

Opioid receptor agonistic characteristics of mitragynine pseudoindoxyl in comparison with mitragynine derived from Thai medicinal plant *Mitragyna speciosa*

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Abstract

We have previously elucidated the opiate-like action of mitragynine, an active principle isolated from the Thai medicinal plant *Mitragyna speciosa*. In the present study, effects of the related compound, mitragynine pseudoindoxyl on electrically stimulated contraction in guinea pig ileum and mouse vas deferens, and on its binding affinity in the guinea pig brain membranes were studied. Mitragynine pseudoindoxyl inhibited the electrically stimulated ileum and mouse vas deferens contractions in a concentration-dependent manner. In the ileum, the effective concentration is in an nM order, being nearly equivalent to reported concentrations of the μ -opioid receptor agonist [D-Ala², Met-Phe⁴, Gly-ol⁵] enkephalin (DAMGO), and is 100- and 20-fold smaller than those of mitragynine and morphine, respectively. In the vas deferens, it is 35-fold smaller than that of morphine. The inhibitory action of mitragynine pseudoindoxyl in the ileum was antagonized by the non-selective opioid receptor antagonist naloxone and the μ -receptor antagonist naloxonazine. It was also antagonized by the δ -receptor antagonist naltrindole in the vas deferens. Mitragynine pseudoindoxyl showed a similar binding affinity to DAMGO and naltrindole at μ - and δ -receptors, respectively. However, the affinity at κ -receptors was negligible. The present study demonstrates that mitragynine pseudoindoxyl, a novel alkaloid structurally different from other opioid agonists, acts on opioid receptors, leading to a potent inhibition of electrically stimulated contraction in the ileum through the μ -receptors and in mouse vas deferens through δ -receptors. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Guinea pig ileum; Mitragynine; Mitragynine pseudoindoxyl; Morphine; Mouse vas deferens; Opioid receptor

Mitragynine pseudoindoxyl is an alkaloid synthesized from mitragynine, which is the principal indole alkaloid isolated from the leaves of Thai folk medicine *Mitragyna speciosa* Korth (*kratom* in Thai language). The leaf of kratom had been used in Thailand because of its opium-like action (Burkill, 1935), and for its coca-like stimulant action for combating fatigue or enhancing tolerance to hard work under a scorching sun (Grewal, 1932; Suwanlert, 1975). Additionally, it had been used

to treat diarrhea and to replace morphine in treating addicts (Suwanlert, 1975; Jansen and Prast, 1988).

We have so far studied the pharmacological characteristics of indole alkaloids such as hirsutine (Yano et al., 1991; Horie et al., 1992) and yohimbine (Watanabe et al., 1987; Horiuchi et al., 1988; Ito et al., 1990) with various smooth muscle preparations. In the course of these studies, we found that mitragynine inhibits electrically elicited guinea pig ileum contraction through the opioid receptors, and that mitragynine inhibits morphine withdrawal response in the ileum (Watanabe et al., 1997).

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Some pharmacological investigations with mitragynine in the *in vivo* experiments have shown that mitragynine exerts antinociceptive actions in rodents (Macko et al., 1972; Watanabe et al., 1992; Matsumoto et al., 1996a, 1996b) and antitussive action in dogs (Macko et al., 1972). Mitragynine is comparable with codeine as an analgesic and an antitussive drug. It has been clarified that mitragynine shows antinociceptive action through the opioid receptors, and that the action is dominantly mediated by μ - and δ -receptor subtypes (Matsumoto et al., 1996a; Tohda et al., 1997; Thongpradichote et al., 1998).

Zarembo et al. (1974) subjected mitragynine to biotransformation by the fungus *Helminthosporium sp.* and isolated mitragynine pseudoindoxyl as a metabolite (Fig. 1). Although no concrete pharmacological data about mitragynine pseudoindoxyl is available, its antinociceptive potency is described to be 10-fold greater than that of mitragynine (Zarembo et al., 1974).

