

# Prostatic relaxation induced by loperamide is mediated through activation of opioid $\mu$ -2 receptors *in vitro*

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**Abstract.** The merit of opioid  $\mu$ -receptor activation in the improvement of benign prostatic hyperplasia (BPH) remains obscure. In the present study, we used loperamide to identify the subtype of opioid  $\mu$ -receptors involved in prostatic relaxation and investigate the possible mechanism of this relaxation. Prostate strips were isolated from 12-week-old male Wistar rats for identification of isometric tension. The prostate strips were precontracted with either 1  $\mu$ mol/l phenylephrine or 50 mmol/l KCl. The decrease in muscle tone (relaxation) was then characterized after cumulative administration of loperamide (0.1 to 10  $\mu$ mol/l) into the organ bath for the concentration-dependent study. Pretreatment with specific blockers or antagonists was carried out to compare the changes in loperamide-induced relaxation. Loperamide produced a marked relaxation in the isolated prostates precontracted with phenylephrine or KCl in a dose-dependent manner. This relaxation was abolished by cyprodime, a selective opioid  $\mu$ -receptor antagonist, but was not modified by naloxonazine at a dose sufficient to block the opioid  $\mu$ -1 receptors. Treatment with an agonist for opioid  $\mu$ -1 receptors also failed to modify the muscle tone. Moreover, the relaxation by loperamide was attenuated by glibenclamide at a dose sufficient to block ATP-sensitive K<sup>+</sup> channels. In addition, this action of loperamide was abolished by protein kinase A (PKA) inhibitor and enhanced by the inhibitor of phosphodiesterase for cyclic AMP (cAMP). Our results suggest that loperamide induces prostatic relaxation through activation of opioid  $\mu$ -2 receptors via the cAMP-PKA pathway to open ATP-sensitive K<sup>+</sup> channels.

## Introduction

Benign prostatic hyperplasia (BPH) occurs frequently in older men and is associated with lower urinary tract symptoms causing obstruction of the proximal urethra and urinary flow disturbances (1). In clinics, medical treatments for BPH include widely used  $\alpha$ -1 antagonists and 5- $\alpha$ -reductase inhibitors. However, the side effects, such as postural hypotension, erectile dysfunction and ejaculatory difficulty, disturb the quality of life of these patients (2,3). Therefore, development of a more effective therapy for the treatment of BPH is urgent and necessary.

Loperamide is widely used in the clinic for a variety of diarrheal syndromes, including acute and nonspecific (infectious) diarrhea (4,5). Recently, we identified opioid  $\mu$ -receptor expression in rat prostates, and prostatic relaxation was induced by the activation of opioid  $\mu$ -receptors using loperamide (6). Loperamide was introduced as a peripheral agonist of opioid  $\mu$ -receptors with poor ability to penetrate the blood-brain barrier (7,8). Some analgesic agents have also revealed relaxant effects in smooth muscle (9,10). (+)-Tramadol was found to activate peripheral opioid  $\mu$ -receptors to exhibit a concentration-dependent relaxation of aorta (11). Basically, the opioid  $\mu$ -receptors have been divided into 3 subtypes, including  $\mu$ -1,  $\mu$ -2 and  $\mu$ -3 opioid receptors (12). However, activation of opioid  $\mu$ -1 receptors was reported to be link mainly with the PLC-PKC pathway (13). Since PLC-PKC signals increase intracellular calcium to induce vasoconstriction or bladder contraction (14,15), prostatic relaxation is not considered to be induced by the activation of opioid  $\mu$ -1 receptors.

ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels have been found to be involved in the relaxation of urethral smooth muscle (16). Actually, the opening of the K<sub>ATP</sub> channel is introduced to lower intracellular Ca<sup>2+</sup> concentrations (17,18). Moreover, impairment of the K<sub>ATP</sub> channel appears to be associated with the dysfunction of the lower urinary tract (19). However, the role of the K<sub>ATP</sub> channel in prostatic relaxation remains obscure.

In an attempt to clarify the subtype of opioid  $\mu$ -receptor involved in the regulation of prostatic tone, we used loperamide as an agonist in order to induce relaxation in isolated prostates. Specific blockers or antagonists were then applied to investigate the possible mechanism(s) of loperamide.

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## Materials and methods

**Experimental animals.** We obtained 12-week-old male Wistar rats from the Animal Center of National Cheng Kung University Medical College. Rats were maintained in a temperature-controlled room ( $25 \pm 1^\circ\text{C}$ ) under a 12 h light-dark cycle (lights on at 06:00). All rats were given water and fed standard chow (Purina Mills, LLC, St. Louis, MO, USA) *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, as well as the Guidelines of the Animal Welfare Act.

