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In vitro characterization of the effects of endomorphin 1 and 2, endogenous ligands for μ-opioid receptors, on mouse colonic motility

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ABSTRACT

The effects of endomorphin 1 (EM1) and 2 (EM2) in colonic motility remain unknown. We investigated the effects and mechanisms of these endomorphins (EMs) on the colonic motility in vitro by applying various neural blocking agents and various opioid receptor antagonists. EMs (10^{-9} to 10^{-6} M) displayed significant stimulatory effects on the basal tonus or spontaneous activity of mouse colon but not of stomach and small intestine. It is noteworthy that the contractile actions of EMs varied slightly among different regions of colonic longitudinal muscle layers, whereas the contractile responses induced by EMs were significantly different among different regions of circular muscle layers. EMs-induced longitudinal or circular muscle contractions were not significantly affected by atropine, N^G-nitro-L-arginine methyl ester, phentolamine, propranolol and methysergide. Tetrodotoxin, indomethacin and naloxone completely abolished the EMs-induced colonic contractions. Surprisingly, EMs (10^{-7} M)-induced longitudinal muscle contractions were significantly attenuated by nor-binaltorphimine (3×10^{-6} M). By contrast, pretreatment with naltrindole (10^{-6} M) did not significantly affect EMs-induced longitudinal or circular muscle contractions. Interestingly, the circular muscle contractions in response to EM2 (10^{-7} M) were not fully blocked by β-funaltrexamine (6×10^{-6} M). Naloxonazine (10^{-6} M) almost fully antagonized the EMs-induced longitudinal or circular muscle contractions, and these effects could be only partially reversed by extensive washing. All the results indicated that the mechanisms and sites of actions of EMs were region-specific. Furthermore, these findings showed that the activation of multiple subtypes of opioid receptors, possibly including μ_1 (naloxonazine-sensitive), μ_2 and even other forms of μORs (β-FNA-insensitive), was required for EMs-induced mouse colonic motility.

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Abbreviations: DAMGO, [D-Ala^2; N-Me-Phe^4; Gly-ol^5]-enkephalin; DMSO, dimethylsulfoxide; EM1, endomorphin 1; EM2, endomorphin 2; EMs, endomorphins; β-FNA, β-funaltrexamine; GI, gastrointestinal; L-NAME, N^G-nitro-L-arginine methyl ester; nor-BNI, nor-binaltorphimine; NTI, naltrindole; ORs, opioid receptors

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1. Introduction

Endogenous opioid peptides and opiate drugs affect a variety of gastrointestinal (GI) functions, including motility, secretion as well as transport of electrolytes and fluids by the activation of three major classes of opioid receptors (ORs), δOR, κOR, and μOR [1–5]. Many endogenous opioids and opioid-related peptides, like dynorphins and orphan FQ/nociceptin (OFQ/NC), have been shown to exhibit profound actions on the GI motility [6–10]. Furthermore, some observations demonstrated that μOR agonists, like morphine and endorphins, significantly affected the GI motility in different species as well as in human [11–14]. However, the potential nonselective activity of these μOR agonists has hampered further interpretation of μOR-mediated effects on GI motility patterns [6,15].

In 1997, Zadina JE et al. isolated two tetrapeptides from the bovine frontal cortex and named them endomorphin1 (Tyr-Pro-Trp-Phe-NH_2, EM1) and endomorphin2 (Tyr-Pro-Phe-Phe-NH_2, EM2), respectively [16]. Both endomorphins (EMs) exhibited the highest specificity for μOR amongst all endogenous ligands. In the radioligand binding assays, EM1 showed remarkable affinity for μOR (0.36 nM) and selectivity of 4000- and 15,000-fold for μOR over δOR and κOR, respectively, and similar high affinity (0.69 nM) and selectivity (>13,000-fold preference over the δOR) for μOR is shown in EM2 [16]. For GI smooth muscle, it was demonstrated that EM1 and EM2 reduced acetylcholine release from the myenteric plexus of the guinea-pig ileum and rat stomach. This reduction could be reversed by μOR antagonists [17,18]. In the rat oesophagus, EMs reduced smooth and striated muscle activity by a μOR-mediated mechanism [19]. Storr et al. also provided evidence that EMs modified the ascending part of myenteric reflex of rat small intestine [15]. However, little if anything is known about the effects of these endogenous μOR ligands on the colonic motility. The constipating effects of μOR agonists, like morphine, are probably of colonic origin since the retention time digesta in the colon takes up about 80% of the oro-anal transit time in animals as well as in humans [20,21]. Although the stimulatory effects of μOR agonists on the colonic motility involve both central and peripheral components, the primary sites of action are in the periphery [22]. Therefore, it is meaningful to investigate the functional roles of these endogenous μOR agonists in different regions of colon in vitro. The present investigation was undertaken to evaluate the effects of the EMs on the colonic motility and to determine the mechanisms of these contractile actions by using various neural blocking agents and various OR antagonists.

2. Methods

2.1. Motility investigations

Experiments were performed as described in previous reports [6,23]. Briefly, adult mice (weighing 25–30 g, Kunming strain) were sacrificed by cervical dislocation. Segments of stomach body, jejum, ileum, proximal colon (immediately distal to cecum, ~1 cm in length), mid colon (~1 cm in length), and distal colon (about 1 cm proximal to the anus, ~1 cm in length) were obtained, flushed of their contents and trimmed of mesentery. Preparations were suspended in the axis of the longitudinal muscle with fine thread or were suspended in the axis of the circular muscle with a metal triangle under 9.8 mN tension [6] in 10 ml siliconised organ baths containing Krebs' solution (NaCl, 118 mM; KCl, 4.74 mM; $CaCl_2$, 2.54 mM; KH_2PO4, 1.19 mM; $MgSO_4$, 1.20 mM; $NaHCO_3$, 25 mM; glucose, 11 mM) maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2, then allowed to equilibrate for 60 min prior to drug addition, with changes of Krebs' solution every 15 min. Isometric responses were recorded using a strain gauge transducer (Machine Equipment Corporation of GaoBeiDian, China) linked to a 6240B recorder system (Machine Equipment Corporation of Chengdu, Chengdu, China). Carbachol (1 μM) was added as an internal contractile control at the end of each assay.