In the present study, we attempted to elucidate the involvement of opioid receptors in its putative analgesic effect in the *in vitro* experiments. We studied the effect of mitragynine pseudoindoxyl on electrically elicited contraction of the guinea pig ileum and mouse vas deferens. The former preparation predominantly contains functional μ - and κ - (Lord et al., 1977; Chavkin and Goldstein, 1981), and the latter preparation contains δ -receptor population (Lord et al., 1977), respectively. In addition, we studied binding affinity and selectivity of mitragynine pseudoindoxyl for specific opioid receptors such as μ -, δ -, and κ -receptors.

1. Materials and methods

Male, albino Dunkin-Hartley guinea pigs (300–400 g) purchased from Takasugi Lab. Animals Co. Ltd. (Saitama, Japan) and male ddY mice (30–37 g) from Japan SLC Inc. (Shizuoka, Japan) were used. The segments of guinea pig ileum were removed in Krebs-Henseleit solution (Cox and Weinstock, 1966). A whole

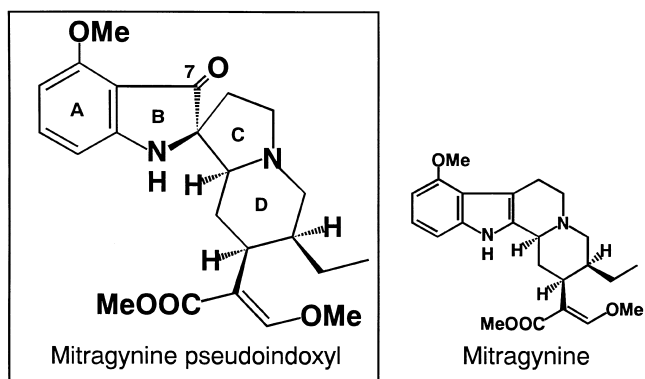


Fig. 1. Chemical structures of mitragynine and mitragynine pseudoindoxyl.

brain (excluding cerebellum) was quickly removed and placed in ice-cold 50 mM Tris HCl buffer, pH 7.4 at 25°C, weighed, and immediately frozen in dry ice containing acetone. Frozen brains were stored at -70°C until the assay. Mouse vas deferens was removed and placed in the modified Krebs-Henseleit solution. The nutrient solution was prepared by eliminating MgSO_4 from the Krebs-Henseleit solution (Cohen et al., 1994).

1.1. Isolated tissue preparations

Male guinea pigs were stunned by a blow on the head and exsanguinated. The ileum was removed and placed in Krebs-Henseleit solution (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. The 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 of 95% O_2 and 5% CO_2 .

Male mice were sacrificed by cervical dislocation. Tissues were dissected free of connective tissue and suspended in modified Krebs-Henseleit solution. The nutrient solution was prepared by eliminating MgSO_4 from the Krebs-Henseleit solution. Segments of the vas deferens were set up longitudinally in a 5-ml organ bath containing the nutrient solution. The bath was maintained at 37°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 .

Tissues were stimulated by platinum needle-ring (a ring was placed 20 mm above the base of the needle 5 mm in length) electrodes using square wave pulses of supra maximal voltage. The ileum was transmurally stimulated (Cox and Weinstock, 1966) with mono-phasic pulses (0.2 Hz) and 0.1 ms duration; and vas deferens was transmurally stimulated with train of 10 pulses (10 Hz), 1.5 ms duration every 30 sec by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isotonic and isometrically recorded in the ileum and in the vas deferens, respectively, by using a displacement transducer (NEC, San-ei Instruments Ltd., Type 45347, Tokyo, Japan).

Concentration–response curves for the inhibition of electrically elicited contractions were constructed in a cumulative manner. pD_2 and pA_2 values were determined as previously described (Watanabe et al., 1997).

Antagonistic action of naloxonazine on inhibition of the twitch contraction by mitragynine pseudoindoxyl (100 nM), mitragynine (3 μM) or morphine (3 μM) were determined as reversal (%) by the following equation: $\text{Reversal (\%)} = (\text{Naloxonazine})/(\text{Agonist}) \times 100$; (Naloxonazine) means the restoration of the twitch contraction by naloxonazine in mm; (Agonist) means the inhibition of the twitch contraction elicited by agonists in mm. Moreover, the specificity of naloxone action on opioid receptors in the ileum was evaluated by studying the effect of naloxone on inhibition of twitch

contraction induced by clonidine (100 nM) and nicaldipine (100 nM).