**Preparation of isolated prostate strips.** In all prostatic experiments, the isolated prostates from Wistar rats were used. Each rat was sacrificed by decapitation under anesthesia with pentobarbital (50 mg/kg). Following our previous study, the prostate strips were rapidly removed and placed in oxygenated Krebs' buffer (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). After the prostate strips were carefully freed from fat and connective tissue, the strips were then mounted in organ baths filled with 10 ml of oxygenated Krebs' buffer (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) at  $37^\circ\text{C}$  containing (in mmol/l) NaCl 135; KCl 5;  $\text{CaCl}_2$  2.5;  $\text{MgSO}_4$  1.3;  $\text{KH}_2\text{PO}_4$  1.2;  $\text{NaHCO}_3$  20; and D-glucose 10 (pH 7.4).

Each preparation was connected to strain gauges (FT03; Grass Instrument, Quincy, MA, USA). 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.

**Prostatic relaxation caused by loperamide.** After the resting tension had stabilized, a solution of phenylephrine (Sigma-Aldrich, St. Louis, MO, USA) or KCl prepared in distilled water was added into bathing buffer to induce a rapid increase in prostatic tone followed by stable constriction (tonic contraction). The final concentration in the organ bath for phenylephrine was  $1 \mu\text{mol/l}$  and for KCl, 50 mmol/l, respectively. Prostate strips in the treatment group were exposed to loperamide (0.1–10  $\mu\text{mol/l}$ ) to observe the decrease in tonic contraction (vasodilatation). Relaxation was expressed as the percent decrease in maximal tonic contraction. Concentration-relaxation curves were generated in cumulative fashion.

**Effects of blockers on loperamide-induced prostatic relaxation.** Prostate strips were exposed to glibenclamide (Research Biochemical, Wayland, MA, USA) or opioid  $\mu$ -receptor antagonist, cyprodime or naloxonazine (Tocris Cookson, Bristol, UK), for 15 min before the addition of loperamide into the organ bath. In addition, the inhibitor of cyclic AMP phosphodiesterase (IBMX) or protein kinase A (H-89) was administered in the same manner. Changes in relaxation caused by loperamide were compared with that in the vehicle (distilled water)-treated controls.

**Statistical analysis.** All values are presented as the mean  $\pm$  SEM for a given number of animals or samples. Analysis of variance and Dunnett's post hoc test were used to evaluate the

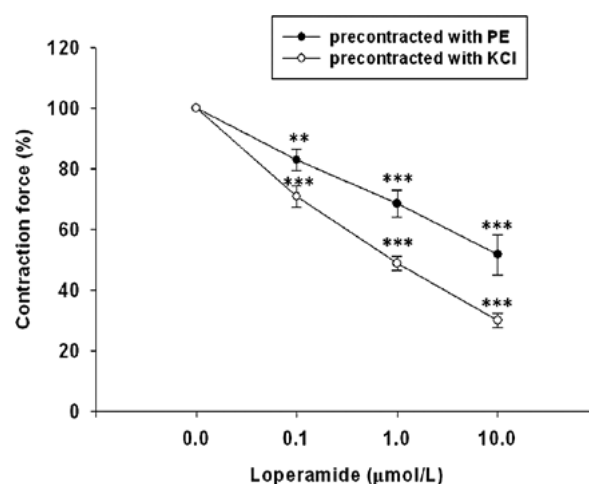


Figure 1. Concentration-dependent relaxation induced by loperamide in isolated prostate strips precontracted with  $1 \mu\text{mol/l}$  phenylephrine or 50 mmol/l KCl. Data represent the mean  $\pm$  SEM of eight animals. \*\*\* $P < 0.001$  compared with the vehicle (distilled water)-treated control.

significance between groups.  $P < 0.05$  was considered to be a significant difference.

