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Effects of KW-5617 (Zaldaride Maleate), a Potent and Selective Calmodulin Inhibitor, on Secretory Diarrhea and on Gastrointestinal Propulsion in Rats

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ABSTRACT—KW-5617 (zaldaride maleate), 1,3-dihydro-1-[1-[(4-methyl-4*H*,6*H*-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one maleate, is a selective calmodulin inhibitor. We studied the effects of KW-5617 on secretory diarrhea and gastrointestinal propulsion in rats, as compared with those of loperamide, a conventional anti-diarrheal drug. Diarrhea was induced in rats either by 16,16-dimethyl prostaglandin E_2 (500 μg/kg, i.p.) or by castor oil (1 ml/100 g body weight, p.o.). In the 16,16-dimethyl prostaglandin E_2 model, KW-5617 at the doses of 3 mg/kg (p.o.) and higher ameliorated the diarrhea. Similarly, loperamide improved the diarrhea, the activity of loperamide being equivalent to that of KW-5617. In the castor oil model, KW-5617 significantly delayed the onset of diarrhea at the doses of 3 mg/kg (p.o.) and higher, while loperamide delayed the onset of diarrhea at the doses of 0.3 mg/kg (p.o.) and higher. KW-5617 only at the high doses of 30 and 100 mg/kg (p.o.) reduced gastric emptying, small intestinal propulsion, proximal colonic propulsion and distal colonic propulsion. In contrast, loperamide at its anti-diarrheal doses inhibited gastrointestinal propulsion. Our results show that KW-5617, unlike loperamide, at its anti-diarrheal doses does not exert anti-propulsive effects in rats. KW-5617 may be a useful drug for the treatment of diarrhea in terms of less side effects such as constipation.

Keywords: KW-5617, Loperamide, Diarrhea, Zaldaride, Gastrointestinal propulsion

Calmodulin is a ubiquitous Ca^{2+}-binding protein that is involved in the intracellular Ca^{2+} messenger system in various tissues. Calmodulin located in the intestinal epithelium (1) plays important roles in the secretion of electrolytes and fluid in the intestinal tract (2).

The phenothiazine calmodulin inhibitors such as chlorpromazine and trifluoperazine have been shown to ameriolate diarrhea in the rodent (3, 4); however, these neuroleptic drugs induce sedation and, for this reason, these calmodulin inhibitors have not been used as anti-diarrheal therapy (5).

KW-5617 (zaldaride maleate), 1,3-dihydro-1-[1-[(4-methyl-4*H*,6*H*-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one maleate, is a selective and potent inhibitor of calmodulin, its activity being approximately 4-times more potent than that of trifluoperazine (6), and has less sedative effect (3). In contrast to trifluoperazine, KW-5617 does not inhibit protein kinase C and has much less affinity for dopamine receptors (6). Moreover, KW-5617 is reported to ameliorate 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$)-induced diarrhea in rats and castor oil-induced diarrhea in mice, without compromising the rate of intestinal propulsion (3).

Loperamide hydrochloride (loperamide), an opioid mu- and delta-agonist, is a widely prescribed and effective anti-diarrheal drug. The anti-diarrheal effect of loperamide is excuted by its anti-motility and anti-secretory actions. On the other hand, Merrit et al. reported that loperamide also inhibits the activity of calmodulin (7), suggesting that the inhibition of calmodulin is at least partly responsible for the anti-diarrheal effect of this drug. In fact, the anti-diarrheal effects of loperamide and phenotiazine derivatives, which are known to inhibit calmodulin activity, parallel their affinity for calmodulin (4). Furthermore, Reynolds et al. have reported that loperamide can block Ca^{2+} channels, an action that may also be involved in its anti-diarrheal effect (8). However,

loperamide is known to prolong the duration of severe secretory diarrhea caused by a bacteria or virus such as *Vibrio cholera*, *Escherichia coli*, *Salmonella* and *Rotavirus*, since this drug inhibits the intestinal propulsion, resulting in the prolonged excretion of the bacteria or virus (9). Thus, loperamide is usually contraindicated for the treatment of infectious diarrhea.

In the present study, we compared the anti-diarrheal effects of KW-5617 to those of loperamide in rat models of secretory diarrhea. Moreover, we examined the possible inhibitory effects of KW-5617 and loperamide on the various parts of gastrointestinal propulsion in rats. From the results, the clinical implication was inferred, especially focusing on the clinical utility of KW-5617.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (Charles River, Atsugi), weighing 193−265 g, were used for the present study. The animals were housed under the following environmental conditions: temperature of 23±1℃, humidity of 55±5% and a 12-hr light/dark cycle. Commercial rat chow and water were given ad libitum. The present experiments were conducted in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society, and the experimental protocols were approved by the Ethical Committee of the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd.