The concentration-response curves of EM1 (10^{-9} to 10^{-6} M), EM2 (10^{-9} to 10^{-6} M), DAMGO ([D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin) (10^{-9} to 10^{-6} M) and morphine (10^{-8} to 10^{-5} M) were determined non-cumulatively, since preliminary experiments revealed tissue desensitization to drugs when administered with a cumulative protocol. One dose of drugs was applied every 15 min to prevent tachyphylaxis.

2.2. Effects of various neural blocking agents

To investigate the neural pathways responsible for the contractile actions of EMs, we examined the effects of various antagonists on EMs-induced contractions. Segments of colon were pre-incubated with each antagonist for 10 min followed by incubation with EMs. The chemicals used were as follows: atropine (10^{-5} M), hexamethonium (10^{-4} M), indomethacin (3×10^{-6} to 10^{-4} M), methysergide (3×10^{-5} M), L-NAME (10^{-4} M), phentolamine (10^{-5} M), propranolol (10^{-6} M) and tetrodotoxin (10^{-7} M). Each antagonist investigation was carried out with at least eight segments from eight different mice.

2.3. Effects of various OR antagonists

To investigate the roles of ORs responsible for the contractile actions of EMs, the effects of naloxone (10^{-5} M), nor-binaltorphimine (nor-BNI) (10^{-7} to 3×10^{-6} M) and naltrindole (NTI) (10^{-6} M) were determined. These antagonists were equilibrated with tissues for at least 10 min before they are tested for interactions with ORs.

Naloxonazine is a relatively selective μ_1OR affinity label in binding studies, whereas it can reversibly bind to a number of OR subtypes with a potency similar to naloxone [24,25]. It is noteworthy that only the irreversible actions of naloxonazine could demonstrate the unique selectivity for μ_1 sites [24,25]. We now present two detailed sets of procedures characterizing the μ_1 sites-selectivity of naloxonazine according to the previous report [24]. In the first set of experiments, segments of colon were pre-incubated with naloxonazine (10^{-6} M) for 20 min followed by incubation with EMs (10^{-7} M), from which we are able to determine the non-selective antagonist potency of naloxonazine. By contrast, in the second set of experiments, segments of colon were treated with naloxonazine (10^{-6} M) for 20 min followed by an extensive washing (6 intermediate

washings of preparation during a 18 min period, to give maximal washout of non-covalently binding affinity), and then EMs (10^{-7} M) were administrated, from which we can gather information about the role of μ_1OR in the regulation of contractile responses to EMs. On the other hand, β-funaltrexamine (β-FNA), another irreversible μORs antagonist which is able to irreversibly alkylate μORs binding sites, was also investigated. To evaluate irreversible antagonist effects, washing and non-washing methods were also applied to β-FNA [24]. By contrast, in each experiment, the time of pretreatment with β-FNA (3×10^{-6} M) was prolonged to 30 min. In several experiments, the concentration of β-FNA was up to 6×10^{-6} M.

Each method mentioned above was carried out with at least eight segments from eight different mice. In all experiments, each tissue acted as its own control.

2.4. Animals

Animals were housed in an animal room that was maintained at 22 ± 2 °C with a 12-h light/dark cycle. Food and water were available ad libitum. All animals were cared for and experiments were carried out in accordance with the principles and guidelines of the Ethics Committee of Lanzhou Medical College of Lanzhou University.

2.5. Chemical

EM1, EM2 and DAMGO were synthesized by a liquid method. The purified peptides were characterized by RP-HPLC, TLC, ESI-TOF MS, $[\alpha]_D$, melting-point, elemental analyses and ^{1}H NMR [26]. The following compounds were used for functional studies: carbachol, nor-BNI hydrochloride, NTI isothiocyanate hydrochloride, naloxonazine dihydrochloride, β-FNA hydrochloride, methysergide maleate salt, propranolol hydrochloride, indomethacin and phentolamine hydrochloride from Sigma Chemical Company (USA); naloxone hydrochloride dehydrate and L-NAME hydrochloride from Fluka; atropine sulfate from Dongting Lake Drug Factory of Hunan of China; tetrodotoxin from the People's Hospital of Gansu Province of China. Preparation of all stock solutions (except indomethacin and β-FNA hydrochloride) and their subsequent dilutions were performed in saline. Stock solutions were stored frozen in aliquots, thawed and diluted daily. Indomethacin was dissolved in dimethylsulfoxide (DMSO). The final concentrations of DMSO in the bath solutions were always less than 1%. Control trials were performed in the presence of corresponding concentration of DMSO to rule out any possible nonspecific action of this solvent on tonus or contractility of the preparation. β-FNA aqueous solution was promptly used because of its instability. All concentrations of drugs are expressed as their final concentration in the organ bath.

2.6. Statistical analysis

To quantitatively evaluate contractions elicited by drugs, the changes (tension, in mN) of maxium amplitude of tonic or phasic component were measured using 6240B system (Machine Equipment Corporation of Chengdu, Chengdu, China) and expressed as motility index. To measure the potencies of EMs, DAMGO and morphine in longitudinal muscle of colon, EC_{50} (the concentration of an agonists that produced 50% maximal effect) were determined by using the graded dose-response procedure, in which at least seven drugs doses were used with at least 8 mice at each dose. E_{max} (the maximal effect induced by agonists expressed as a percentage of contractile effect elicited by 10^{-6} M carbachol) of all μOR agonists were the arithmetic mean of data obtained from independent experiments. Quantitative data are presented as means ± S.E. of n experiments, where n refers to the number of mice used in the test. Responses were analyzed with a one-way analysis of variance (ANOVA) and Scheffe's *F*-test. For mechanisms studies, the paired *t*-test was used to determine whether there were significant differences in the absence or presence of an antagonist; $p < 0.05$ was used as the criterion for statistical significance. Computer software packages utilized were BL310 for Windows (Taimen, Chengdu Technology & Market. Corp., Ltd.), OriginPro, Release 7.5 (OriginLab Corporation) and SPSS for Windows, Release 10.0.