1.2. Receptor binding assay

For each experiment, frozen brains from two animals were thawed and homogenized with a homogenizer (Kinematica GmbH LITTAU, Polytron, PT 10-35, Switzerland) for 60 sec in 40 vol of 50 mM Tris HCl (pH 7.4) and centrifuged at $49,000 \times g$ for 10 min (Pasternak and Snyder, 1975; Childers et al., 1979). The pellet was re-homogenized and centrifuged again. For the binding assays, membrane fractions were suspended in assay buffer at a protein concentration of 50 mg/ml. Protein was measured by the method of Gornali et al. (1949).

Saturation binding isotherms were produced by incubating each labeled compound at nine or ten different concentrations (10 pM–2 nM) with 2 mg of membrane protein. To start the reaction, 0.1-ml aliquots of protein were added to 0.9 ml of 50 mM Tris-HCl (pH 7.4) assay buffer containing 1 nM of [^3H]DAMGO, [^3H]DPDPE, or [^3H]U-69593 and appropriate concentrations of competing unlabeled ligands in a total volume of 1 ml. The incubated periods were 1.5, 2, and 1 hr for [^3H]DAMGO, [^3H]DPDPE, and [^3H]U-69593, respectively at 25°C. The reaction was terminated by rapid filtration under reduced pressure through glass fiber filters (Whatman GF/B, presoaked in Tris-HCl buffer) followed by the addition of 4 ml ice-cold Tris-HCl buffer. Filters were further washed with 4 ml ice-cold buffer and left to dry for 12 hr. Radioactivity bound to the filters was quantitated by liquid scintillation spectrometry (ALOKA LSC-5100, Japan). Nonspecific binding for [^3H]DAMGO, [^3H]DPDPE, and [^3H]U-69593 was determined in the presence of 1 μM unlabeled DAMGO, naltrindole, and U-69593, respectively. All values were presented as the mean \pm SEM.

The apparent dissociation constant (K_D) and maximum binding site density (B_{max}) for radioligands were estimated by Scatchard analysis of the saturation data over a concentration range of 10 pM to 2 nM. The ability of unlabeled drugs to inhibit specific radioligand binding was expressed as the IC_{50} value, which was the molar concentration of unlabeled drug necessary to displace 50% of the specific binding. Competitive inhibition studies were carried out in the presence of six to nine different inhibitor concentrations.

1.3. Data analysis

Results are expressed as a mean \pm SEM. The difference between the means of two groups was estimated by Student's *t*-test.

1.4. Drugs

Chemicals were obtained from the following sources: acetylcholine chloride (Dai-ichi Pharmaceutical Co., Ltd., Tokyo, Japan), clonidine hydrochloride, yohimbine hydrochloride, hexamethonium dichloride (Wako

Pure Chemical Industries, Osaka, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), atropine sulfate (Nacal Tesque Inc., Tokyo, Japan), [D-Ala², Met-Phe⁴, Gly-ol⁵] enkephalin (DAMGO), naltrindole hydrochloride, U-69593, naloxone hydrochloride, (Sigma Chemical Co., St. Louis, MO), naloxonazine hydrochloride (BIOMOL Res. Labs. Inc., Plymouth Meeting, PA), [D-Pen^{2,5}] enkephalin (DPDPE) (BACHEM Feinchemikalien, Switzerland), morphine hydrochloride (Takeda Chemical Industries, Japan), [^3H]DAMGO, [^3H]DPDPE, [^3H]U-69593 (Du Pont NEN Life Sci. Prods, Boston, MA), nicaldipine hydrochloride kindly given by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Mitragynine was isolated from extract of leaves of *M. speciosa* and mitragynine pseudoindoxyl was synthesized as described previously (Ponglux et al., 1994; Takayama et al., 1996). Mitragynine and mitragynine pseudoindoxyl were at first dissolved in 50% and 75% dimethyl sulfoxide, respectively, to yield 1 mM solution, and then subsequent dilution of both compounds were made with distilled water or 50 mM Tris HCl for bioassay and binding assay, respectively. Other drugs were dissolved in distilled water.

