-2 in longitudinal muscles of the guinea-pig duodenum and ileum. Representative contraction caused by gpMLN-2 in the duodenum (a) and caused by gpMLN-1 in the ileum (b). The number under each triangle indicates the concentration of gpMLNs (log M).

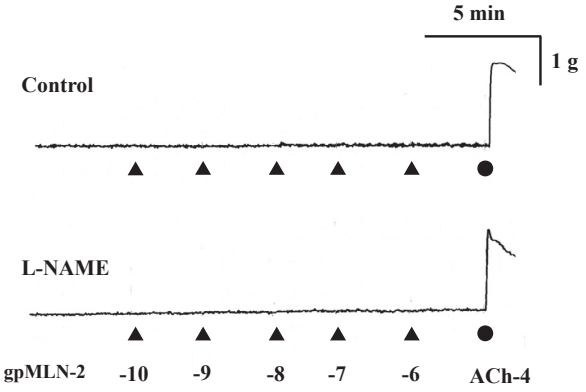

Fig. 8. Effects of L-NAME treatment on responses to gpMLN-2 in longitudinal muscle of the guinea-pig ileum. Each trace indicates typical mechanical response to gpMLN-2 (▲) in the absence (Control) and presence of L-nitroarginine methylester (L-NAME, 10^{-4} M for 15 min). ACh (10^{-4} M, ●) caused obvious contraction in each condition.

responses in the duodenal preparations as in the case of the gastric antrum. However, in one of six duodenal strips, a small contraction (over 10% of ACh-induced contraction) caused by gpMLN-2 was observed (Fig. 6a).

DMPP (10^{-5} M) caused a contraction of the ileal longitudinal muscle and the responses was significantly decreased by atropine (10^{-6} M, 39.6 ± 15%, n = 5, $P = 0.02$) and abolished by TTX (10^{-6} M, 9 ± 2.6%, n = 5), indicating the activation of enteric cholinergic neurons by DMPP (Fig. 7a). As in the case of the duodenal preparations, although one of five preparations showed a small response to gpMLN-1 causing over 10% of the ACh-induced contraction (Fig. 6b), neither gpMLN-1 nor gpMLN-2 caused mechanical responses in the ileal strips (Figs. 4c and 5c). Treatment of gpMLN-1 did not change the DMPP (10^{-5} M)-induced neural contractions. Relative contractions of DMPP were 100 ± 3% for 10^{-9} M, 101 ± 4% for 10^{-8} M, 95 ± 9% for 10^{-7} M and 104 ± 2% for 10^{-6} M (n = 5), respectively (Fig. 7b). In some experiments (n = 3), to investigate the responses to gpMLN-2 in the absence of inhibitory nitrergic innervation, the effect of gpMLN-2 in ileal strips treated with L-NAME (10^{-4} M) was examined. gpMLN-2 also caused no mechanical responses in the muscle strips (Fig. 8). Ileal preparations with intact mucosa (n = 4) also did not respond to gpMLN-2 (data not shown), and the relative changes in muscle tonus were −0.4 ± 0.4% for 10^{-10} M, −0.3 ± 0.3% for 10^{-9} M, 1.5 ± 1.2% for 10^{-8} M, 0.5 ± 0.5% for 10^{-7} M and 0.8 ± 0.8% for 10^{-6} M (n = 4).

Proximal colon longitudinal muscle strips without mucosa were used to examine the responses to gpMLN-1 (Fig. 4d) and gpMLN-2 (Fig. 5d). As in the case of gastric and small intestinal strips, neither gpMLN-1 nor gpMLN-2 caused contraction of colonic strips. The same results were obtained in the distal colon longitudinal muscles (data not shown).

3.3. RT-PCR cloning of gpMLN

In the experiments, we used several primer sets (Table 1) to clone the gpMLN-2 gene reported by Xu et al. (2001). However, despite various PCR trials, no specific target products were obtained in any of the RT-PCR conditions (data not shown).

