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INHIBITORY EFFECT OF MITRAGYNINE, AN ALKALOID WITH ANALGESIC EFFECT FROM THAI MEDICINAL PLANT *Mitragyna speciosa*, ON ELECTRICALLY STIMULATED CONTRACTION OF ISOLATED GUINEA-PIG ILEUM THROUGH THE OPIOID RECEPTOR

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Summary

Effect of mitragynine, an indole alkaloid isolated from Thai medicinal plant kratom (*Mitragyna speciosa*), on electrically stimulated contraction was studied in the guinea-pig ileum. Mitragynine (1 nM - 3 μM) inhibited the ileum contraction elicited by electrical stimulation, and its pD_2 value was 6.91 ± 0.04 (n = 5). Morphine (1 nM - 1 μM) also inhibited the electrically stimulated contraction in a concentration-dependent manner (pD_2 7.68 ± 0.11; n = 5). Mitragynine was 10 fold less potent than morphine. Mitragynine (3 - 10 μM) did not show any effect on the smooth muscle contraction induced by acetylcholine or histamine. Naloxone (10 - 300 nM) reversed the inhibitory effect of mitragynine on electrically stimulated contraction. Furthermore, naloxone showed a shift of concentration-response curve of mitragynine to the right. There was no significant difference in the affinity of naloxone (i.e. pA_2) in the presence of mitragynine or morphine. Mitragynine (3 - 10 μM) inhibited the naloxone-precipitated withdrawal contraction following a brief (5 min) exposure of the ileum to morphine. Tetrodotoxin (1 μM) and atropine (1 μM) inhibited the withdrawal contraction. The present results suggest that mitragynine inhibits the electrically stimulated contraction of guinea-pig ileum through the opioid receptor.

Key Words: **mitragynine, guinea pig ileum, electrical stimulation, opioid receptor, morphine, naloxone, withdrawal**

Mitragyna speciosa (Thai name "Kratom") is a tree indigenous to the southeastern Asia. Owing to its opioid properties, the leaves of kratom had been previously used most commonly in Thailand (1, 2, 3). There are some descriptions about its use as a kind of tonic, cure for fever, treatment for diarrhea and substitution for morphine in treating addicts (2, 4, 5), but its use is now prohibited in Thailand. Kratom has been reported to be a central nervous system stimulant rather than depressant, which is seemingly contradictory (6). Some resemblances to cocaine such as increase in work efficiency and tolerance to hard work under a scorching sun are also described (2, 6).

Mitragynine (MG) is the main component of the leaves of Kratom (3). The chemical structure of MG contains indoloquinolizidine moiety with methoxy group at C-9 position (Fig. 1). MG is comparable with codeine as an analgesic and an antitussive drugs in dog. Unlike codeine at equivalent doses, it did not cause emesis or dyspnoea (4). No opiate-like syndrome or antagonism by nalorphine was observed. A negligible anti-cholinergic action and a minimal effect on gastric motility were observed (4).

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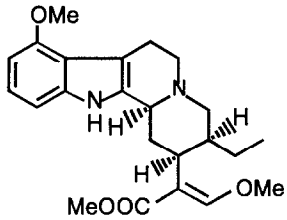

Fig.1
Chemical structure of mitragynine

We have also reported that MG showed similar potency to aminopyrine and it was 5 fold weaker than morphine in analgesiometric tests in mice (PBQ writhing test and hot plate test). We have studied pharmacological characteristics of indoloquinolizidine-related natural compounds such as hirsutine and yohimbine with various smooth muscle preparations (7, 8, 9, 10, 11). We studied the effect of MG on smooth muscle, and found that MG inhibited guinea-pig ileum and vas deferens contraction by inhibiting the neurotransmitter release from the nerve endings partly through the blockade of the neuronal Ca^{2+} channel (12).

A good correlation between the opioid agonistic potencies in the guinea-pig ileum and their potencies for human analgesia has been established in a large number of opioid analgesic drugs (13). Moreover, the enteric nervous structures contain types of opiate receptor most of which are very similar, if not identical, to those found centrally. The purpose of our present study was to examine the involvement of opioid receptor in the pharmacological actions of MG in *in vitro* experiments.