3. Results

3.1. In vitro actions of EMs on the longitudinal muscle of stomach, small intestine and colon

In vitro studies using longitudinal muscle from various regions of mouse GI tract revealed that EM1 and EM2 (10^{-9} to 10^{-6} M) only induced contractions in the colon (Fig. 1). EMs up to a concentration of 10^{-6} M failed to elicit significant

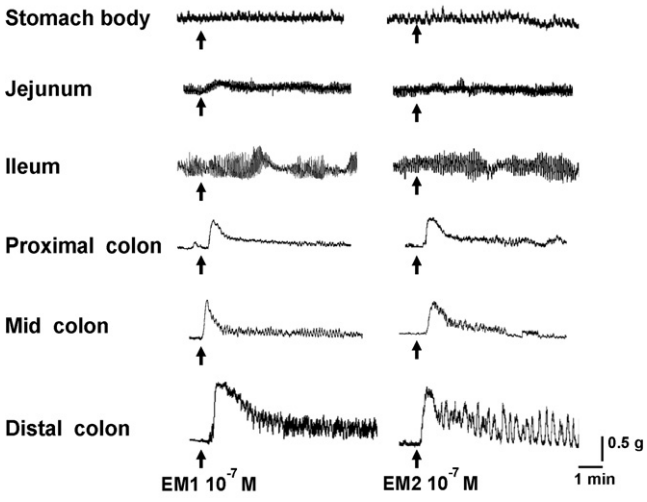

Fig. 1 – Effects of EM1 and EM2 (10^{-7} M) on contractions of the longitudinal muscle in mouse GI tract. After treatment with EM1 or EM2, significant stimulatory effects on the basal tonus or spontaneous activity were mainly seen in the colon but not in the stomach and small intestine. The majority of EMs-induced colonic contractions were characterized by a large initial contraction followed by a modest phasic response. Similar results were obtained in eight different experiments. Arrows indicate time of compound addition to bath.

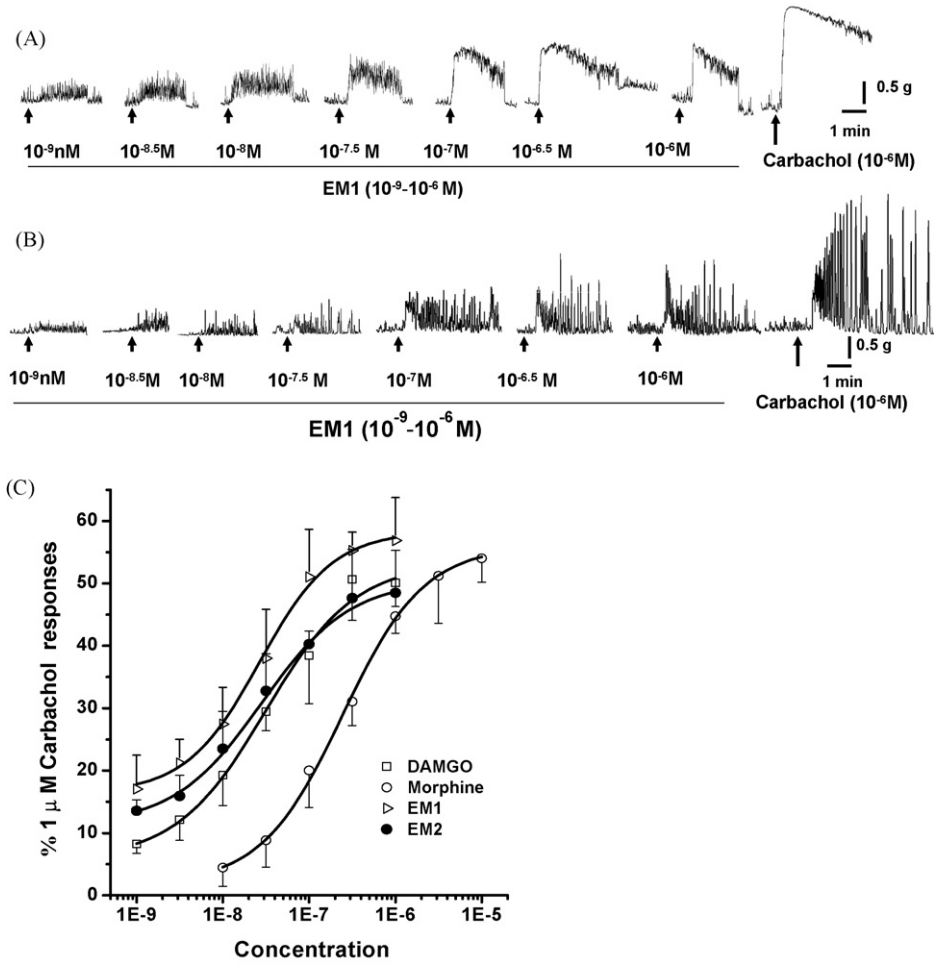

Fig. 2 – (A) Contractions of the longitudinal muscle of distal colon induced by EM1 (10^{-9} to 10^{-6} M) (similar contractions were also observed in response to EM2). Arrows indicate time of compound addition to bath. (B) Contractions of the circular muscle of proximal colon induced by EM1 (10^{-9} to 10^{-6} M) (similar contractions were also observed in response to EM2). Arrows indicate time of compound addition to bath. (C) Concentration–response curves of longitudinal muscle contractions induced by EM1, EM2, DAMGO (10^{-9} to 10^{-6} M, in half-log increments) and morphine (10^{-8} to 10^{-5} M, in half-log increments). Data are expressed as percentages of carbachol (10^{-6} M)-induced contraction. EMs, DAMGO and morphine caused significantly colonic contractions in a dose-dependent manner (means ± S.E., n = 8–13).