2. Results

2.1. Effect of mitragynine pseudoindoxyl on electrical stimulation-induced contraction

The inhibitory effect of mitragynine pseudoindoxyl on contraction evoked by single pulse electrical stimulation in the guinea pig ileum is shown in Fig. 2. The mean amplitude of electrical contraction was about 20% of the maximal response to acetylcholine (3 μM). The solvent (dimethyl sulfoxide 0.1%) used in the experiment did not affect the contraction ($0.3 \pm 1.6\%$ inhibition, $n = 3$).

Mitragynine pseudoindoxyl inhibited the electrically stimulated ileum contraction in a concentration-dependent manner as did mitragynine and morphine (Fig. 3) and their pD_2 values were 8.96 ± 0.09 , 6.92 ± 0.05 , and 7.67 ± 0.06 , respectively (Table 1)(see Arunlakshana and Schild, 1959). Mitragynine pseudoindoxyl potency was about 100- and 20-fold greater than that of mitragynine and morphine, respectively. On the other hand, mitragynine pseudoindoxyl (3 μM) did not affect the concentration–response curve for acetylcholine in the ileum (data not shown). Moreover, the electrically stimulated ileum contraction was abolished by tetrodotoxin (1 μM) and atropine (100 nM) (data not shown). However, hexamethonium (100 μM) did not affect the contraction ($6 \pm 3\%$ inhibition, $n = 4$).

2.2. Reversal effect of naloxone and naloxonazine on mitragynine and mitragynine pseudoindoxyl decreased neurogenic twitch contraction

To investigate the involvement of opioid receptors in the inhibitory effect of mitragynine pseudoindoxyl, the

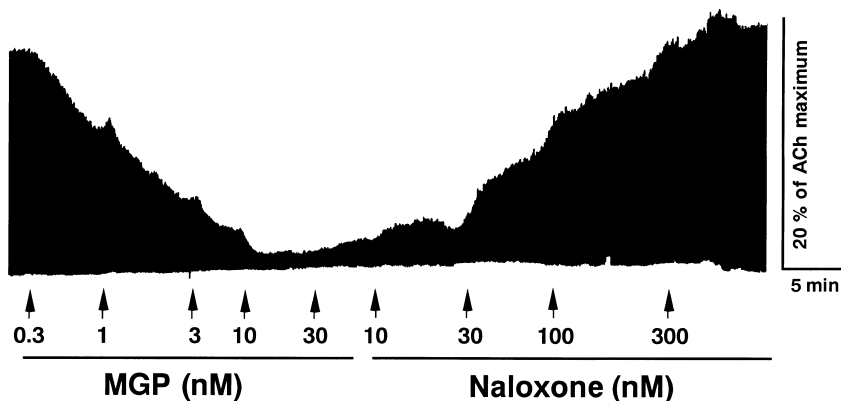


Fig. 2. Typical tracing showing effect of mitragynine pseudoindoxyl and successive naloxone on contraction induced by the transmural stimulation in the guinea pig ileum. Ordinate scale represents 20% of the maximum response to acetylcholine (3 μ M). Abscissa represents the time.

reversal effects of the non-selective opioid receptor antagonist naloxone and the μ -receptor antagonist naloxonazine on the decreased contraction were examined. Mitragynine pseudoindoxyl (30 nM) displayed a $77 \pm 3\%$ inhibition of the electrically stimulated ileum contraction 20 min after drug administration and this response was similar to that of morphine (1 μ M, $81 \pm 5\%$ inhibition). Naloxonazine (10–300 nM; Fig. 4) reversed the inhibitory effect of mitragynine pseudoindoxyl (100 nM) in a concentration-dependent manner. The similar observation was obtained by the addition of naloxone (Fig. 2). Naloxone and naloxonazine also restored the inhibition by morphine and mitragynine (Watanabe et al., 1997). On the other hand, naloxone (0.3 μ M) did not show any effect on the inhibition by clonidine, an α_2 -adrenoceptor agonist, or nicardipine, an L-type Ca^{2+} channel antagonist. Yohimbine, an α_2 antagonist, reversed the inhibition elicited by clonidine (data not shown). Naloxone (100 nM) increased the electrically

