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BRIEF ARTICLE

Opiate-induced constipation related to activation of small intestine opioid μ2-receptors

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Abstract

AIM: To investigate the role of opioid μ-receptor subtype in opiate-induced constipation (OIC).

METHODS: The effect of loperamide on intestinal transit was investigated in mice. Ileum strips were isolated from 12-wk-old male BALB/c mice for identification of isometric tension. The ileum strips were precontracted with 1 μmol/L acetylcholine (ACh). Then, decrease in muscle tone (relaxation) was characterized after cumulative administration of 0.1-10 μmol/L loperamide into the organ bath, for a concentration-dependent study. Specific blockers or antagonists were used for pretreatment to compare the changes in loperamide-induced relaxation.

RESULTS: In addition to the delay in intestinal transit, loperamide produced a marked relaxation in isolated ileum precontracted with ACh, in a dose-dependent manner. This relaxation was abolished by cyprodime, a selective opioid μ-receptor antagonist, but not modified by naloxonazine at a dose sufficient to block opioid μ-1 receptors. Also, treatment with opioid μ-1 receptor agonist failed to modify the muscle tone. Moreover, the relaxation by loperamide was attenuated by glibenclamide at a dose sufficient to block ATP-sensitive K^+ (K_{ATP}) channels, and by protein kinase A (PKA) inhibitor, but was enhanced by an inhibitor of phosphodiesterase for cyclic adenosine monophosphate (cAMP).

CONCLUSION: Loperamide induces intestinal relaxation by activation of opioid μ-2 receptors via the cAMP-PKA pathway to open K_{ATP} channels, relates to OIC.

Key words: ATP-sensitive K^+ channels; Isometric tension; Loperamide; Opioid μ-receptors; Small intestine

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INTRODUCTION

Opiate-induced constipation (OIC) is widely observed among patients receiving chemotherapy[1,2]. In the gastrointestinal system, the opioid peptides are released and activate opioid receptors, which regulate the enteric circuitry by controlling motility and secretion, resulting in an increase in sphincter tone, inhibition of gastric emptying, and induction of stationary motor patterns. Together with the inhibition of ion and fluid secretion, these effects result in constipation, one of the most troublesome

side effects of opiate analgesic treatment[3]. The development of a better therapy for treating OIC is urgent and necessary.

Loperamide is widely used clinically to treat a variety of diarrheal syndromes, including acute and nonspecific (infectious) diarrhea[4,5]. Loperamide is a peripheral agonist of opioid μ-receptors with poor ability to penetrate the blood-brain barrier[6,7]. Some analgesic agents have been shown to have relaxant effects on smooth muscle[8,9]. (+)-Tramadol activates peripheral opioid μ-receptors, inducing concentration-dependent relaxation of the aorta[10]. Opioid μ-receptors are divided into three subtypes: μ-1, μ-2 and μ-3[11]. The activation of opioid μ-1 receptors has been reported to be associated primarily with the phospholipase C (PLC)-protein kinase C (PKC) pathway[12]. PLC-PKC signals can increase the intracellular calcium concentration, inducing gastrointestinal or bladder contraction[13,14]. Therefore, it is unlikely that intestinal relaxation is induced by the activation of opioid μ-1 receptors.

ATP-sensitive K^+ (K_{ATP}) channels are involved in the regulation of intestinal smooth muscle[15]. In addition, the opening of K_{ATP} channels has been reported to reduce intracellular Ca^+ concentration[16-18]. The K_{ATP} channel opener diazoxide has been shown to have the ability to attenuate indomethacin-induced small intestinal damage in rats[19]. However, the role of K_{ATP} channels in loperamide-induced gastrointestinal transit remains obscure.

In an attempt to determine the subtype of opioid μ-receptors involved in the regulation of intestinal tone, we used loperamide as an agonist to induce intestinal relaxation in the present study. In addition, specific blockers or antagonists were applied to investigate the potential mechanisms of action of loperamide.

MATERIALS AND METHODS

Experimental animals

We obtained 12-wk-old male BALB/c mice from the Animal Center of National Cheng Kung University Medical College. Mice were maintained in a temperature-controlled room (25 ± 1 ℃) under a 12-h light-dark cycle (lights on at 06:00 h). All mice were given water and fed standard chow (Purina Mills, LLC, St Louis, MO, United States) *ad libitum*. All animal-handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the Animal Welfare Act.