Effect of MG on naloxone-precipitated withdrawal contraction was also studied to evaluate its action on opiate dependence. Naloxone elicited withdrawal contraction in the ileum after a 5 min exposure of the tissue to morphine (14, 15, 16, 17). The mechanism underlying the development of acute morphine dependence *in vivo* has been assumed to be similar to that observed *in vitro* (14, 18).

Materials and Methods

Ileum preparation: Male, albino guinea-pigs (Dunkin-Hartley) weighing 300 to 400 g were stunned by a blow on the head and exsanguinated. The ileum was removed to Krebs-Henseleit solution of the following composition (mM): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00 and glucose, 11.49. Ileum was set up under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37°C and continuously bubbled with a gas mixture composed of 95% O_2 and 5% CO_2. At the start of each experiment a maximum response to acetylcholine (ACh, 3 μM) was obtained in each tissue to check its suitability.

Electrical transmural stimulation: The preparation was successively stimulated through the platinum ring-rod electrodes using square wave pulses of supra maximal voltage, 0.1 ms duration, delivered once every 5 s from a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Tension changes were isotonically recorded by using a displacement transducer (NEC, San-ei Instruments Ltd., Type 45347, Tokyo, Japan), DC strain amplifier (San-ei 6M92, Tokyo, Japan) and a DC recorder (Hitachi, Mod 056, Tokyo, Japan).

Concentration-response curve: All concentration-response curves were constructed in a cumulative manner. Then, the tissue was washed thoroughly and allowed to equilibrate for 1 h. The second response curve to the same agonist in the presence of the samples was constructed. Because the concentration-response curve for MG is unstable at the second challenge in the same tissue, the MG concentration-response curves in the presence of different concentration of naloxone were obtained from another ileum segments of the same animal.

Withdrawal contraction: The withdrawal contraction was induced by the administration of naloxone (1 μM) to the ileum after a 5 min incubation with morphine (500 nM). MG was

administered 3 min before addition of morphine.

Data analysis of concentration-response curve: The data are expressed as the mean ± S.E.M. and the agonist IC_{50} is shown as pD_2. In experiments with MG and morphine, concentration-response curves were constructed in the absence and presence of naloxone. Naloxone was applied 10 min before cumulative agonist administration. The twitch contraction amplitude elicited by transmural stimulation in the presence of naloxone was used as control value. Since naloxone shifted the concentration-response curves rightward, Schild plots were made. pA_2 values and slopes of the linear regression were calculated. The differences between the means of two groups were estimated using the paired t-test.

Drugs: Chemicals were obtained from the following sources: morphine hydrochloride (Takeda Chemical Industries Japan); naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-Enkephalin (DAMGO, Sigma Chemical Co., St. Louis, MO, USA); acetylcholine chloride (Dai-ichi Seiyaku Co., Ltd., Tokyo, Japan); clonidine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan); yohimbine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan);tetrodotoxin (Sankyo, Tokyo, Japan); atropine sulfate (Nacalai Tesque, Kyoto, Japan) and hexamethonium dichloride (Wako Pure Chemical Industries, Osaka, Japan). Nicardipine hydrochloride was kindly given by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). MG was isolated from extract of leaves of *Mitragyna speciosa* as described previously (19). MG was dissolved in 50% dimethyl sulfoxide and other drugs were dissolved in distilled water. The solvent itself had no effect.

Results

Response to MG on electrical stimulation-induced contraction

The effects of MG and morphine on contraction evoked by a single pulse electrical transmural stimulation were studied in the guinea-pig ileum. The mean amplitude of ileum contraction evoked by electrical stimulation was about 20% of the maximal response to ACh (3 μM). This contraction was abolished by tetrodotoxin (1 μM) and atropine (100 nM). However, hexamethonium (100 μM) did not affect the contraction (6 ± 3% inhibition).