## Results

**Effect of opioid receptor blockade on loperamide-induced prostatic relaxation.** Prostate strips were strongly contracted by the application of phenylephrine ( $1 \mu\text{mol/l}$ ) or KCl (50 mmol/l). As shown in Fig. 1, loperamide dilated both phenylephrine- and KCl-contracted prostate strips in a concentration-dependent manner. At the maximal concentration tested (10  $\mu\text{mol/l}$ ), loperamide significantly attenuated the tonic contraction of prostate strips induced by phenylephrine to  $52.79 \pm 6.10\%$  of the maximal contraction. Similarly, 10  $\mu\text{mol/l}$  loperamide also lowered KCl-induced tonic constriction to  $30.06 \pm 2.19\%$  of the maximal contraction. Cyprodime (0.01–0.1  $\mu\text{mol/l}$ ) produced a significant and concentration-dependent attenuation of the relaxant effect of loperamide on the tonic contraction of phenylephrine-precontracted prostate strips. The prostatic relaxation due to loperamide in KCl-pretreated prostate strips was also abolished in a similar manner in the presence of cyprodime (Table I). In addition, naloxonazine failed to abolish the relaxant effect of loperamide on tonic contraction in phenylephrine ( $1 \mu\text{mol/l}$ )-precontracted prostate strips at a higher concentration (0.1  $\mu\text{mol/l}$ ). As shown in Table I, the prostatic relaxation by loperamide in KCl (50 mmol/l)-precontracted prostate strips was also not reversed by naloxonazine even at a higher concentration. Also, treatment with stevioside at a dose sufficient to activate the opioid  $\mu$ -1 receptor as described previously (20) failed to modify muscle tone in either the phenylephrine- or KCl-contracted prostate strips (Fig. 2).

**Role of ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels in loperamide-induced prostatic relaxation.** Glibenclamide produced a concentration-dependent (0.01–1  $\mu\text{mol/l}$ ) attenuation of the relaxant effect of loperamide on tonic contraction of phenylephrine ( $1 \mu\text{mol/l}$ )-precontracted prostate strips. The

Table I. Inhibitory effect of cyprodime or naloxonazine on the relaxation of loperamide (10  $\mu$ mol/l) in isolated prostates precontracted with 1  $\mu$ mol/l phenylephrine (PE) or 50 mmol/l KCl.

	PE (%)	KCl (%)
Loperamide (10 $\mu$ mol/l)		
+ Vehicle	55.49 $\pm$ 6.16	35.18 $\pm$ 3.30
+ Cyprodime		
0.01 $\mu$ mol/l	76.48 $\pm$ 1.75 <sup>b</sup>	72.58 $\pm$ 1.47 <sup>c</sup>
0.10 $\mu$ mol/l	90.69 $\pm$ 0.80 <sup>c</sup>	87.59 $\pm$ 0.47 <sup>c</sup>
+ Naloxonazine		
0.01 $\mu$ mol/l	57.90 $\pm$ 2.73	38.58 $\pm$ 3.38
0.10 $\mu$ mol/l	66.97 $\pm$ 1.31	41.17 $\pm$ 3.93

Data represent the mean  $\pm$  SEM of eight animals. <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 compared with the vehicle-treated control.

Table II. Effects of the inhibitors for cAMP phosphodiesterase or protein kinase A (PKA) on relaxation induced by loperamide (10  $\mu$ mol/l) or forskolin (10  $\mu$ mol/l) in isolated prostates precontracted with 1  $\mu$ mol/l phenylephrine (PE) or 50 mmol/l KCl.

	PE (%)	KCl (%)
Loperamide (10 $\mu$ mol/l)		
+ Vehicle	59.69 $\pm$ 3.54	33.08 $\pm$ 1.89
+ IBMX (10 $\mu$ mol/l)	46.58 $\pm$ 1.87 <sup>b</sup>	25.12 $\pm$ 0.86 <sup>b</sup>
+ H-89 (1 $\mu$ mol/l)	86.22 $\pm$ 2.10 <sup>c</sup>	85.00 $\pm$ 0.58 <sup>c</sup>
Forskolin (10 $\mu$ mol/l)		
+ Vehicle	28.47 $\pm$ 1.83	24.48 $\pm$ 1.92
+ Glibenclamide (1 $\mu$ mol/l)	83.17 $\pm$ 2.17 <sup>c</sup>	80.21 $\pm$ 1.77 <sup>c</sup>
+ IBMX (10 $\mu$ mol/l)	11.69 $\pm$ 1.29 <sup>c</sup>	10.47 $\pm$ 0.28 <sup>c</sup>
+ H-89 (1 $\mu$ mol/l)	86.08 $\pm$ 1.69 <sup>c</sup>	83.03 $\pm$ 1.17 <sup>c</sup>
IBMX (10 $\mu$ mol/l)	94.64 $\pm$ 0.88 <sup>c</sup>	90.26 $\pm$ 1.01 <sup>c</sup>
H-89 (1 $\mu$ mol/l)	95.78 $\pm$ 1.07 <sup>c</sup>	91.25 $\pm$ 1.18 <sup>c</sup>
Glibenclamide (1 $\mu$ mol/l)	95.79 $\pm$ 1.16 <sup>c</sup>	90.32 $\pm$ 0.69 <sup>c</sup>

Data represent the mean  $\pm$  SEM of eight animals. <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 compared with the vehicle-treated control, respectively.

prostatic relaxation by loperamide in KCl (50 mmol/l)-pre-treated prostate strips was also abolished in a similar manner by the treatment of glibenclamide (Fig. 3).