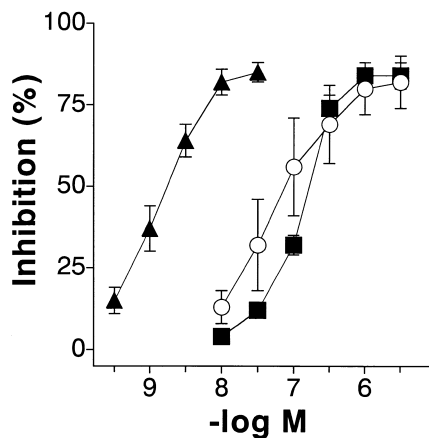


Fig. 3. Concentration–response curves for inhibitory effects of mitragynine pseudoindoxyl (▲), mitragynine (■), and morphine (○) on electrical stimulation-induced contraction in the guinea pig ileum. Responses are expressed as inhibition percentage of the transmurally stimulated contractions before the addition of agonist. Values are means \pm SEM. $n = 4$ –7.

stimulated ileum contraction ($50 \pm 7\%$, $n = 5$), but naloxonazine (3 μ M) itself had no effect on the contraction ($6 \pm 5\%$ contraction, $n = 3$). This means that endogenous opioid substances inhibit the electrically stimulated contraction through opioid receptors except μ -receptors.

Exposure of the ileum to naloxone (3–30 nM) shifted the concentration–response curves for mitragynine pseudoindoxyl to the right in a competitive manner. The pA_2 values of naloxone were 8.73 ± 0.15 for mitragynine pseudoindoxyl, 8.77 ± 0.12 for mitragynine, and 8.61 ± 0.15 for morphine, the differences being statistically insignificant. The slope factors were not significantly different from a unity (Table 1).

Fig. 5 shows that mitragynine pseudoindoxyl inhibited the electrically elicited mouse vas deferens contractions in a dose-dependent manner, as did [D-Pen^{2,5}] enkephalin (DPDPE), a δ -opioid receptor selective agonist (Mosberg et al., 1987) and morphine, a μ -agonist.

The concentration–response curves for mitragynine pseudoindoxyl were shifted to the right in the presence of naltrindole (1–3 nM), a δ -opioid receptor selective antagonist (Portoghese et al., 1988), giving an apparent pA_2 value of 9.93 ± 0.20 . This value was similar to that for DPDPE (Table 2).

Table 1

pD_2 values for mitragynine pseudoindoxyl (MGP), mitragynine (MG), and morphine on inhibiting electrically stimulated contraction in the guinea pig ileum, and pA_2 values of naloxone against MG, MGP, and morphine

Agonist	pD_2	pA_2	Slope
MGP	8.96 ± 0.09	8.73 ± 0.15	0.82 ± 0.13
MG	6.92 ± 0.05	8.77 ± 0.12	0.93 ± 0.13
Morphine	7.67 ± 0.06	8.61 ± 0.15	1.14 ± 0.20

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values were calculated from parallel shifts of the curves for the agonists MG, MGP, and morphine by the method of Arunlakshana and Schild (1959). Slope: slope of the Schild plot. Each value represents the mean \pm SEM, $n = 4$ –5.

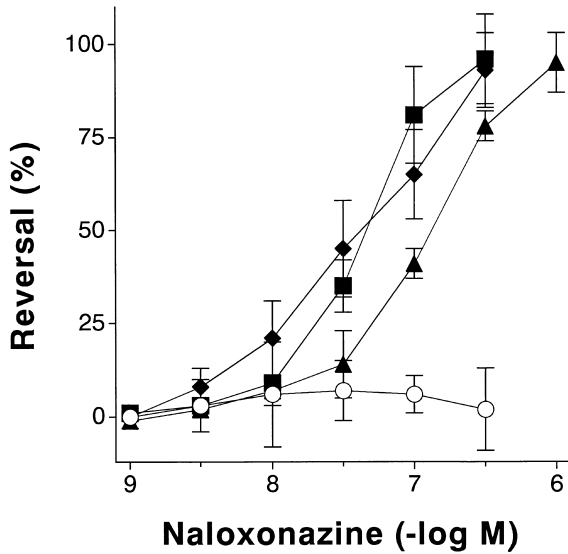


Fig. 4. Effect of naloxonazine on the transmurally stimulated ileum contraction previously inhibited by morphine (3 μ M, ■), mitragynine (3 μ M, ◆) and mitragynine pseudoindoxyl (100 nM, ▲). Responses are indicated as percent reversal. Results are means \pm SEM. $n = 3-5$. Effect of naloxonazine alone on the stimulated contraction is presented as the control group (○).