contractions in the longitudinal muscle layers of the stomach and small intestine. These are similar to the contractile responses induced by OFQ/NC in rat GI tract [7]. The threshold concentration of EM1 and EM2 to induce colonic contractions was 10^{-9} M (Fig. 2A). In the longitudinal muscle layer of colon, the maximal contractile actions (tension, in mN) of EM1 and EM2 (10^{-7} M) varied slightly among proximal-, mid- and distal-region, in which the contractions induced by EMs (10^{-7} M) in the longitudinal muscle of the distal colon were about 1.3- and 2-fold greater than those of the mid colon and proximal colon, respectively. Among the 107 isolated distal colon tissues examined, the majority (91.6%) showed a large initial tonic contraction followed by a modest phasic response (Fig. 1, the sixth line tracings). Occasionally, some muscle strips (8.4%) showed a relatively modest initial tonic contraction followed by a pronounced phasic response (Fig. 3C).

3.2. *Comparison of the effects of EMs, morphine and DAMGO on the longitudinal muscle of distal colon*

EMs-induced longitudinal muscle contractions were further investigated in the distal colon. EM1 (Fig. 2A and C) and EM2 (Fig. 2C) (10^{-9} to 10^{-6} M, in half-log increments) induced contractions in the longitudinal muscle of mouse distal colon in a dose-dependent manner. The concentration–response curves of DAMGO (10^{-9} to 10^{-6} M, in half-log increments) and morphine (10^{-8} to 10^{-5} M, in half-log increments) were also shown as controls (Fig. 2C). EC_{50} and E_{max} of EMs, DAMGO and morphine were calculated from the concentration–response curves. The E_{max} evoked by EM1 (56.9 ± 4.96% of control), EM2 (48.5 ± 9.54% of control) and DAMGO (50.2 ± 6.92% of control) were not significantly different from that evoked by morphine (54.1 ± 3.18% of control) ($p > 0.05$), which was about 50% of the contractile effect elicited by 10^{-6} M carbachol. However, The

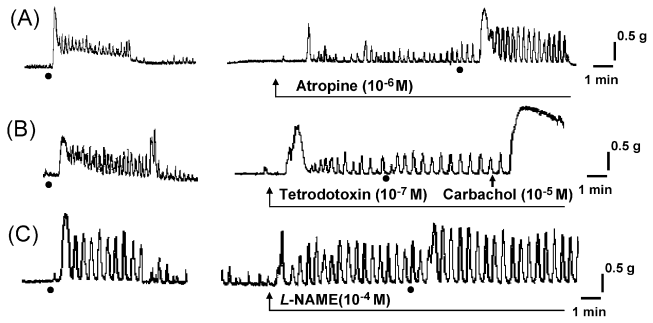

Fig. 3 – Effects of atropine (10^{-5} M) (A), tetrodotoxin (10^{-7} M) (B) and L-NAME (10^{-4} M) (C) on the EM1 (10^{-7} M)-induced contractions of the longitudinal muscle of the distal colon. Increased baseline contractions after exposure to EM1 were represented by (●) and were shown on the left. Contractions of muscle strips after preincubation with various antagonists and further treatment with EM1 were shown on the right. The contractile responses induced by EM1 were not significantly modified by atropine (10^{-5} M). Tetrodotoxin itself enhanced spontaneous contractions in the longitudinal muscles of the colon because of the removal of inhibitory neural influence on the smooth muscle cells. In the presence of tetrodotoxin, EM1 (10^{-7} M) failed to elicit additional contractions in distal colon, whereas carbachol (10^{-5} M) caused further contractions. L-NAME (10^{-4} M) significantly enhanced spontaneous contractions. EM1-induced contractions were preserved in the presence of L-NAME. All the results mentioned above were also observed in responses to EM2 (10^{-7} M). Results were reproducible at least from eight segments of eight different mice. Arrows indicate the time of compound addition to bath.

EC_{50} values of EM1 (8.27 ± 3.21 nM) and EM2 (7.97 ± 2.97 nM) were about thirty times lower than that of morphine (229 ± 31 nM).

3.3. *Effects of various neural blocking agents on the longitudinal muscle of distal colon*

The contractile responses induced by EM1 and EM2 (10^{-7} M) were reproducible at 15 min intervals and not significantly modified by atropine (10^{-5} M), hexamethonium (10^{-4} M), methysergide (3×10^{-5} M), phentolamine (10^{-5} M) and propranolol (10^{-6} M) ($p > 0.05$) (Table 1, Fig. 3). However, indomethacin (3×10^{-6} to 10^{-4} M, in half-log increments) was able to dose-dependently reduce the contractions induced by EMs (10^{-7} M) (Fig. 4A and C). To determine whether the activity of EMs was mediated by neural pathways or whether it was a direct myogenic effect, EMs-induced contractions were examined in the presence of tetrodotoxin (10^{-7} M). Spontaneous contractions were enhanced by tetrodotoxin in the longitudinal muscle layer of mouse colon, as a result of the removal of inhibitory neural pathways that inhibit the myogenic properties of muscle cells [27]. In the presence of tetrodotoxin (10^{-7} M), EMs (10^{-7} M) failed to elicit additional contractions (Fig. 3B). To exclude the possibility that the muscle had achieved maximal contractility induced by tetrodotoxin and therefore EMs would not enhance contractions, 10^{-5} M carbachol was applied after tetrodotoxin treatment. Carbachol caused further contractions in the presence of tetrodotoxin (Fig. 3B), suggesting that the muscle had not reached maximal contractility. The responses to EMs were restored 30 min after washing out tetrodotoxin (six intermediate washings of the preparation at 5-min intervals) [11]. Although L-NAME (10^{-4} M) alone caused significant colonic contractions, additional contractions were induced by EMs in the presence of L-NAME (Fig. 3C).