**Role of cAMP and PKA in loperamide-induced prostatic relaxation.** In the present study, forskolin (10  $\mu$ mol/l), a direct activator of adenylate cyclase, was used as a positive control to increase cyclic AMP (cAMP) as described previously (21). In prostate strips precontracted with phenylephrine (1  $\mu$ mol/l) or KCl (50 mmol/l), forskolin-induced relaxation was also abolished by pretreatment with glibenclamide (1  $\mu$ mol/l).

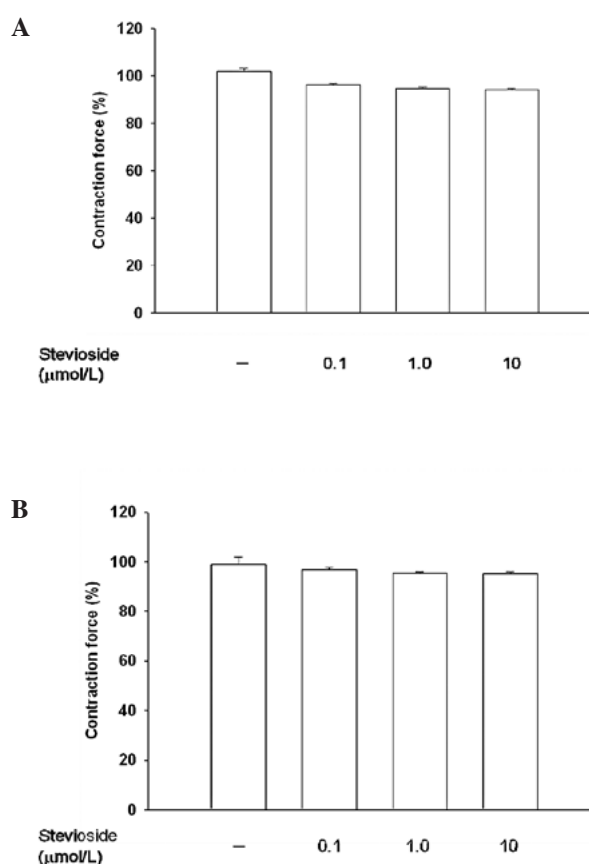


Figure 2. Effect of stevioside on the muscle tone in isolated prostate strips precontracted with 1  $\mu$ mol/l phenylephrine (A) or 50 mmol/l KCl (B). Data represent the mean  $\pm$  SEM of eight animals. No difference was observed as compared with the vehicle (distilled water)-treated control.

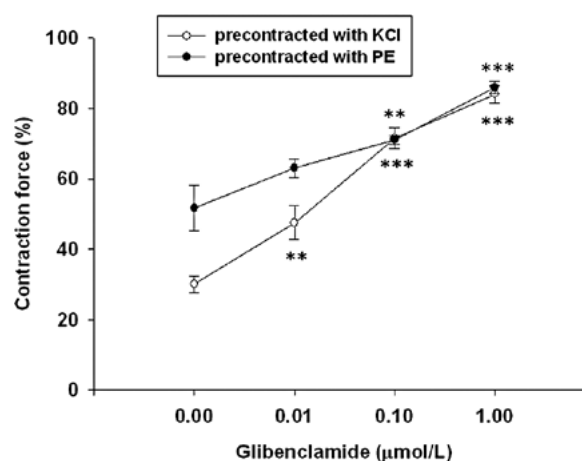


Figure 3. Concentration-dependent inhibitory effect of glibenclamide on the relaxation induced by loperamide (10  $\mu$ mol/l) in isolated prostates precontracted with 1  $\mu$ mol/l phenylephrine or 50 mmol/l KCl. Data represent the mean  $\pm$  SEM of eight animals. <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 compared with the vehicle-treated control.