Materials

KW-5617 was a gift from Novartis Consumer Health (Nyon, Switzerland). Loperamide hydrochloride and $dmPGE_2$, a stable prostaglandin E_2 analogue, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Castor oil was purchased from Kanto Chemical Co., Inc. (Tokyo). Gum arabicum was purchased from Nacalai Tesque Inc. (Tokyo). Phenol red, cornstarch and charcoal were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other reagents and solvents were analytical grade. KW-5617 and loperamide hydrochloride were suspended in the vehicle consisting of 0.3 w/v% cornstarch, 5 w/v% polyethylene glycol #400 and 0.34 w/v% Tween 80 before experimental use; each drug was orally administered to the rats at a volume of 5 ml/kg body weight. Before the experiments, $dmPGE_2$ was diluted with distilled water for intraperitoneal injection.

Methods

Anti-diarrheal action

$DmPGE_2$-induced diarrhea: The animals had free access to food and water until 3 hr prior to the experiment. On the day of the experiment, rats were screened to exclude the animals with preexisting diarrhea. Diarrhea was induced in rats with a slight modification of the method of Shook et al. (3). KW-5617, loperamide or the vehicle was orally administered at 60 min before receiving 500 μg/kg (i.p.) of $dmPGE_2$. All the rats were then continuously observed for 90 min after $dmPGE_2$ challenge. The fecal output was scored according to the arbitrary scoring criteria as follows: hard stools or no stools, score 0; normal stools, score 1; wet but formed stools, score 2; unformed stools, score 3 and severe watery diarrhea, score 4. The average of their score was defined as the fecal output index. In addition, the evacuated feces were dried and weighed.

Castor oil-induced diarrhea: Prior to the experiment, the animals were fasted, but allowed free access to drinking water, for 18−20 hr. On the day of the experiment, rats were screened to exclude the animals with preexisting diarrhea. KW-5617, loperamide or the vehicle was orally administered at 60 min before they received castor oil (1 ml/100 g body weight). Thereafter, individual cages for each rat were inspected for the presence or absence of diarrhea at 30-min intervals for 3 hr after they received castor oil. At 3 hr, rats without diarrhea were killed by cervical dislocation, and autopsies were performed for examination of the intracolonic contents.

Gastrointestinal propulsion

Gastric emptyting: The animals were fasted, but free access to drinking water was provided for 18−20 hr before the experiments. Gastric emptying was determined by the method of Scarpignato et al. (10). KW-5617, loperamide or the vehicle was orally administered at 40 min before receiving the test meal (0.05 w/v% phenol red in 1.5 w/v% aqueous sodium carbxymethyl cellulose) at a volume of 1.5 ml per rat. Fifteen minutes after administration of the test meal, the rat was sacrificed by cervical dislocation, and the stomach was carefully removed to determine the amount of phenol red remaining in the stomach. The content in the stomach was solubilized in 40 ml of 0.1 M NaOH. One milliliter of the mixture was added to 2 ml of 7.5 w/v% trichloroacetic acid solution to precipitate the proteins. This mixture was then centrifuged at 3000×*g* for 15 min. To 2 ml of the supernatant solution, 1 ml of 1 M NaOH was added. The absorbance value of this solution was read with a spectrophotometer (U-1080; Hitachi, Tokyo) at 560 nm against distilled water. The gastric emptying for each rat was calculated according to the following formula:

$$\text{Gastric emptying (\%)} = \left[1 - \frac{\text{the amount of phenol red recovered from the test stomach}}{\text{the amount of phenol red recovered from the standard stomach}}\right] \times 100$$

Small intestinal propulsion: The animals were fasted, but allowed free access to drinking water, for 18–20 hr before use. KW-5617, loperamide or the vehicle was orally administered at 40 min before receiving 1 ml/100 g body weight of the charcoal meal (10 w/v% suspension of activated charcoal in 5 w/v% aqueous gum arabicum). After 15 min, the rat was sacrificed by cervical dislocation, and the small intestine was carefully removed without stretching. The length of the small intestine (pyloric sphincter to caecum) and the distance traveled by the charcoal meal front were measured. The small intestinal propulsion for each rat was calculated according to the following formula:

$$\text{Small intestinal propulsion (\%)} = \left[\frac{\text{the distance of travel by charcoal meal}}{\text{the length of small intestine}}\right] \times 100$$