Table 1 – Effects of various neural blocking agents on EMs-induced longitudinal muscle contractions in the mouse distal colon in vitro

Neural blocking agents	Control (%)[a]	
	EM1[b]	EM2[b]
Atropine (10^{-5} M)	92 ± 3	92 ± 5
L-NAME (10^{-4} M)	93 ± 7	97 ± 2
Propranolol (10^{-6} M)	92 ± 4	93 ± 7
Phentolamine (10^{-5} M)	104 ± 8	95 ± 3
Hexamethonium (10^{-4} M)	93 ± 2	92 ± 4
Methysergide (3×10^{-5} M)	91 ± 3	92 ± 4

[a] Results were calculated as percent of control experiments (without antagonists) of EM1 or 2 (10^{-7} M)-induced contractions (tension, in mN) (means ± S.E., n = 8–12).
[b] EMs-induced colonic contractions were not significantly affected by these agents ($p > 0.05$).

3.4. *Effects of various ORs antagonists on the longitudinal muscle of distal colon*

In order to examine which OR mediates the longitudinal muscle contractions in distal colon caused by EMs, the effects of EMs before and after OR antagonist addition were compared. Naloxone, a competitive antagonist at δOR, κOR and μOR, fully abolished the contractile responses induced by EMs (data not shown), indicating that ORs were involved. To further investigate which ORs are involved in the response in the longitudinal muscle of distal colon, the effects of NTI (a δOR antagonist), nor-BNI (a high selective κOR antagonist), β-FNA (an irreversible μOR antagonist) and naloxonazine (an irreversible μ_1-OR antagonist) were determined. The contractile responses induced by EM1 and EM2 (10^{-7} M) were not significantly modified by NTI (10^{-6} M) (data not shown). Surprisingly, EMs (10^{-7} M)-induced longitudinal muscle contractions were significantly attenuated by nor-BNI (10^{-7} to 3×10^{-6} M, in half-log increments) in a dose-dependent manner (Fig. 4B and D), indicating κORs were involved. β-FNA (3×10^{-6} M) completely abolished the EMs-induced contraction (Fig. 5A), which was not reversed by extensive washing (Fig. 5B). Naloxonazine (10^{-6} M) fully abolished the EM1-induced contractions (Fig. 5C), which were only partially reversed (39.98 ± 11.54% of the first contraction induced by EM1) by extensive washing (Fig. 5D), indicating not only μ_1OR but also other μOR subtypes, possibly μ_2OR, are involved in the contractile responses evoked by EM1. Similar results were also observed in EM2 (data not shown).

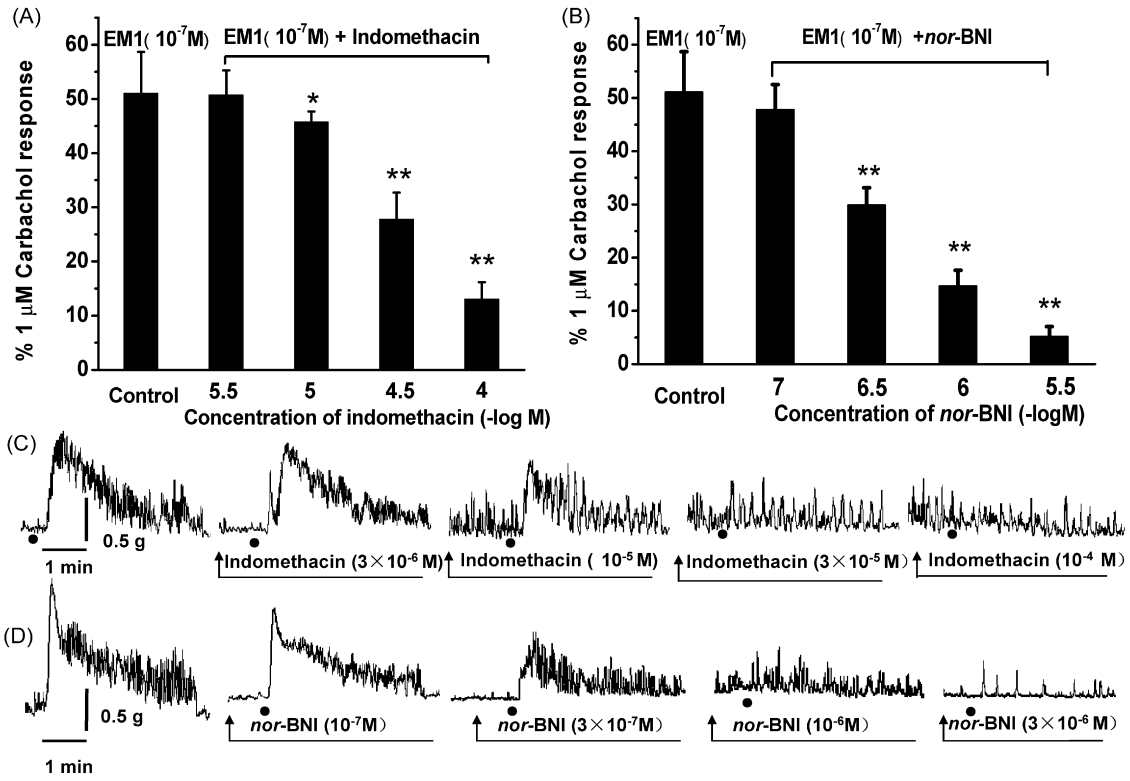

Fig. 4 – Effects of indomethacin (A and C) and nor-BNI (B and D) on the EM1-induced contractions in the longitudinal muscle of the distal colon. Increased baseline contractions after exposure to EM1 were represented by (●). The contractile responses induced by EM1 (10^{-7} M) were significantly modified by indomethacin (A and C) in a dose-dependent manner (3×10^{-6} to 10^{-4} M, in half-log increments). nor-BNI (10^{-7} to 3×10^{-6} M, in half-log increments) also significantly attenuated the EM1 (10^{-7} M)-induced contractions. All the results mentioned above were also observed in responses to EM2 (10^{-7} M). Results were obtained from at least eight segments of eight different mice. Arrows indicate the time of compound addition to bath. Data present mean ± S.E. of 5–12 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control. The error bar indicated the S.E. of the mean.