Moreover, prostatic relaxation by forskolin was increased by 3-isobutyl-1-methylxanthine (IBMX) at a concentration (10  $\mu$ mol/l) sufficient to inhibit cAMP-phosphodiesterase (22), and was decreased by H-89 at a concentration (1  $\mu$ mol/l) enough to abolish the protein kinase A (PKA) (23). The

loperamide-induced prostatic relaxation was also modified by these agents in a similar manner. Results showed that prostatic relaxation induced by loperamide was increased by IBMX and attenuated by H-89 (Table II).

## Discussion

In the present study, we found that loperamide caused a dose-dependent relaxation in prostate strips of rats precontracted with phenylephrine or KCl. The action of loperamide appears to be related to the activation of opioid receptors in peripheral tissue as loperamide does not cross the central nervous system (8). Moreover, loperamide-induced action is effectively abolished by cyprodime, suggesting an activation of opioid  $\mu$ -receptors by loperamide in prostatic relaxation. However, the action of loperamide was not reversed by naloxonazine even at the dose sufficient to block opioid  $\mu$ -1 receptors. In addition, relaxation was not induced by agonist specific for opioid  $\mu$ -1 receptors (Fig. 2). Thus, mediation of opioid  $\mu$ -1 receptors was unlikely involved in the prostatic relaxation of loperamide.

Thus, another opioid  $\mu$ -receptor involved in this action of loperamide was considered. There is no doubt that loperamide is an agonist of peripheral opioid  $\mu$ -receptors (8,24). Recently, the opioid  $\mu$ -receptors have been divided into 3 subtypes, including  $\mu$ -1,  $\mu$ -2 and  $\mu$ -3 (25-27). Analgesic action through the activation of opioid  $\mu$ -1 receptors has been reported to exert spinal antinociception (28,29). In addition, activation of the opioid  $\mu$ -1 receptors seems to be related to smooth muscle contraction via the PLC-PKC pathway (14,15). Activation of the opioid  $\mu$ -2 receptors was found to participate in the relaxation of guinea pig ileum and inhibition of gastrointestinal transit (30,31). Moreover, opioid  $\mu$ -3 receptors are mostly present in endothelial cells associated with the production of nitric oxide to induce vasodilatation (32). Therefore, mediation of opioid  $\mu$ -1 or  $\mu$ -3 receptors in prostatic relaxation seems unlikely. Taken together, an activation of opioid  $\mu$ -2 receptors is more likely to participate in the action of loperamide in the relaxation of isolated prostate strips. Since there is no suitable tool or agent which can aid us in identifying this receptor, we focused on the subcellular signals of this receptor as an alternative method.

Potassium channels play an important role in the regulation of prostatic contractility in guinea-pig prostate smooth muscle cells (33). In a previous study, potassium channel expression was reduced in human BPH and prostate cancer (34). Moreover, the ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels are composed of four inwardly rectifying  $K^+$  channel subunits and four regulatory sulphonylurea receptors (35). The activation of  $K_{ATP}$  channels causes hyperpolarization of the cell membrane and consequently relaxes smooth muscle. In our previous study (36,37), the relaxation of loperamide in rat prostate strips was abolished by the pretreatment with glibenclamide at a concentration sufficient to block  $K_{ATP}$  channels. Thus, we focused on the involvement of  $K_{ATP}$  channels in the prostatic relaxation induced by loperamide. We then applied forskolin as a positive reference since forskolin was introduced as a direct activator of adenylate cyclase which can increase the intracellular cyclic AMP (cAMP) to activate cAMP-dependent protein kinase (PKA) for opening  $K_{ATP}$  channels (23).

As shown in Table II, we determined that forskolin-induced prostatic relaxation was also blocked by glibenclamide. The prostatic relaxation of forskolin was abolished by H-89 at a concentration sufficient to block PKA (23) and was enhanced by IBMX at a concentration sufficient to inhibit cAMP-phosphodiesterase (22). Similar results were also observed in prostate strips relaxed by loperamide (Table II). These data suggest that the possible mechanism for loperamide-induced prostatic relaxation is mediated through the cAMP-PKA pathway to open  $K_{ATP}$  channels, which can explain previous phenomenon for loperamide-induced prostatic relaxation (6). Therefore, the obtained results provide novel insights into the action mechanisms of loperamide particularly in the understanding of prostatic relaxation.

In conclusion, we suggest that activation of opioid  $\mu$ -2 receptors to open  $K_{ATP}$  channels is responsible for loperamide-induced prostatic relaxation. Therefore, activation of peripheral opioid  $\mu$ -2 receptors may be a new target in the development of agents for the management of benign prostatic hyperplasia.

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