Gastrointestinal transit assay

Gastrointestinal tract (GIT) in mice was measured according to the method used in a previous study[20]. Briefly, 18 h before the experiment, food was withheld from the animals but free access to water was allowed. The mice received 0.25 mL of a suspension of charcoal consisting of 10% vegetable charcoal in 5% gum acacia (Sigma-Aldrich, St Louis, MO, United States) that was administered by an intragastric cannula. In subsequent experiments, the effects of loperamide or other compounds on GIT were evaluated 20 min after administration of the marker. At that time, the animals were sacrificed, the stomach and small intestine removed and the omentum was separated, avoiding stretching. The length of the intestine from the pyloric sphincter to the ileocecal junction and the distance travelled by the charcoal front were measured and recorded. We also recorded the time at which the mice started to drain stool after the administration of the charcoal meal.

We evaluated the effects of loperamide, stevioside (an agonist of opioid μ-1 receptor) or vehicle on GIT using subcutaneous injection into mice. Loperamide, stevioside and vehicle were given 30 min before the charcoal meal. The opioid antagonists cyprodime and naloxonazine were intraperitoneally injected at 60 min before the charcoal meal.

Preparation of isolated ileum

In the *in vitro* experiments, isolated ileum from BALB/c mice was used. Each mouse was killed by decapitation under anesthesia with pentobarbital (50 mg/kg). After the ileum strips had been carefully freed from the fat and connective tissue, the strips were mounted in organ baths filled with 10 mL oxygenated Krebs' buffer (95% O_2, 5% CO_2) at 37 ℃ containing: 135 mmol/L NaCl; 5 mmol/L KCl; 2.5 mmol/L $CaCl_2$; 1.3 mmol/L $MgSO_4$; 1.2 mmol/L KH_2PO_4; 20 mmol/L $NaHCO_3$; and 10 mmol/L D-glucose (pH 7.4). Each preparation was connected to strain gauges (FT03; Grass Instruments, Quincy, MA, United States). The isometric tension was recorded using chart software (MLS023, Powerlab; ADInstruments, Bella Vista, NSW, Australia). Strips were mounted and allowed to stabilize for 2 h. Each preparation was then gradually stretched to achieve an optimal resting tension of 0.5 g.

Intestinal relaxation induced by loperamide

After the resting tension had stabilized, a solution of acetylcholine (ACh; Sigma-Aldrich) prepared in distilled water was added to the bathing buffer to induce a rapid increase in ileum tone followed by stable constriction (tonic contraction). The final ACh concentration in the organ bath was 1 μmol/L. Ileum strips in the treatment group were exposed to loperamide (0.1-10 μmol/L) to observe the decrease in the tonic contraction (ileum relaxation). In addition, stevioside, an opioid μ-1 receptor agonist[21], was also used to investigate the effect on tonic contraction. Relaxation was expressed as the percentage decrease in the maximum tonic contraction. Concentration-relaxation curves were generated in a cumulative fashion.

Effects of antagonists on loperamide-induced intestinal relaxation

Ileum strips were exposed to glibenclamide (Research Biochemicals, Wayland, MA, United States), a specific opioid μ-1 receptor antagonist (naloxonazine) or a general opioid μ receptor antagonist (cyprodime) (Tocris Cookson, Bristol, United Kingdom), for 15 min before addition of loperamide to the organ bath. The strips were treated with an inhibitor of cyclic adenosine monophosphate (cAMP) phosphodiesterase (3-isobutyl-1-methylxanthine; IBMX) or an inhibitor of protein kinase A (PKA) (H-89)