As illustrated in Fig. 2, MG and morphine inhibited the electrically stimulated contraction in a concentration-dependent manner and their pD_2 values were 6.91 ± 0.04 and 7.68 ± 0.11, respectively.

Responses to acetylcholine and histamine

As shown in Fig. 3, MG (25 μM) significantly affected the concentration-response curves for acetylcholine (ACh) and histamine (His), but these effects of MG on smooth muscle were negligible.

Effect of naloxone on MG-decreased neurogenic twitch contraction

To investigate the involvement of opioid-receptor in the inhibitory effect of MG, the effect of naloxone on the decreased contraction was examined (Fig. 4). Naloxone (10 - 300 nM) reversed the inhibitory effect of MG on electrically stimulated twitch contraction in a concentration-dependent manner. The same effect of naloxone was obtained in the experiment with morphine.

On the other hand, naloxone did not show any effect on the inhibition induced by clonidine, an α_2-adrenoceptor agonist, or nicardipine, an L-type Ca^{2+} channel antagonist (Fig. 5). Yohimbine, an α_2-antagonist, inhibited the clonidine effect (data not shown).

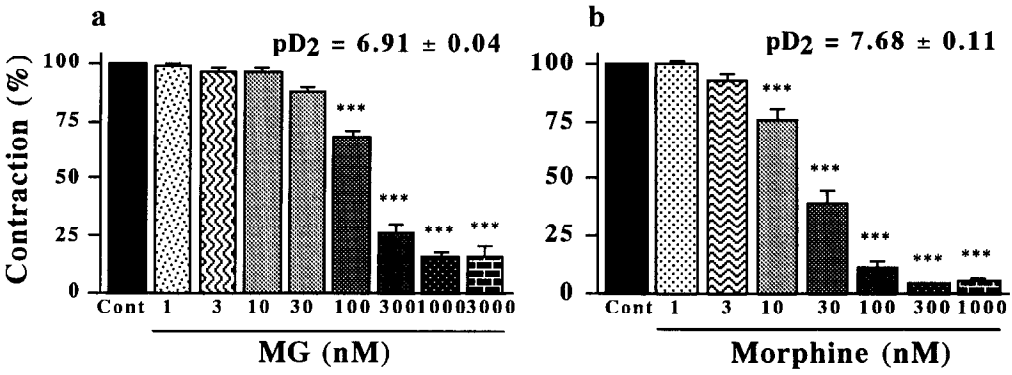

Fig. 2

(a) Effects of mitragynine (MG) and (b) morphine on transmural stimulation-induced contraction in the guinea-pig ileum. The solid columns represent the control group (Cont). The increasing doses of MG and morphine are shown below the columns. Results are expressed as contraction percentage of the twitch contraction before agonist addition. Each column represents the mean ± S.E.M of the values obtained from 4-7 animals. *** $P < 0.001$, significantly different from the Cont.

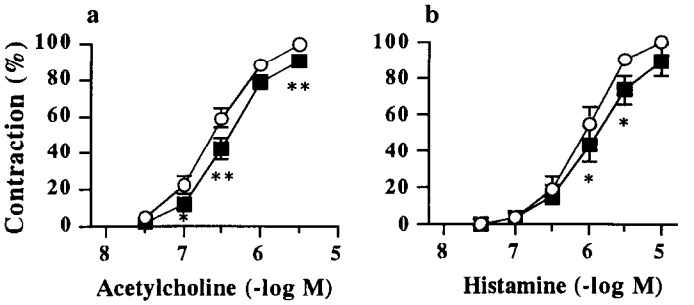

Fig. 3

Concentration-response curves for (a) acetylcholine (ACh) and (b) histamine (His) in the absence (O) or presence of MG (25 μM, ■) in the guinea-pig ileum. Results are expressed as percentage of maximum response to ACh or His. Values are means ± S.E.M. (n = 5). * $P < 0.05$ and ** $P < 0.01$, significantly different from the control.