Proximal colonic propulsion: Proximal colonic propulsion in the rat was evaluated according to the procedure described by Ueda et al. (11). Each animal was anesthetized with pentobarbital sodium (60 mg/kg, i.p.), and a polyethylene tube (1.57 mm in diameter) was implanted into the caecum at the beginning of the colon. The other end of the tube was then taken out and fixed. The animal was housed in an individual cage for 3 days and fasted, but free access to drinking water was provided for 18–20 hr before the experiment. KW-5617, loperamide or the vehicle was orally administered 40 min before the colonic transit vehicle, a suspension of 10 w/v% activated charcoal in 5 w/v% aqueous gum arabicum (1 ml/100 g body weight), was injected into the cannula. One hour later, the animal was sacrificed by cervical dislocation, and the colon was carefully removed without stretching. The length of the colon and the distance traveled by the colonic transit marker front were measured, and the proximal colonic propulsion for each rat was calculated according to the following formula:

$$\text{Proximal colonic propulsion (\%)} = \left[\frac{\text{the distance of travel by colonic transit marker}}{\text{the length of colon}}\right] \times 100$$

Distal colonic propulsion: The animals were fasted, but free access to drinking water was provided for 18–20 hr before the experiment. Distal colonic propulsion was evaluated according to the modified method of Koslo et al. (12). A teflon ball (3.17 mm in diameter) was inserted retrograde by 3 cm into the rectum of the rat slightly anesthetized with ether, and the time for expulsion of the ball was measured.

Statistical analyses

Each value indicates a mean±S.E.M. The data of $dmPGE_2$ diarrhea, gastric emptying, small intestinal propulsion, proximal colonic propulsion and distal colonic propulsion were analyzed by the Kruskal-Wallis test followed by the Shirley-Williams test or by analysis of variance (ANOVA) followed by the Steel test. In the experiment of castor oil-induced diarrhea, the χ^2 test was used to determine the significance of the difference between the vehicle-treated group and the drug-treated group. A P-value less than 0.05 was considered to be statistically significant.

RESULTS

Anti-diarrheal action

Effects of drugs on $dmPGE_2$-induced diarrhea: Intraperitoneal administration of $dmPGE_2$ (500 μg/kg) produced an increase in diarrhea score. The diarrhea score in the control animals (3.9±0.1; mean±S.E.M., n=10) was higher than that in normal animals (diarrhea

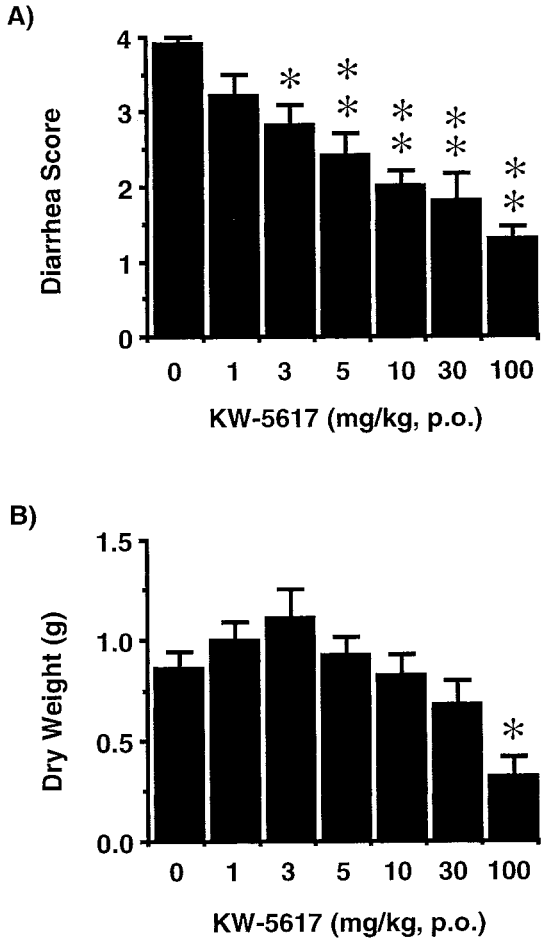

Fig. 1. Effects of KW-5617 on $dmPGE_2$-induced diarrhea in rats. Each column with bar represents the mean±S.E.M. of 10 animals, except for 100 mg/kg (p.o.) (n=7). A: diarrhea score, B: dry weight of feces. *P<0.05, **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