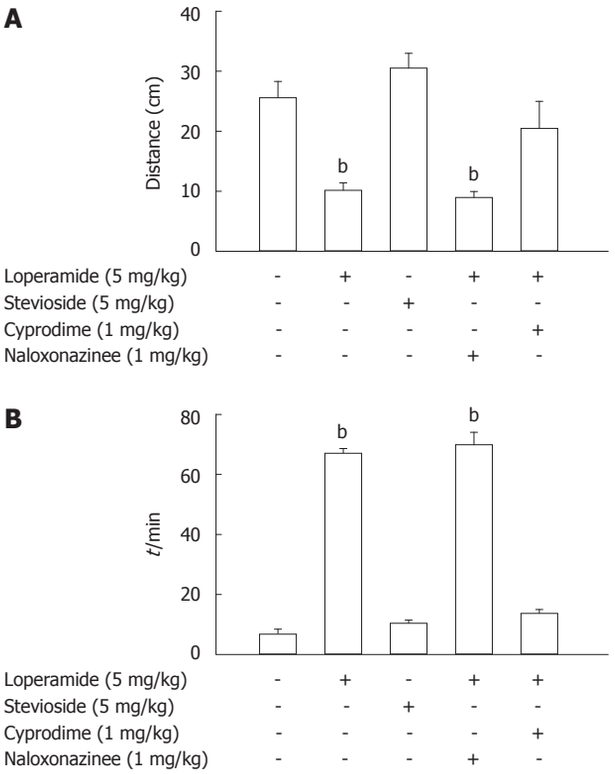

Figure 1 Role of opioid μ-receptors in gastrointestinal tract using charcoal as an indicator. The data represent the distance (A) and time (B) for the transit of charcoal. Data represent the mean ± SEM of eight animals. $^{b}P < 0.01$ *vs* the distilled water (vehicle)-treated control.

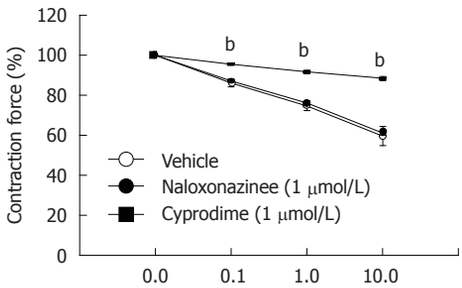

Figure 2 Inhibitory effect of cyprodime or naloxonazine on relaxation induced by loperamide (10 μmol/L) in isolated ileum contracted with 1 μmol/L acetylcholine. Data represent the mean ± SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. $^{b}P < 0.01$ *vs* the distilled water (vehicle)-treated control.

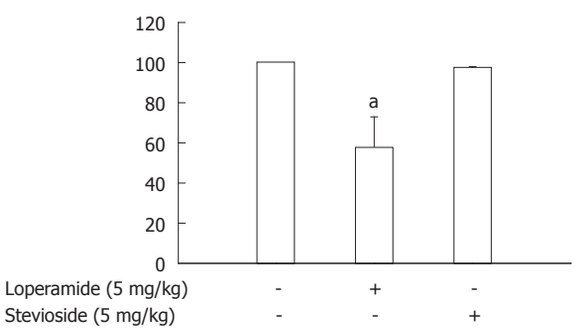

Figure 3 Effect of stevioside on the tone of isolated ileum strips contracted with 1 μmol/L acetylcholine. Data represent the mean ± SEM of the percentage changes in the acetylcholine (ACh)-induced tonic contraction of ileum from eight animals. $^{a}P < 0.05$ *vs* the distilled water (vehicle)-treated control, shown in the first column.

in the same manner. Forskolin (Sigma-Aldrich) was used as a control. The changes in the relaxation caused by antagonists or blockers were compared with those of the vehicle-treated control.

Statistical analysis

All values are presented as the mean ± SEM for a given number of animals or samples. Analysis of variance and Dunnett's post hoc test were used to evaluate the significance between groups. $P < 0.05$ was considered significant.

RESULTS

Role of opioid receptor in loperamide-induced gastrointestinal transit

As shown in Figure 1A, the distance travelled by charcoal in the loperamide-treated group (5 mg/kg) was shorter than that in the vehicle-treated group. However, the distance travelled in the stevioside-treated (5 mg/kg) group was similar to that in the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) significantly abolished the effect of loperamide on GIT, but naloxonazine (1 mg/kg) failed to produce the same effect. Moreover, the time for transit of charcoal from the stomach to the anus stool drain in the loperamide-treated group (5 mg/kg) was longer than that in the vehicle-treated group. The transit time of the stevioside-treated (5 mg/kg) group was the same as that of the vehicle-treated group. In addition, pretreatment with cyprodime (1 mg/kg) attenuated the loperamide-induced delay in charcoal transit, but naloxonazine (1 mg/kg) failed to exhibit the same action (Figure 1B).

Effect of opioid receptor blockade on loperamide-induced intestinal relaxation

Ileum strips strongly contracted in response to the application of ACh at 1 μmol/L. As shown in Figure 2, loperamide relaxed the ACh-contracted ileum strips in a concentration-dependent manner. At the maximum concentration tested (10 μmol/L), loperamide significantly attenuated the tonic contraction of ileum strips to 63.59% ± 5.60% of the contraction induced by ACh. Cyprodime (1 μmol/L) produced a marked attenuation of the relaxation induced by loperamide. However, naloxonazine failed to modify the action of loperamide, even at a higher concentration (1 μmol/L). In addition, treatment with stevioside at a dose sufficient to activate the opioid μ-1 receptor[21] failed to modify the intestinal tone in ACh-contracted ileum (Figure 3).