As shown in Fig. 6, naloxone (3 - 30 nM) shifted the concentration-response curves for MG and morphine to the right. pA_2 and 95% confidence limits of naloxone was 8.77 ± 0.04 (n = 3 - 6) for MG and 9.00 ± 0.04 (n = 4) for morphine. The statistical significance between two pA_2 values was not observed. The slope factors were not significantly different from the unity (0.93 ± 0.29 for MG and 1.72 ± 0.28 for morphine).

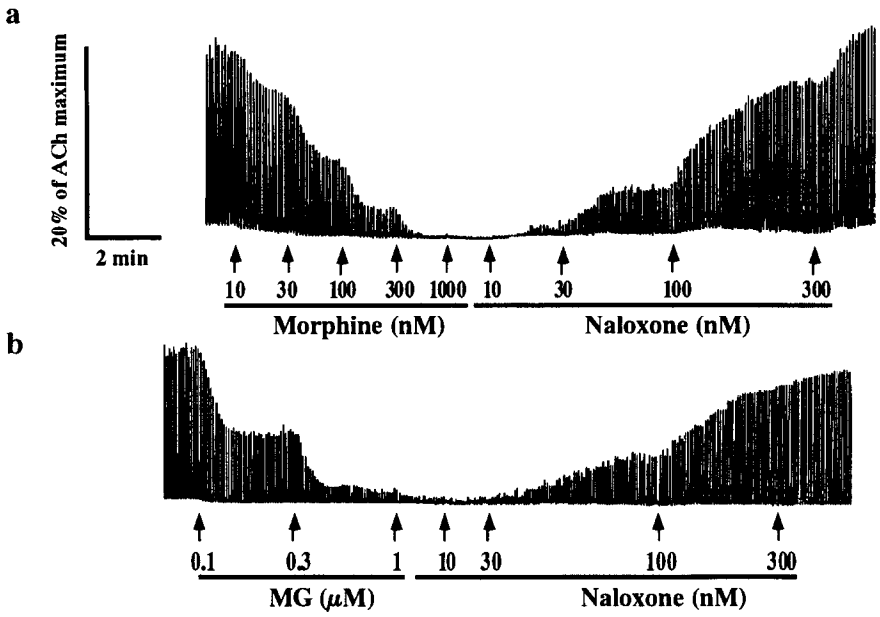

Fig. 4

Typical tracings of effects of naloxone on relaxant responses to (a) morphine or (b) mitragynine (MG) in the guinea-pig ileum contracted by electrical transmural stimulation.

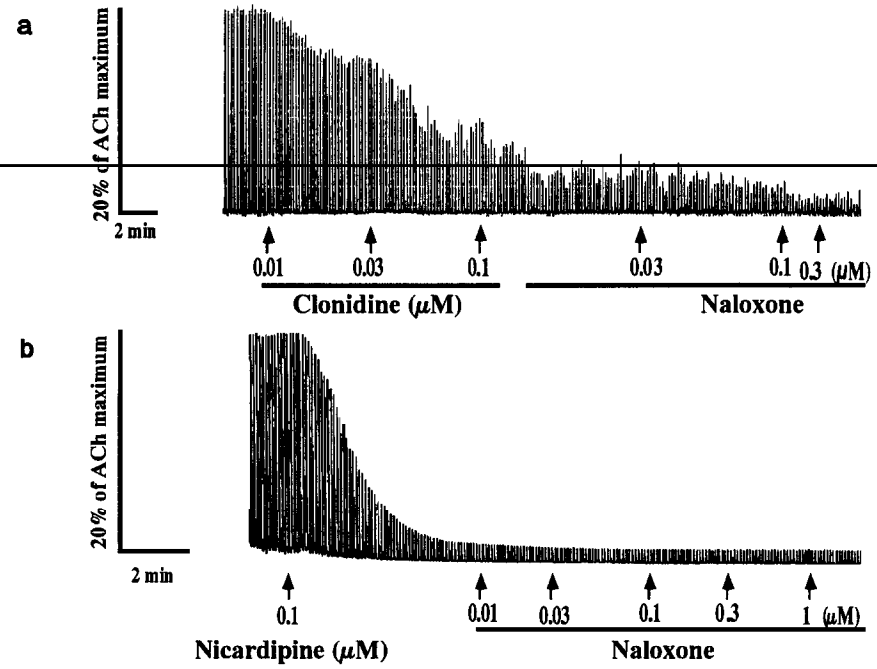

Fig. 5

Typical tracings of effects of naloxone on relaxant responses to (a) clonidine or (b) nicardipine in the guinea-pig ileum contracted by electrical transmural stimulation.