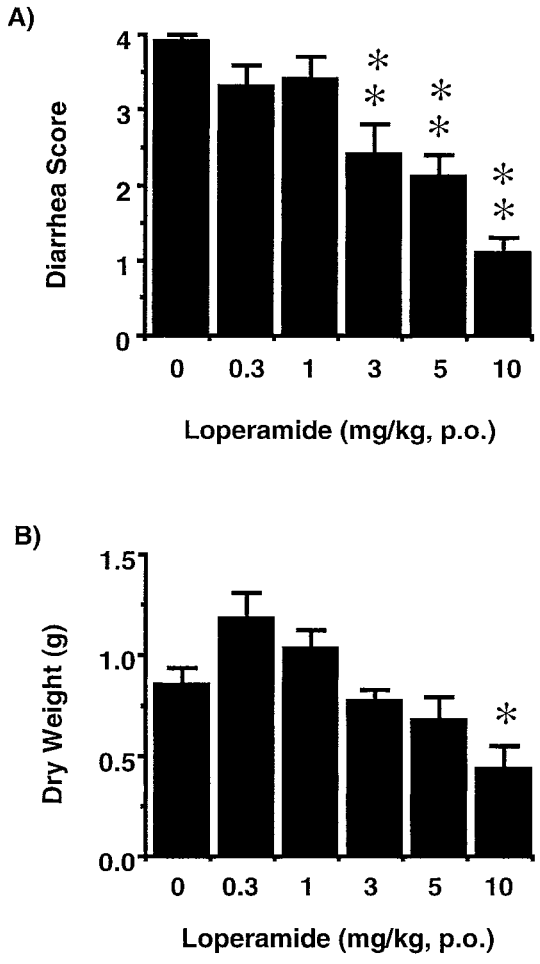

Fig. 2. Effects of loperamide on dmPGE$_2$-induced diarrhea in rats. Each column with bar represents the mean±S.E.M. of 10 animals. A: diarrhea score, B: dry weight of feces. *P<0.05, **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

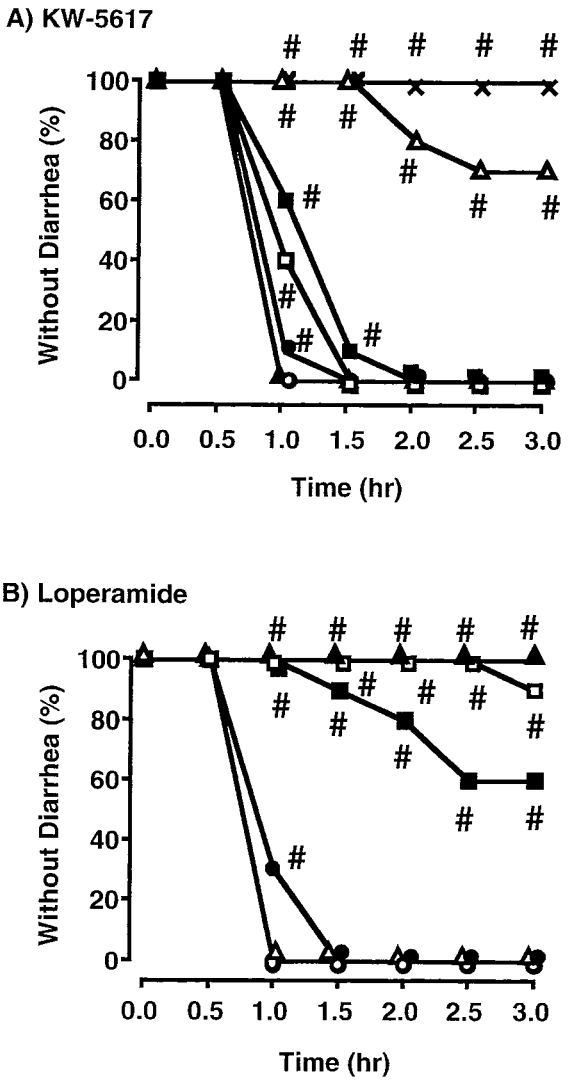

Fig. 3. Effects of KW-5617 (A) and loperamide (B) on castor oil-induced diarrhea in rats. Ten animals were used for each group. #P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group. A: KW-5617 was orally administered to rats at 0 (○), 1 (▲), 3 (●), 5 (□), 10 (■), 30 (△) and 100 (×) mg/kg. B: Loperamide was orally administered to rats at 0 (○), 0.1 (△), 0.3 (●), 1 (■), 3 (□) and 10 (▲) mg/kg.

score: 1). In the vehicle-treated group, dmPGE$_2$ induced secretory diarrhea in all the animals within 60 min after the challenge. KW-5617, when orally administered at 3 to 100 mg/kg 60 min before dmPGE$_2$ challenge, significantly ameliorated the dmPGE$_2$-induced diarrhea, when this drug at 100 mg/kg (p.o.) significantly reduced fecal evacuation (Fig. 1). On the other hand, loperamide at 3 to 10 mg/kg (p.o.) significantly prevented the dmPGE$_2$-induced diarrhea, when this drug at 10 mg/kg (p.o.) significantly decreased fecal evacuation (Fig. 2).