3.5. *In vitro actions of EMs on the circular muscle of stomach, small Intestine and colon*

Similar to the actions of EMs on the longitudinal muscle from various regions of mouse GI tract, in vitro studies using circular muscle from various regions of mouse GI tract revealed that EM1 and EM2 (10^{-9} to 10^{-6} M) only induced contractions in the colon (Fig. 6). EMs up to a concentration of 10^{-6} M failed to elicit significant contractions in the longitudinal muscle layers in the stomach and small intestine. The threshold concentration of EM1 and EM2 to induce colonic contractions was 10^{-9} M (Fig. 2B). However, the contractile actions of EMs on the circular muscle from various regions of mouse colon were significantly different. It is noteworthy that EMs (10^{-7} M) could only induce contractions in the proximal colon (Fig. 6 the fourth line tracings) and in one region of mid colon which is proximal to proximal colon (about 1.3 cm distal to the cecum, ~1 cm in length) (Fig. 6 the fifth line tracings), but not distal colon (Fig. 6 the seventh line tracings) and another region of mid colon which is proximal to distal colon (about 2.3 cm distal to the cecum, ~1 cm in length) (Fig. 6 the sixth line tracings). Furthermore, among the 83 isolated proximal colon tissues examined, the majority (78.3%) showed a modest initial tonic contraction followed by a pronounced phasic response (Fig. 6, the fourth tracing; Fig. 7A). Occasionally, some muscle strips (21.7%) showed a large initial contraction followed by a modest phasic response (Fig. 7B). These characters are different from those of EMs-induced longitudinal muscle contraction in mouse colon. Moreover, EMs-induced contractions in the circular muscle of mouse distal colon were also in a dose-dependent manner (Fig. 2B).

3.6. *Effects of various neural blocking agents on the circular muscle of proximal colon*

The contractile responses induced by EM1 and EM2 (10^{-7} M) in the circular muscle of proximal colon were not significantly modified by atropine (10^{-5} M), hexamethonium (10^{-4} M), methysergide (3×10^{-5} M), phentolamine (10^{-5} M), L-NAME (10^{-4} M) and propranolol (10^{-6} M) ($p > 0.05$), but were fully antagonized by tetrodotoxin (10^{-7} M) and indomethacin (10^{-4} M) ($p < 0.05$) (data not shown).

3.7. *Effects of various OR antagonists on the circular muscle of proximal colon*

In the present investigation, effects of various OR antagonists on the circular muscle of proximal colon were also investigated.

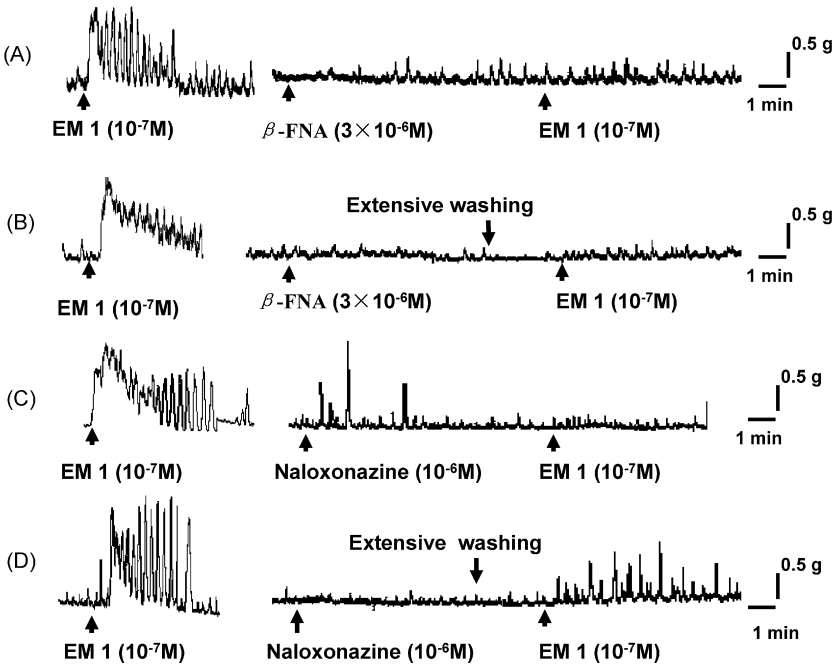

Fig. 5 – Effects of naloxonazine and β-FNA on the EM1-induced contractions of the longitudinal muscle of the distal colon. Naloxonazine (10^{-6} M) fully abolished the EM1-induced contractions (A), which was partially reversed by extensive washing (B). β-FNA (3×10^{-6} M) completely abolished the EMs-induced contractions (C), which was not reversed by extensive washing (D). Results were obtained from at least eight segments of eight different mice. All the results mentioned above were also observed in responses to EM2 (10^{-7} M).

Naloxone (10^{-5} M) completely abolished the contractile responses induced by EMs in the circular muscle of proximal colon (data not shown). The contractile responses induced by EM1 and EM2 (10^{-7} M) were not significantly modified by NTI (10^{-6} M) (data not shown), but were antagonized by nor-BNI (10^{-7} to 3×10^{-6} M, in half-log increments) in a dose-dependent manner (data not shown). Interestingly, β-FNA almost fully blocked the EM1 (10^{-7} M)-induced contractions (Fig. 7A),