4. Discussion

The guinea-pig belongs to rodentia as do rats and mice, and its GI

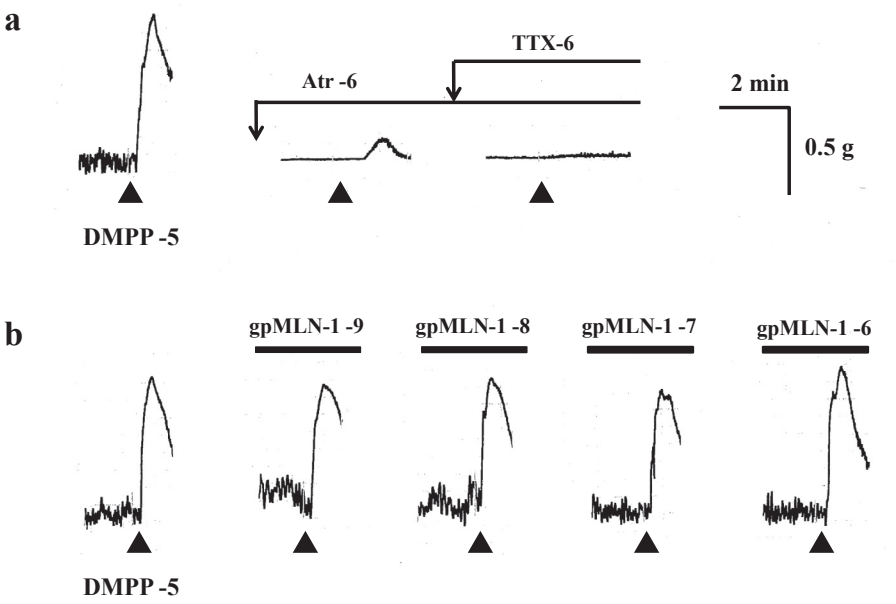

Fig. 7. Effects of gpMLN-1 on the DMPP-induced contraction of guinea-pig ileal longitudinal muscle. (a) DMPP (10^{-5} M) caused a contraction and atropine (Atr, 10^{-6} M) and tetrodotoxin (TTX, 10^{-6} M) decreased the DMPP-induced responses. (b) The effects of treatment with gpMLN-1 (10^{-9} M– 10^{-6} M for 3 min) on the DMPP-induced (10^{-5} M) contraction of the guinea-pig ileum. Each concentration of gpMLN-1 did not cause any mechanical changes. The number indicates the concentration of each drug (log M).

tract has been widely used for physiological and pharmacological studies on GI smooth muscles and enteric neurons. It has been reported that rats and mice lack the genes for the MLN system (MLN and MLN-R) (He et al., 2010; Sanger et al., 2011). In previous studies, Xu et al. (2001) determined the cDNA sequence of the MLN precursor from the duodenal mucosa of the guinea-pig (GenBank accession No. AF323752). Furthermore, Katayama et al. (2005) reported functional activity of hMLN in the guinea-pig. However, the effect of gpMLN determined by Xu et al. (2001) has never been examined in the guinea-pig GI tract itself. Previous comparative studies indicated species-dependent efficacy of homologous MLN peptide for eliciting GI smooth muscle contractions (Kitazawa et al., 1997; Poitras et al., 1987). Moreover, we recently found the possible presence of another MLN molecule (ENSCPOT00000008024), which differs from that reported by Xu et al (2001), by searching the Ensemble Genome Database. Therefore, in the present study, we examined the biological activity of these two gpMLNs (gpMLN-1 and gpMLN-2) by investigating GI contractility in the rabbit duodenum, which is known to be contracted by MLN (positive control), and in guinea-pig GI strips, in which the action is unknown.

In the rabbit duodenum, both gpMLN-1 and gpMLN-2 caused concentration-dependent contractions. The maximum amplitudes of contractions were comparable to that of hMLN, but EC_{50} of gpMLNs was slightly higher than that of hMLN. The MLN-R antagonist GM109 and MLN-R desensitization significantly decreased the responses to gpMLNs. These results clearly indicate that both gpMLN candidates function through the MLN-R that exists in the rabbit duodenum. The N-terminal region of hMLN, which corresponds to 1–6 amino acids (FVPIFT), has been thought to be essential for eliciting the biological activity of MLN (Peeters et al., 1992; Poitras et al., 1992). In this regard, gpMLN-2 (FVPIFT) is identical to it, and gpMLN-1 (FIPIFT) has only one substitution at position 2 from V to I, suggesting that this substitution of gpMLN-1 did not markedly affect the contractile efficacy. These two gpMLNs are able to bind to the rabbit MLN-R as a full agonist, but different sequences in the middle and C-terminal amino acids might be related to the slight change in affinity (EC_{50} values).

hMLN caused the region-dependent facilitation of electrically evoked cholinergic contraction and the increase in the tonus of human GI strips through activation of muscle and neural MLN-Rs (Broad et al., 2012), but hMLN was ineffective to cause contraction (Strunz et al., 1975) and to modify the neural responses of guinea-pig GI strips (Minocha and Galligan, 1991). The results of the present study using gpMLNs correspond to those of the previous studies in the guinea-pig. The potential action of gpMLN on the enteric neurons, was tested in the present study examining the responses to DMPP. DMPP caused neurally mediated contraction of ileal longitudinal muscles and of the gastric circular muscles after blocking nitrergic transmission with L-NAME. The lack of action of gpMLN-1 in these preparations is consistent with those of contraction studies in the guinea-pig (Strunz et al., 1975; Minocha and Galligan, 1991) but is different from the actions of MLN in the human stomach (Broad et al., 2012) and chicken stomach (Kitazawa et al., 1995). The present results indicate that gpMLN does not influence the enteric neurons and smooth muscles, and is not involved in regulation of GI motility in the guinea-pig GI tract. We also examined another GI preparation with intact mucosa because GHRL, a MLN-related peptide, has been reported to act on intrinsic primary afferent neurons in the gastric mucosa of the *Suncus* (Mondal et al., 2013). However, neither of the gpMLNs caused contraction in the gastric circular muscle preparations with mucosa or whole intestinal preparations in this study. Furthermore, chicken MLN-induced contraction was potentiated by L-NAME in chicken gastric preparations, indicating that MLN acts on nitrergic inhibitory nerves in addition to cholinergic excitatory nerves in the chicken stomach (Kitazawa et al., 2002). Xu et al. (2005) detected MLN-R immunoreactivity in neural nitric oxide synthase-positive neurons of the guinea-pig intestine. Therefore, it might be possible that simultaneous excitation of inhibitory nitrergic neurons could attenuate the contractile responses to MLN in the guinea-pig GI tract. However, no contractile response to gpMLN was seen in L-NAME-treated gastric and intestinal muscle preparations.