Role of cyclic adenosine monophosphate and protein kinase A in loperamide-induced intestinal relaxation

In the present study, forskolin (5 μmol/L), a direct activator of adenylate cyclase, was used as a positive control to increase the activity of cAMP, based on the findings of a previous study[22]. In ileum strips contracted with

Table 1 Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation

	ACh (%)
Loperamide (10 μmol/L)	
+ Vehicle	63.59 ± 5.60
+ H-89 (1 μmol/L)	80.49 ± 3.07[a]
+ IBMX (10 μmol/L)	41.02 ± 2.57[b]
+ Glibenclamide (1 μmol/L)	83.52 ± 0.89[a]
Forskolin (5 μmol/L)	
+ Vehicle	31.64 ± 7.39
+ H-89 (1 μmol/L)	79.29 ± 2.76[b]
+ IBMX (10 μmol/L)	27.77 ± 1.40[b]
+ Glibenclamide (1 μmol/L)	80.98 ± 2.75[b]
IBMX (10 μmol/L)	93.41 ± 2.15[b]
H-89 (1 μmol/L)	91.31 ± 3.47[b]
Glibenclamide (1 μmol/L)	92.45 ± 3.29[b]

Effects of inhibitors of cyclic adenosine monophosphate-phosphodiesterase or protein kinase A on the relaxation induced by loperamide (10 μmol/L) or forskolin (5 μmol/L) in isolated ileum contracted with 1 μmol/L acetylcholine (ACh). IBMX: 3-isobutyl-1-methylxanthine. [a]$P < 0.05$, [b]$P < 0.01$ *vs* vehicle treated control.

ACh (1 μmol/L), forskolin-induced relaxation was also abolished by pretreatment with glibenclamide (1 μmol/L). Moreover, intestinal relaxation induced by forskolin was increased by the addition of IBMX at a concentration (10 μmol/L) sufficient to inhibit cAMP-phosphodiesterase[23], and was decreased by addition of H-89 at a concentration (1 μmol/L), which was sufficient to inhibit the activity of PKA[24]. The loperamide-induced intestinal relaxation was also modified by these agents in the same manner. Our results showed that intestinal relaxation induced by loperamide was increased by IBMX and attenuated by H-89 (Table 1).

DISCUSSION

In the present study, we found that loperamide caused a dose-dependent delay in GIT using charcoal as an indicator in mice. In addition, loperamide induced relaxation in the ileum strips contracted with stimulant. This action of loperamide seems to be related primarily to the activation of opioid receptors in peripheral tissue, because loperamide does not cross into the central nervous system[7]. The loperamide-induced action was effectively abolished by cyprodime, suggesting that opioid μ receptors were involved. However, this action of loperamide was not reversed by naloxonazine even at a dose sufficient to block opioid μ-1 receptors. In addition, as shown in Figure 2, relaxation was not induced by stevioside, which is an agonist specific for opioid μ-1 receptors[21]. The involvement of opioid μ-1 receptors in the intestinal relaxation mechanism of loperamide seems unlikely.

Thus, another opioid μ receptor must be involved in this action of loperamide. There is no doubt that loperamide is an agonist of peripheral opioid μ receptors[7,25]. Opioid μ receptors have been divided into three subtypes[11]: μ-1, μ-2 and μ-3[26-28]. The analgesic action mediated by the activation of opioid μ-1 receptors has been reported to exert spinal antinociception[29,30]. In addition, the activation of opioid μ-1 receptors seems to be related to smooth muscle contraction *via* the PLC-PKC pathway[14,31]. Moreover, opioid μ-3 receptors are present predominantly in endothelial cells associated with the production of nitric oxide to induce vasodilatation[32]. Therefore, the involvement of opioid μ-1 or μ-3 receptors in intestinal relaxation seems unlikely. Taken together, our results suggest that the activation of opioid μ-2 receptors is more likely to participate in the action of loperamide with respect to intestinal relaxation. The activation of opioid μ-2 receptors has been reported to be involved in the relaxation of guinea pig ileum and in the inhibition of GIT[33,34]. In addition, opioid μ-receptor-expressing myenteric neurons are distributed primarily in the small intestine, followed by the stomach and the proximal colon[35]. Although constipation is predominantly a large bowel disorder, the presence of opioid μ receptors in the colon and the longer GIT time of charcoal to the anus stool drain in mice that received loperamide support the role of opioid μ-2 receptors in opiate-induced constipation. Unfortunately, there is no suitable tool or agent that can be used to provide further evidence supporting this hypothesis. Therefore, we focused on the subcellular signals as an alternative experimental approach.