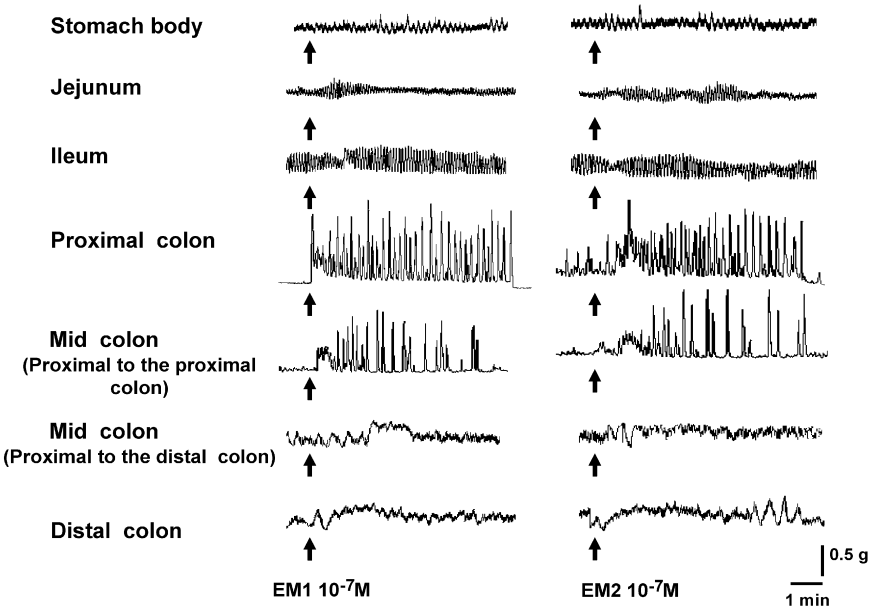

Fig. 6 – Effects of EM1 and EM2 on the baseline contractions of the circular muscle in mouse GI tract. After treatment with EM1 or EM2, significant stimulatory effects on the basal tonus or spontaneous activity were mainly seen in the colon but not in the stomach and small intestine. Similar results were obtained in ten different experiments. Arrows indicate the time of compound addition to bath.

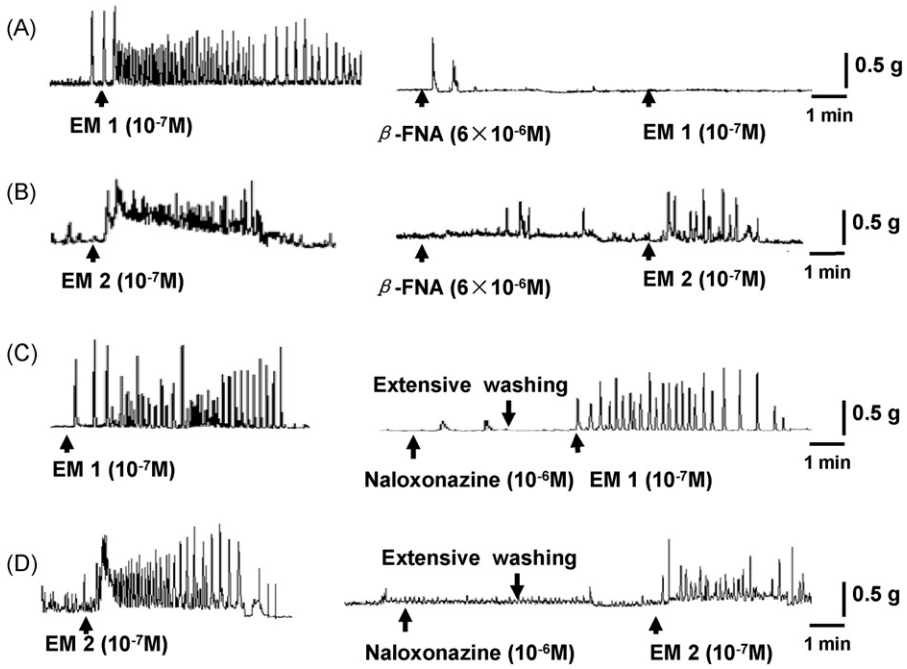

Fig. 7 – (A and B) Effects of β-FNA on EMs-induced circular muscle contractions in proximal colon. Interestingly, β-FNA (6×10^{-6} M) almost fully blocked the EM1 (10^{-7} M)-induced contractions (A), whereas could not completely abolish EM2-induced contraction (B) even at a very high concentration (6×10^{-6} M). (C and D) Effects of naloxonazine on EMs-induced circular muscle contractions in proximal colon. Naloxonazine (10^{-6} M) fully abolished the EM1(C)- and EM2 (D)-induced contractions, which was partially reversed by extensive washing. Results were obtained from at least eight segments of eight different mice.

whereas could not completely abolish (63.81 ± 12.91%) EM2-induced contraction even at a very high concentration (6×10^{-6} M) (Fig. 7B), indicating that EM2-evoked contractions in circular muscle of proximal colon may partially act by a mechanism of activation of β-FNA-non-sensitive μOR-binding sites. Furthermore, none of the blocking effects induced by β-FNA were reversed by extensive washing (data not shown). Naloxonazine (10^{-6} M) fully abolished the EM1 or 2-induced contraction, which was only partially reversed by extensive washing (23.56 ± 15.12% and 34.76 ± 9.63% of the first contraction induced by EM1 and EM2, respectively) (Fig. 7C and D), indicating not only μ_1OR but also other μOR subtypes, possibly μ_2OR, are involved in the EMs-evoked contractions in the circular muscle of proximal colon.

4. Discussion

The present investigation confirmed the effects of EMs on mouse colonic motility. In terms of the actions of contractions in mouse colon, EMs displayed relatively higher potencies than that of morphine, which is in good agreement with the results of binding assays [16]. EM1 and EM2 at the concentration of 10^{-6} M induced contractions only in the colon, but not in the stomach and small intestine, though the μOR expression has been found not only in the colon but also in the stomach and small intestine [11,30]. The mechanisms responsible for this phenomenon are largely unknown. It is possibly induced by the different sensitivities to extrinsic neural input among the stomach, small intestine and colon [28,29]. It is also possible that the different subtypes of opioid receptors have different distributions in the guts wall; or perhaps different balance exists in stomach, small intestine and colon. In the longitudinal muscle layer of colon, the maximal contractile actions of EMs varied slightly among proximal-, mid- and distal-region. However, in the circular muscle layer of colon, it is noteworthy that EMs could only induce contractions in the proximal-region and in one region of mid colon, but not in the distal-region. Furthermore, EMs induced a large initial tonic contraction followed by a modest phasic response in the longitudinal muscle layer of distal colon at a time when a reverse response was observed in the circular muscle layer of proximal colon after EMs being treated. All the results mentioned above indicated that the sites of actions of EMs were region-specific. The mechanisms responsible for these differences remain to be investigated. Conceivably, different distributions of opioid receptor subtypes may cause different amplitudes and different modes of colonic contractions.