Naloxone (100 nM–1 μ M) also shifted the concentration–response curves of mitragynine pseudoindoxyl and DPDPE to the right. The concentration–response curve for morphine was also shifted to the right by naloxone (30–56 nM) or naltrindole (3–30 nM). These results are shown as pA_2 values in Table 2. On the other hand, the inhibitory effect of mitragynine on mouse vas deferens

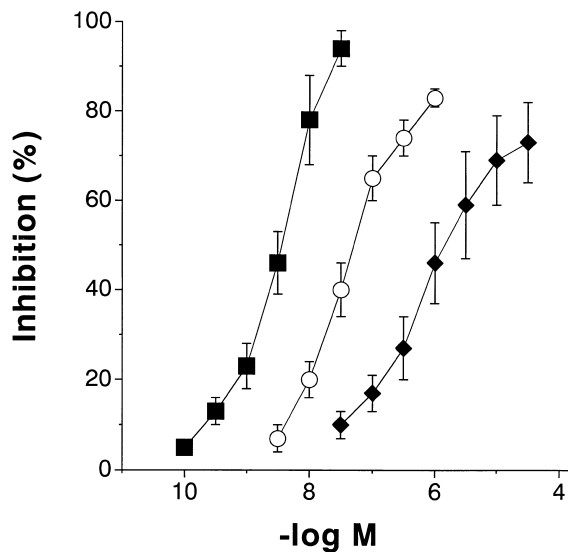


Fig. 5. Concentration–response curves of inhibitory effects of DPDPE (■), morphine (◆), and mitragynine pseudoindoxyl (○) on electrical stimulation-induced contraction in the mouse vas deferens contraction. Responses are expressed as inhibition percentage of the transmurally stimulated contractions before agonist addition. Values are means \pm SEM. $n = 5$.

Table 2

pD_2 values of mitragynine pseudoindoxyl (MGP), mitragynine, DPDPE, and morphine on inhibiting electrically stimulated contractions in the mouse vas deferens, and pA_2 values of naltrindole and naloxone against MGP, DPDPE, and morphine

Agonist	pD_2	pA_2 (naltrindole)	pA_2 (naloxone)
MGP	7.40 \pm 0.11	9.93 \pm 0.20	7.02 \pm 0.42
MG	4.57 \pm 0.14	<6	<6
DPDPE	8.53 \pm 0.16	9.48 \pm 0.16	7.19 \pm 0.11
Morphine	5.85 \pm 0.08	7.74 \pm 0.20	8.14 \pm 0.15

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values were calculated from parallel shifts of the curves for the agonists MGP, DPDPE, and morphine by the method of Arunlakshana and Schild (1959). Each value represents the mean \pm SEM, $n = 4-6$.

was not affected by the above antagonists, even at a high dose of 1 μ M (Table 2).

2.3. Effect of mitragynine pseudoindoxyl on opioid-receptor binding in brain homogenate

The effects of mitragynine pseudoindoxyl on three types of opioid receptors were determined by evaluating the inhibition of binding of specific ligands at μ -, δ -, and κ -opioid receptors in the guinea pig brain membranes. Specific bindings of these radioligands for the specific opioid receptors were saturable and Scatchard plots were linear. The K_D values of [3 H]DAMGO, [3 H]DPDPE, and [3 H]U-69593 were 0.44 \pm 0.04 nM, 0.83 \pm 0.05 nM, and 0.69 \pm 0.10 nM, respectively. Further, their B_{max} values were 2.90 \pm 0.59 fmol/mg protein, 2.24 \pm 0.21 fmol/mg protein, and 5.08 \pm 0.51 fmol/mg protein, respectively.