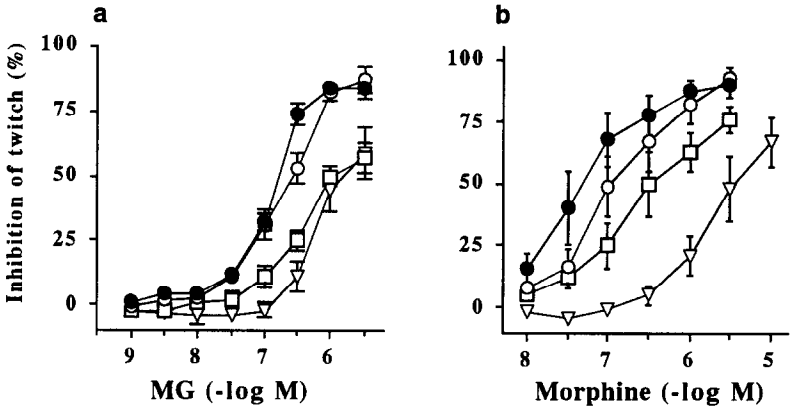

Fig. 6
Concentration-response curves for (a) MG and (b) morphine in the electrically stimulated guinea-pig ileum in the absence (●) or the presence of naloxone (3 nM, ○; 10 nM, □; and 30 nM, ▽). Responses are expressed as inhibition percentage of the twitch contractions before agonist addition. Values are means ± S.E.M. (n = 4).

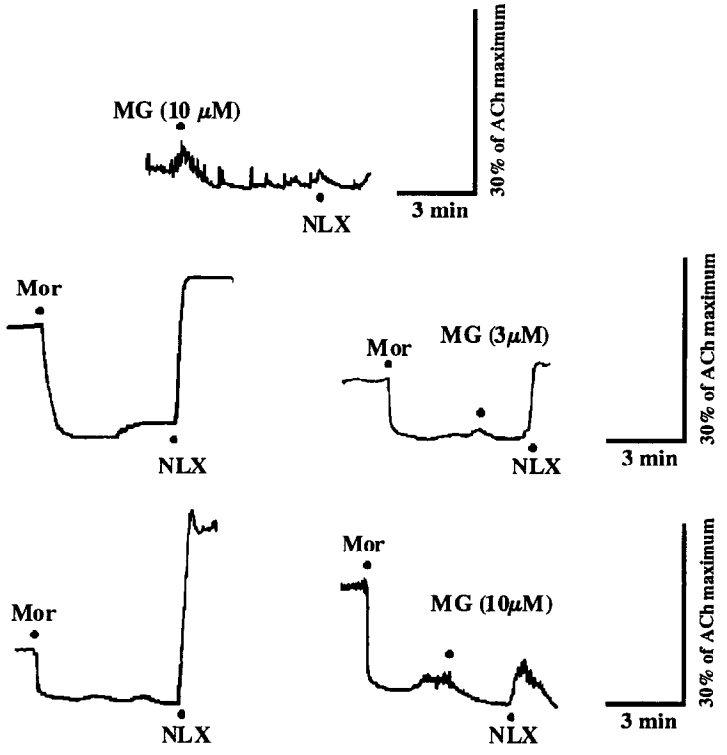

Fig. 7
Typical tracings of effect of mitragynine (MG) on naloxone (NLX, 1 μM)-precipitated withdrawal response after a 5 min exposure of guinea-pig ileum to morphine (Mor, 500 nM).