Effects of drugs on castor oil-induced diarrhea: In the vehicle-treated rat, oral administration of castor oil (1 ml/100 g body weight) produced severe secretory diarrhea from 30 to 60 min after the challenge. Pretreatment with KW-5617 at 3 to 10 mg/kg (p.o.) significantly delayed the onset of diarrhea, and this drug at 30 and 100 mg/kg (p.o.) reduced or abolished the incidence of diarrhea (Fig. 3A). Loperamide at 0.3 mg/kg (p.o.) significantly delayed the onset of diarrhea, and this drug at 1 to 10 mg/kg (p.o.) reduced or abolished the incidence of diarrhea (Fig. 3B). In autopsy, all the rats treated with each drug and without diarrhea were found to contain normal stools in the colon.

Gastrointestinal propulsion

Effects of drugs on gastric emptying: The gastric emptying of the vehicle control group for each drug was 49.9±4.8% (mean±S.E.M., n=10). KW-5617 at up to 10 mg/kg (p.o.) did not affect gastric emptying, whereas

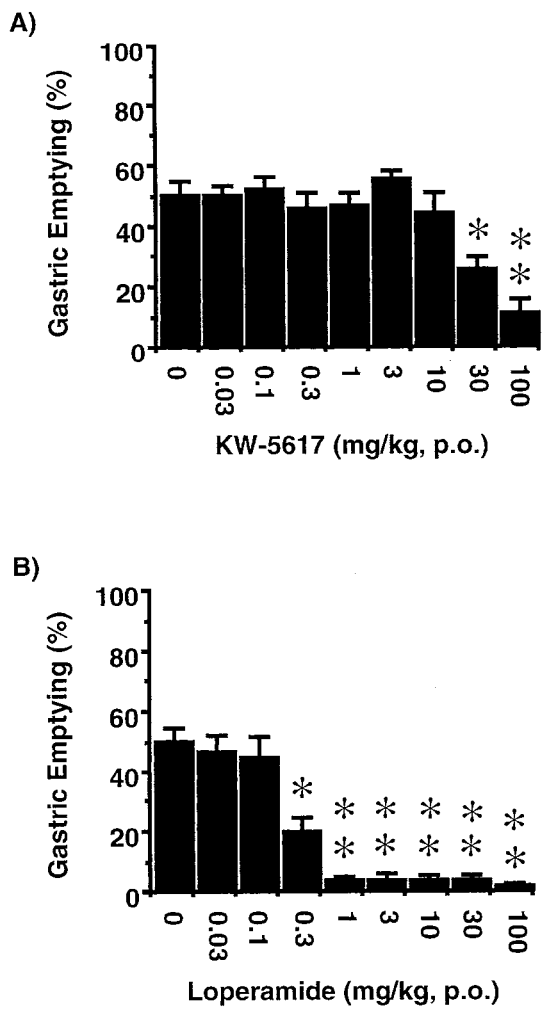

Fig. 4. Effects of KW-5617 (A) and loperamide (B) on gastric emptying in rats. Each column with bar represents the mean±S.E.M. of 10 animals. *P<0.05, **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