The mechanisms of EMs-induced mouse colon contractions were also investigated. In the presence of tetradotoxin, EMs failed to evoke additional contractions, suggesting that actions of EMs are mediated by neural pathways. The enteric nervous system lies entirely within the wall of the gut from the oesophagus to the anus, and is organized into two major plexuses: the myenteric plexus and the submucosal plexus [2]. Because the myenteric plexus lies between the longitudinal and circular muscle layers and extends the length of the intestine, it is involved primarily with the control over motor

activity within the gut. Generally, stimulation of the myenteric plexus increases the tone of the GI wall, the intensity and rhythm of contractions, and the conduction velocity [2]. The submucosal plexus controls local secretory and absorptive activity. Furthermore, the distribution of μOR in the myenteric plexus has been demonstrated by Sternini et al. [1]. Thus, it is possible that EMs-induced colonic contractions were evoked by a myenteric plexus activation mechanism. The inability of methysergide (3×10^{-5} M), hexamethonium (10^{-4} M), phentolamine (10^{-5} M), propranolol (10^{-6} M) and L-NAME to antagonize the EMs-induce contractions allows discarding serotonergic, preganglionic, adrenergic and NO-releasing effects of the contractions. According to the observations of Fontaine and Reuse [11], the contractile effect evoked by opioids in the mouse colon is not modified by atropine, which excludes the possibility of involvement of acetylcholine in the contractile response, whereas Contreras et al. [31] reported that contractions induced by morphine were inhibited by atropine. Furthermore, Bueno et al. [32] thought that the incomplete inhibition of morphine-induced colonic contractions in conscious dogs by atropine was probably due to too low a dose of atropine. These discrepancies between these data may be due to the differences among species and the different doses applied in the experiment. In the present study, atropine only slightly reduced (9–17%) the EMs-induced contractions even at a very high concentration (10^{-5} M). Fontaine and Reuse [11] reported that morphine-induced mouse colon contractions were potently inhibited by indomethacin, implying the involvement of a prostaglandin-mediated pathway. In our observation, prostaglandins may be involved in the contractile responses evoked by EMs, since indomethacin affected the colon's responses to EMs in a dose-dependent manner, which is good in agreement with the results of Fontaine and Reuse.

The present investigation also showed the roles of ORs in EMs-induced mouse colon contractions. Naloxone, a non-selective competitive antagonist at δOR, κOR and μOR, fully abolished the contractile responses induced by EMs, indicating that ORs are involved. The contractile responses induced by EMs were not significantly modified by NTI, indicating δOR is not involved. Surprisingly, nor-BNI, the highly selective κOR antagonist, significantly attenuated EMs-induced mouse colon contractions. This observation seems to oppose the low affinity of EMs to κOR in binding assays. The close relationships between the κOR and μOR in the GI tract may offer some implications to explain the role of κOR in EMs-induced longitudinal contractions. In the guinea-pig ileum, electrophysiological studies suggested a co-existence of κOR and μOR on a single neuron [33]. Other published results suggested an even stronger interaction between κOR and μOR [34,35]. It has been demonstrated that chronic treatment with U-50,488, the selective κOR agonist, led to the development of tolerance not only to the inhibitory effects of U-50,488 but also to those of μOR agonist such as DAMGO and morphine [34]. Thus, the stronger interactions between κOR and μOR in the gut may be one of the reasons why nor-BNI reduced EMs-induced colonic contractions. On the other hand, Tseng et al. [36] demonstrated that dynorphin A (1–17) was involved in EM2-induced antinociception and that the analgesic effects were blocked by nor-BNI. This observation also implied that endogenous κOR agonists released from mouse colon after applying EMs might be another reason why nor-BNI reduced EMs-induced colonic contractions. Naloxonazine is a relatively selective μ_1OR-affinity-label in binding studies, whereas it can reversibly bind to a number of OR subtypes with a potency similar to that of naloxone [24,25]. It is noteworthy that only the irreversible actions of naloxonazine could demonstrate the unique selectivity for μ_1 sites [24,25]. In our present observation, naloxonazine fully abolished the EMs-induced longitudinal and circular muscle contractions, which was only partially reversed by extensive washing, indicating not only μ_1OR but also other μOR subtypes, possibly μ_2OR, are involved in the contractile responses evoked by EMs. β-FNA, another irreversible μORs antagonist, is able to irreversibly alkylate μORs binding sites. In the present investigation, β-FNA completely abolished both the EMs-induced longitudinal muscle contractions and EM1-induced circular muscle contractions, but only partially reduced EM2-induced circular muscle contractions even at the concentration of 6 μM. Several studies have demonstrated μORs to be either β-FNA sensitive or insensitive, the latter representing 30% of μOR receptor sites in brain [37]. Furthermore, in competitive experiments μOR ligands are unable to differentiate between β-FNA sensitive and insensitive sites, confirming that the β-FNA insensitive sites are a form of μORs [38]. Thus, it is possible that EM2-induced circular muscle contractions were evoked by a mechanism of not only μ_1OR, μ_2OR but also other forms (β-FNA-insensitive) of μORs activation.

In conclusion, all the results indicated that the mechanisms and sites of actions of EMs were region-specific. Furthermore, these findings showed that the activation of multiple subtypes of opioid receptors, possibly μ_1 (naloxonazine-sensitive), μ_2 and even other forms of μORs (β-FNA-insensitive), was required for EMs-induced mouse colonic motility.

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