In the present study, however, some preparations from the small intestine showed a weak contractile response to gpMLNs (Fig. 6). We have observed similar small contractions in the guinea-pig ileum by GHRL (Kitazawa et al., 2011), but we could not conclude whether it is a specific action or an artifact. The mechanisms of contraction caused by gpMLNs were not examined in the present study because of the small amplitude and infrequent appearance of contractile responses.

The effect of MLN is elicited through ligand binding to the specific receptor, MLN-R. Is MLN merely ineffective for GI motility? In this regard, it is critical whether a functional MLN-R exists or not. As mentioned before, Xu et al. (2005) detected MLN-R immunoreactivity in the guinea-pig intestine. To explore the possible presence of the MLN system in the guinea-pig, we searched for the MLN-R cDNA sequence in the Ensemble Genome Database. No sequence was hit by a simple search, but we found a candidate cDNA sequence of which the amino acid sequence shares 42.5% homology with human MLN-R when human MLN-R was used as a query for the TBLASTN search. This homology was lower than that of chickens (59.1%, Yamamoto et al., 2008) and was close to that of zebrafish (47%, Liu et al., 2013), and there is a great difference compared with mammalian MLN-R sequences (rabbit: 84%, *Suncus*: 76%, dog: 71%, Dass et al., 2003; Ohshiro et al., 2008; Suzuki et al., 2012). We tried to clone the MLN-R candidate that is consistent with the second exon of the MLN-R gene. However, we failed to amplify the correspondent part of exon-1 by 5′RACE PCR. This result suggests a MLN-R gene is not present in the guinea-pig as mentioned by He et al. (2010) and Sanger et al. (2011).

Xu et al. (2001) cloned a cDNA encoding the MLN precursor and deduced a mature 22-amino-acid MLN peptide as FVPIF TYSEL RRTQE REQNK RL (namely gpMLN-2 in this study). However, in the Ensemble Genome Database, we could not find this gpMLN-2 sequence. Instead, we found another cDNA candidate encoding a deduced mature MLN peptide as FIPIF TYSEL RRTQE REQNK GL (gpMLN-1), in which amino acids at positions 2 and 21 differed from those of gpMLN-2. We tried to amplify these two gpMLNs, especially for gpMLN-2 (Xu et al., 2001), using various RT-PCR primer sets (Table 1). However, no specific target product was obtained in any of the PCR conditions, suggesting that gpMLN-2 mRNA reported by Xu et al. (2001) is not expressed in the guinea-pig. In addition, gpMLN-1 could also not be amplified using the duodenal first-strand cDNA as a template by various PCR conditions. We do not know the reason why the target product found in the genomic sequence of the guinea-pig is not amplified. gpMLN-1 has been annotated by *in silico* estimation. Our results indicate the possibility that actual transcription of gpMLN-1 does not proceeds *in vivo*. The results suggest that although MLN gene might be present, a functional MLN peptide is not transcribed and translated in the guinea-pig. Expression of the MLN and MLN-R genes in several rodentia including, kangaroo rat, guinea-pig, mouse and rat have been compared (He et al., 2010). Mouse and rat are thought to be animal species lacking both MLN and MLN-R genes by pseudogenization, but kangaroo rat has an intact open reading frame for the MLN gene but lacks the MLN-R gene. In the case of guinea-pig, the MLN gene is present but the MLN-R gene is degenerated, similar with other rodent species (squirrel, kangaroo rat, mouse and rat) (He et al., 2010). Therefore, guinea-pig is a species in a state of evolution away from a functionally-viable MLN system like a kangaroo rat.

In conclusion, two gpMLN candidates caused contractions in the rabbit duodenum but did not show any mechanical response and modification of neural responses in the guinea-pig GI tract, probably due to the lack of a functional MLN-R. The results indicate that the guinea-pig is an animal in the transition period for evolution of the MLN system prior to the loss of the MLN-R gene in rats and mice.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.01.010.

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