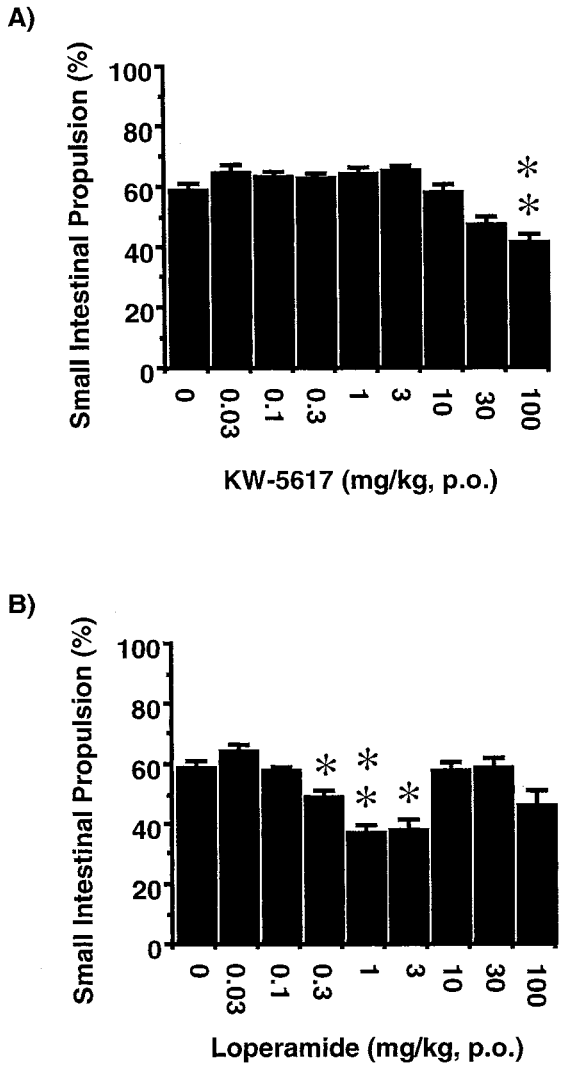

Fig. 5. Effects of KW-5617 (A) and loperamide (B) on small intestinal propulsion in rats. Each column with bar represents the mean±S.E.M. of 10 animals. *P<0.05, **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

this drug at 30 and 100 mg/kg (p.o.) significantly reduced gastric emptying (Fig. 4A). On the other hand, loperamide at the doses of 0.3 mg/kg (p.o.) and higher significantly and prominently reduced gastric emptying (Fig. 4B).

Effects of drugs on small intestinal propulsion: The small intestinal propulsion in the vehicle control group was 58.4±2.6% (mean±S.E.M., n=10). KW-5617 at 100 mg/kg (p.o.) significantly inhibited small intestinal propulsion (Fig. 5A). Loperamide at 0.3 to 3 mg/kg (p.o.) significantly delayed small intestinal propulsion (Fig. 5B). However, the higher doses (10 to 100 mg/kg, p.o.) of loperamide did not significantly affect small intestinal propulsion (Fig. 5B).

Effects of drugs on proximal colonic propulsion: The values of proximal colonic propulsion in the vehicle control group for KW-5617 and loperamide were 64.9±4.4% and 65.3±5.0% (mean±S.E.M., n=10), respectively. KW-5617 at 30 and 100 mg/kg (p.o.) significantly reduced proximal colonic propulsion (Fig. 6A). On the other hand, loperamide at the doses of 0.3 mg/kg (p.o.) and higher significantly reduced proximal colonic propulsion (Fig. 6B).

Effects of drugs on distal colonic propulsion: The time for expulsion of the ball, i.e., the distal colonic propulsion, in the vehicle control group was 37.6±6.5 min (mean±S.E.M., n=10). KW-5617 at 30 and 100 mg/kg (p.o.) significantly prolonged the evacuation time, indicating reduced distal colonic propulsion (Fig. 7A). On the other hand, loperamide at the doses of 0.3 mg/kg (p.o.) and higher significantly reduced distal colonic propulsion (Fig. 7B).

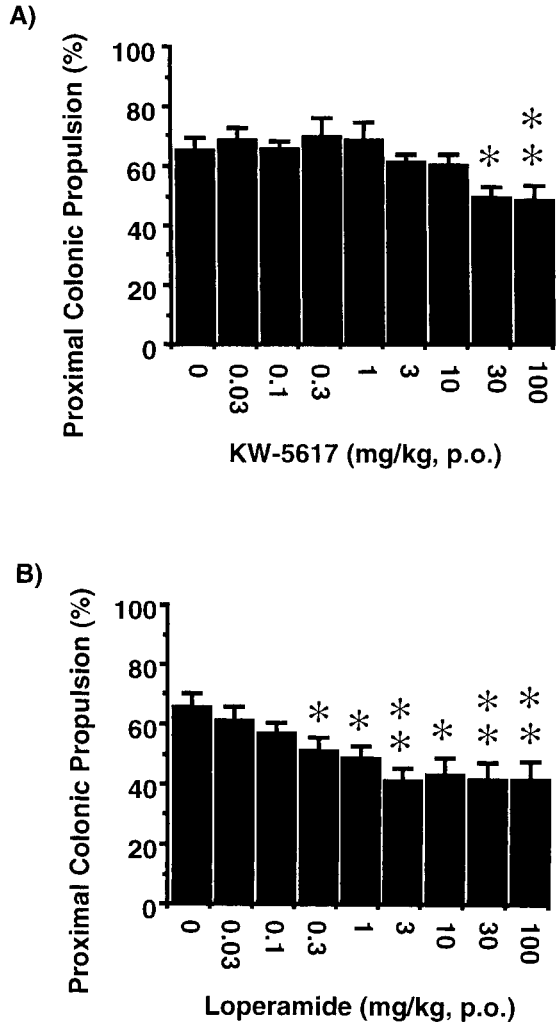

Fig. 6. Effects of KW-5617 (A) and loperamide (B) on proximal colonic propulsion in rats. Each column with bar represents the mean±S.E.M. of 10 animals. *P<0.05, **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