Effect of MG on withdrawal contraction after a brief exposure to morphine

MG (3 - 10 μM) inhibited the naloxone-induced withdrawal contraction of the ileum in a concentration-dependent manner (Fig. 7, 8). The withdrawal contraction was also abolished by tetrodotoxin (1 μM) and atropine (1 μM) while hexamethonium (100 μM) showed a slight inhibition. Naloxone alone did not show any effect in the guinea-pig ileum and it did not show any effect when morphine was replaced by MG 10 μM. The same results were obtained with tissue exposed to MG or morphine for 1 hr before naloxone administration, as seen with the tissue exposed for 5 min. Furthermore, the addition of morphine or DAMGO 1 μM in the tissue exposed to 500 nM morphine (1 μM) for 5 min or 1 hr did not elicit any change on naloxone-induced withdrawal response (data not shown).

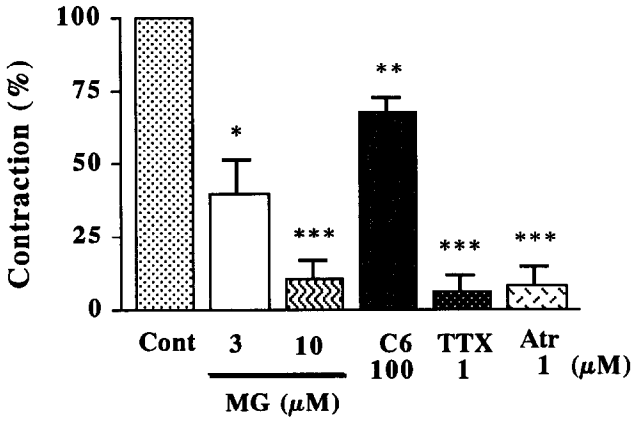

Fig. 8

Effects of mitragynine (MG), hexamethonium (C6), tetrodotoxin (TTX) and atropine (Atr) on naloxone (1 μM)-precipitated withdrawal response after a 5 min exposure of guinea-pig ileum to morphine (Mor, 500 nM). Results are expressed as percentage of maximum response to naloxone-precipitated withdrawal contraction The first column represents the control (Cont). Each column represents the mean ± S.E.M. of the values obtained from 4 animals. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$, significantly different from the Cont.

Discussion

The leaves of kratom have been used by the natives of Thai for decades because of its opioid and stimulant properties. We and others reported that MG had analgesic effect in animal experiments. In spite of its strong analgesic effect, a morphine-like character had not been confirmed in the previous reports.

Electrical stimulation elicits ileum contraction by the release of neurotransmitters, such as ACh and substance P, from the nerve endings (20). In the guinea-pig ileum, the agonists acting on α_2-adrenoceptors or μ-opioid receptors inhibit ACh release and nerve-mediated cholinergic contractions by a prejunctional action (21,22). In the present study, the electrical stimulation-induced twitch contraction was abolished by tetrodotoxin and atropine, but hexamethonium had no effect. These results show that electrical stimulation-induced contraction was elicited by ACh released from post-ganglionic cholinergic neurons. Like morphine, MG inhibits the electrically stimulated ileum contraction in a concentration-dependent manner. On the other hand, MG hardly affected the contraction induced by direct stimulation of the receptor on the smooth muscle by ACh or His. Taken together, it is supposed that MG acts on the nerve endings to inhibit the release of neurotransmitters.

The involvement of opioid receptors in the effect of MG was suggested by the reversal action of naloxone. On the other hand, the rightward shift of concentration-response curve for MG in the presence of naloxone confirms the opioid-like effect of MG. Moreover, the lack of significant difference between pA_2 values for naloxone with MG and morphine as agonists suggests MG inhibits the electrically stimulated ileum contraction through the opioid receptor. When effects of MG and morphine were compared, their potency ratio of inhibitory effect on ileum contraction was comparable to that shown with analgesic activity in which MG was 6 fold less potent than that of morphine.