Potassium channels play an important role in the regulation of intestinal smooth muscle cells[36,37]. ATP-sensitive K^+ (K_{ATP}) channels are composed of four inwardly rectifying K^+ channel subunits and four regulatory sulfonylurea receptors[38]. The activation of K_{ATP} channels induces hyperpolarization of the cell membrane and consequently relaxes the smooth muscle. Thus, we focused on the involvement of K_{ATP} channels in the intestinal relaxation induced by loperamide. We used forskolin as a positive control because forskolin is a direct activator of adenylate cyclase that can increase intracellular cAMP concentration to activate cAMP-dependent PKA, resulting in the opening of K_{ATP} channels[24]. As shown in Table 1, we observed that forskolin-induced intestinal relaxation was also blocked by glibenclamide. The intestinal relaxation induced by forskolin was abolished by H-89 at a concentration sufficient to block the activity of PKA[24] and was enhanced by IBMX at a concentration sufficient to inhibit the activity of cAMP-posphodiesterase[23]. Similar changes were also observed in ileum strips relaxed by loperamide (Table 1). These data suggest that the potential mechanism responsible for loperamide-induced intestinal relaxation is mediated *via* the cAMP-PKA pathway to open K_{ATP} channels. Therefore, the results provide a novel insight into the mechanism of action of loperamide and increase our understanding of intestinal relaxation. It is reasonable to consider that similar results will be obtained for the parts of the colon that have opioid μ receptors. Further investigations are required in the future.

In conclusion, we suggest that the activation of opioid μ-2 receptors, which induce the opening of K_{ATP} channels, is responsible for loperamide-induced intestinal relaxation. Therefore, peripheral opioid μ-2 receptors will

be a new target in the development of agents for treating OIC.

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COMMENTS

Background

Opioid-induced constipation (OIC) is a frequent disorder in tumor patients receiving morphine-like compounds. Thus, it is important to prevent this disorder. To date, it is still unclear which subtype of opioid receptors should be used for development of suitable agents. Loperamide is a well-known agonist of opioid receptors, without the ability to enter the brain. Many studies have reported that loperamide can be used to treat diarrhea, but the receptor site has not been established.

Research frontiers

Loperamide is a widely used agent in clinics and its effectiveness is believed to arise from peripheral action. In the area of prevention of constipation with loperamide, the research hotspot is how to distinguish the receptor subtype to improve its adverse reactions. Then, it will be useful for prevention of OIC.

Innovations and breakthroughs

In previous studies of loperamide for treatment of diarrhea, it was found that intestinal motility was significantly decreased. In order to decrease the side effects of loperamide, the authors investigated the receptor subtype that is selectively activated, and the results will be useful for the development of new agents with similar side effects. Thus, the authors compared loperamide with stevioside, which is mainly effective against opioid μ-1 receptors. We found that opioid μ-2 receptors linked with ATP-sensitive K^+ channels are responsible for intestinal relaxation. Therefore, agents with less effect on opioid μ-2 receptors will be useful to decrease the side effects of constipation.

Applications

The results suggest that opioid μ-2 receptors are mainly responsible for intestinal relaxation. Clinical application of agents showing less affinity than loperamide to opioid μ-2 receptors could be useful for prevention of constipation.

Terminology

OIC is a frequent side effect in cancer patients who received morphine-like compounds to reduce pain. Loperamide is a widely used agent for treatment of diarrhea in clinics, and it has no effect in the brain. Opioid receptors are the action site of opioids and related agents. Receptors are generally expressed in various tissues and located on the cell membrane. Subtypes of opioid receptors have been established.

Peer review

This is a good descriptive study in which the authors analyzed the subtype of opioid μ receptors in the intestine of mice. The results are interesting and suggest that opioid μ-2 receptors are responsible for intestinal relaxation, which could be useful in preventing constipation by agents with less affinity for this receptor site.

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