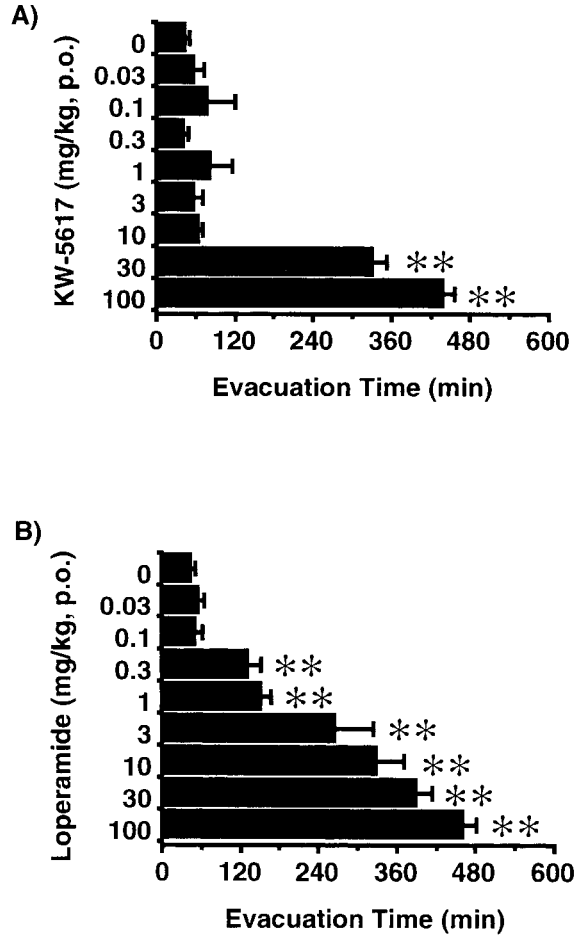

Fig. 7. Effects of KW-5617 (A) and loperamide (B) on distal colonic propulsion in rats. Each column with bar represents the mean±S.E.M. of 10 animals. **P<0.01, statistically significant vs the value in the vehicle control (0 mg/kg, p.o.) group.

DISCUSSION

The present study was performed to compare the anti-diarrheal and anti-gastrointestinal propulsive effects of KW-5617 and loperamide. KW-5617 at 3 mg/kg (p.o.) and higher significantly ameliorated secretory diarrhea in 2 different rat models, and this drug at 30 and 100 mg/kg (p.o.) inhibited gastrointestinal propulsion in rats (Table 1). On the other hand, loperamide at 0.3 mg/kg (p.o.) and higher significantly reduced both the secretory diarrhea and the gastrointestinal propulsion (Table 1). The present results demonstrate that KW-5617 prevents secretory diarrhea without affecting gastrointestinal propulsion, whereas loperamide inhibits both diarrhea and gastrointestinal propulsion.

$DmPGE_2$ is reported to induce diarrhea by the stimulation of adenylate cyclase and cAMP production (13),

Table 1. Summary of anti-diarrheal and anti-gastrointestinal propulsive effects of KW-5617 and loperamide in rats

	Minimun effective dose (mg/kg, p.o.)	
	KW-5617	Loperamide
Anti-diarrheal effect		
PGE_2-induced diarrhea	3	3
Castor oil-induced diarrhea	3	0.3
Anti-gastrointestinal propulsion		
Gastric emptying	30	0.3
Small intestinal propulsion	100	0.3
Proximal colonic propulsion	30	0.3
Distal colonic propulsion	30	0.3

which markedly increases the fluid secretion in the intestine (14). On the other hand, the activity of adenylate cyclase is regulated by Ca^{2+}-calmodulin in the intestinal epithelium (15–17). Both adenylate cyclase and calmodulin are localized along the basolateral membrane of the enterocyte (1). These observations suggest that the anti-diarrheal action of KW-5617 is due to the inhibition of adenylate cyclase, via the blockade of calmodulin activity, in the intestinal epithelium, resulting in the inhibited Cl^- secretion. However, the other mechanisms, such as the inhibition of guanylate cyclase and the K^+ channel blockade, for the anti-diarrheal effect of KW-5617 may be possible. Further studies are required to clarify the precise site of action of this drug.