Activation of opioid receptor (23) as well as α_2-adrenoceptor (24, 25, 26) modulates the neurotransmitter release and consequently the ileum contractility. The present study shows that the opioid agonist morphine and α_2-adrenoceptor agonist clonidine inhibit the electrically stimulated ileum contraction, and that their inhibiting effects are antagonized by their respective antagonists, naloxone and yohimbine. Because naloxone did not show any effect on inhibition induced by clonidine or the Ca^{2+} channel antagonist nicardipine, the naloxone effects were specific to opioid receptors in the ileum. Thus, the action site of MG seems to be different from those of clonidine and nicardipine, at least, in this tissue.

Opiate-dependency can be accomplished in the guinea-pig ileum by a brief incubation with morphine. In this tissue, contraction is observed as washout- or naloxone-induced withdrawal response. The mechanism underlying the development of acute morphine dependence *in vivo* may be similar to that observed *in vitro* (14, 27). In the guinea-pig ileum, the withdrawal response is associated with morphine tolerance that is elicited by hyperpolarization of the myenteric plexus followed by the decline of the membrane potential to the preopioid level as a compensatory reaction. This induces supersensitivity within the myenteric plexus and the subsequent administration of naloxone elicits depolarization which leads to the generation of action potentials (28) and the release of ACh and substance P from cholinergic neurons. In our study, the inhibition of withdrawal contraction by atropine and tetrodotoxin and the small effect of hexamethonium indicates the involvement of post-ganglionic cholinergic neurons and ACh in the withdrawal response.

A difference in pharmacological action between MG and morphine was observed in the ileum, considering that 10 μM of MG is a high concentration enough to inhibit electrically induced ileum contraction, and is of equivalent potency to 1 μM of morphine. However, naloxone did not induce withdrawal response in the ileum exposed to MG at 10 μM. On the other hand, naloxone induced withdrawal response in the ileum exposed to morphine at 1 μM. In addition, a 5 min or a 1 hr exposure of the tissue to MG or morphine showed the similar withdrawal response. Cruz et al (29) pointed out that high concentration of morphine induces an oversaturation at the opioid receptors level, which decreases the sensitivity to the withdrawal response to opiate antagonists This suggests MG would inhibit withdrawal response by the decrease in sensitivity of opioid receptor by the opioid effect itself. However, under our experimental condition, an oversaturation of opiate receptor by addition of high concentration of morphine or DAMGO, μ-opioid receptor selective agonist, in the ileum exposed to morphine did not elicit any change on naloxone-induced withdrawal response. Therefore, the difference of MG and morphine in the withdrawal response seems to result not from the differences of their potencies, durations or opioid receptor oversaturation, but from the differences of the pharmacological character of their effects. Because MG at the concentration that inhibited the withdrawal response did not show a significant effect on ACh-induced contraction in the ileum, it is likely that MG inhibits withdrawal response of ileum through neuronal mechanism. Furthermore, MG is found to block L- and T-type Ca^{2+} channel current in N1E-115 neuroblastoma cells, by using the patch clamp method (12). We speculate that MG inhibits naloxone-induced withdrawal response in the ileum by blocking the neuronal Ca^{2+} channel. Ca^{2+} channel antagonists have been found to increase the antinociceptive effects of morphine and decrease the development of opiate tolerance in animals (30, 31, 32). The present results may explain in part the traditional use of kratom in Thailand as a suppressor of opiate withdrawal syndrome, although a more detailed investigation of MG effects on opioid-withdrawal response in vivo is needed.

It is well-established that opioid receptors in peripheral tissues and in the central nervous system can be classified into at least three different types, namely μ-, δ- and κ- receptors (33, 34). The guinea-pig ileum contains functional populations of both μ- and κ-receptors (35, 36,37). The present

study did not include a detailed study of the subtypes of opioid receptors in the isolated ileum, thus further work including radioligand binding assay is also necessary to determine the selectivity of MG for the opioid receptor.

In conclusion, the present study shows that MG inhibits the nerve-stimulation-elicited twitch contraction through the opioid receptor in the isolated guinea-pig ileum. MG also inhibits naloxone-precipitated withdrawal response. MG seems to have two properties: an opioid-like effect and apparently an inhibitory effect on the withdrawal response.

Acknowledgements

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