In the present study, KW-5617 ameliorated the castor oil-induced diarrhea at similar doses to those inhibiting the $dmPGE_2$-induced diarrhea. Multiple mechanisms have been proposed for the diarrhea induced by castor oil. The castor oil-induced diarrhea is supposed to be due to an action of one of its ingredients, ricinoleic acid. Castor oil or ricinoleic acid causes gastrointestinal damage and diarrhea, which are associated with the increased formation of platelet-activating factor (PAF) (18), prostaglandins (PGs) (19) and nitric oxide (NO) (20, 21) throughout the intestinal tract. PAF (22), PGs (17) and NO (23) are reported to increase electrolyte and fluid secretion in the intestinal tract. Calmodulin is reported to regulate the synthesis of PAF (24), NO (25, 26) and arachidonic acid (27, 28), a precursor of PGs. Moreover, PAF receptor is controlled by the intracellular Ca^{2+}-calmodulin-dependent process (29). These results suggest that the anti-diarrheal action of KW-5617 may partly be associated with the reduction of PGs, NO and/or PAF productions via the inhibition of calmodulin activity in the intestinal tract. In fact, our unpublished data have shown that the castor oil-induced diarrhea is completely suppressed by indomethacin and that KW-5617 exhibits the anti-diarrheal action even after the treatment with the PAF antagonist CV-6209 and the NO synthesis inhibitor L^G-nitro-L-arginine methyl ester hydrochloride. It is thus likely that the anti-diarrheal effect of KW-5617 involves, at least partly, a mechanism other than the inhibitory effect on PAF or NO and that the inhibition of PGs synthesis plays the most probable and provital role in the amelioration by KW-5617 of the castor oil-induced diarrhea.

The anti-diarrheal action of loperamide has been attributed to either the inhibition of the intestinal motility and propulsion or the inhibition of the intestinal secretion. Loperamide has stimulatory effects on both the mu-type and the delta-type of opioid receptors (30), an inhibitory effect on calmodulin activity (7) and a blocking effect on Ca^{2+} channels (8) that relate to the intestinal electrolytes and fluid transport and the intestinal motility and propulsion. It is thus likely that loperamide, by multiple mechanisms of action, improved the castor oil-induced diarrhea, which is due to both the accelerated intestinal propulsion and the augmented secretion of water and electrolytes. On the other hand, the amelioration by loperamide of the $dmPGE_2$-induced diarrhea, which is primarily due to the augmented secretion, seems to be ascribed to the inhibition of calmodulin activity. The discrepancy of the effective doses of loperamide between the $dmPGE_2$-induced and the castor oil-induced diarrhea may exist because the anti-diarrheal effect may be exerted through different mechanisms in these two models of diarrhea. It is likely that the amelioration by loperamide of castor oil-induced diarrhea involves the inhibition of gastric motility or propulsion. In fact, loperamide was shown to inhibit gastrointestinal propulsion at doses showing an inhibitory effect on castor oil-induced diarrhea (Table 1).

In the present study, loperamide at its anti-diarrheal doses induced prominent inhibition of the various parts of gastrointestinal propulsion, whose inhibition is supposed to play a role in the anti-diarrheal effect of this drug. The anti-propulsive effects of loperamide seem to involve the inhibition of acetylcholine and tachykinins release from the enteric nerve, since this drug is reported to inhibit, via the stimulation of opioid receptors, the release of these excitatory mediators (31). The reason why small intestinal propulsion was not changed by the higher doses of loperamide may be because its higher doses augmented the release of acetylcholine. In fact, when the animal was pretreated with atropine sulfate (10 mg/kg, p.o.), loperamide even at its higher doses inhibited small intestinal propulsion (N. Aikawa and A. Karasawa, unpublished observation).

The present study indicated that KW-5617, but not loperamide, exerts anti-diarrheal effects at doses that do not compromise gastrointestinal propulsion. Moreover, in the clinical study, KW-5617 was reported to inhibit secretory activity without affecting the rate of gastrointestinal propulsion in humans (32). In contrast to loperamide, KW-5617 does not possess affinity for opioid receptors (data not shown). This seems to be the reason why KW-5617, unlike loperamide, did not exert anti-propulsion effects at its anti-diarrheal doses. Loperamide can cause constipation as its side effect, and this drug is usually contraindicated for the treatment of infectious diarrhea because of its anti-propulsive action, possibly resulting in the inhibition of the toxin excretion or reduction of toxin excreted from the bacteria or viruses. The present observation suggests that KW-5617 is a potent drug for the treatment of acute secretory diarrhea because it has less side effects of constipation. Furthermore, there

is a possibility that KW-5617 can be used for the treatment of infectious and/or traveler's diarrhea induced by the bacteria or viruses.

In summary, the present study demonstrates that KW-5617 exerts anti-diarrheal effects in rat models of secretory diarrhea and that this drug at its anti-diarrheal doses does not compromise gastrointestinal propulsion. KW-5617 seems to be a useful anti-diarrheal drug with less side effects of constipation.

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