Table 3 shows a close similarity of pIC_{50} values of mitragynine pseudoindoxyl at μ -site to DAMGO. The order of potency of the unlabeled drugs for displacing [3 H]DAMGO was: mitragynine pseudoindoxyl \cong DAMGO > morphine. Mitragynine pseudoindoxyl displayed 4- and 40-fold greater potency than DAMGO and morphine, respectively.

At δ -opioid receptors, mitragynine pseudoindoxyl showed a similar potency to naltrindole in displacing [3 H]DPDPE binding. On the other hand, morphine displayed a similar pIC_{50} value to DAMGO. The order of potency of the unlabeled drugs for displacing [3 H]DPDPE was: naltrindole \cong mitragynine pseudoindoxyl > DAMGO \cong morphine.

All of the compounds examined for their affinity for κ -receptor displayed a relatively lower affinity than U-69593, a selective κ -receptor agonist (Lahti et al., 1985). The order of potency of the unlabeled drugs for displacing [3 H]U-69593 was: U-69593 > mitragynine pseudoindoxyl > morphine > DAMGO.

Mitragynine pseudoindoxyl showed a higher affinity for μ - and δ -receptors. On the other hand, morphine and DAMGO showed a high selectivity for μ -receptors over δ - and κ -receptors (Table 3).

Table 3

pIC₅₀ values of mitragynine pseudoindoxyl (MGP), morphine, [D-Ala², Met-Phe⁴, Gly-ol⁵] enkephalin (DAMGO), naltrindole, and U-69593 in displacing radioligands specific for opioid receptor types in guinea pig brain homogenates

	[³ H]DAMGO (μ-sites)	[³ H]DPDPE (δ-sites)	[³ H]U-69593 (κ-sites)	μ/δ	μ/κ
MGP	9.55 ± 0.39 (4)	8.18 ± 0.22 (5)	6.88 ± 0.32 (4)	23	467
Morphine	7.94 ± 0.29 (4)	6.04 ± 0.13 (5)	6.16 ± 0.17 (4)	80	60
DAMGO	8.90 ± 0.14 (5)	6.37 ± 0.26 (3)	4.87 (2)	339	10715
Naltrindole	ND	8.77 ± 0.20 (4)	ND		
U-69593	ND	ND	8.32 ± 0.10 (4)		

The pIC₅₀ is the molar concentration of unlabeled drug necessary to displace 50% of the specific binding. pIC₅₀, negative logarithm of the IC₅₀ value. Selectivity was calculated from the equations: μ/δ = IC₅₀ (delta)/IC₅₀ (mu); and μ/κ = IC₅₀ (kappa)/IC₅₀ (mu); ND.: not determined. Values are mean ± SEM. The numbers in parentheses are the number of separate experiments.

3. Discussion

The electrically stimulated ileal preparation from guinea pig and vas deferens from mouse, and receptor binding assay with guinea pig brain homogenates were used to study the mode of action of mitragynine pseudoindoxyl in their putative strong analgesic effects in mice.

At first, we studied the effect of mitragynine pseudoindoxyl on electrical stimulation-induced contraction in guinea pig ileum. The contraction was abolished by tetrodotoxin and atropine, but not by hexamethonium. Therefore, the ileum contraction was elicited by acetylcholine released from post-ganglionic cholinergic neurones. Mitragynine pseudoindoxyl inhibited the electrically stimulated ileum contraction in a concentration-dependent manner as did morphine and mitragynine. On the other hand, mitragynine pseudoindoxyl did not affect the contraction induced by direct stimulation of muscarinic receptors on the intestinal smooth muscle. Together, it suggests that mitragynine pseudoindoxyl acts on the post-ganglionic nerves to inhibit the release of neurotransmitters.

The inhibition of the electrically stimulated ileum contraction by mitragynine pseudoindoxyl was antagonized by naloxone and naloxonazine, suggesting the involvement of opioid receptors. This is confirmed by the parallel shift of concentration–response curve for mitragynine pseudoindoxyl by naloxone. No significant difference among the pA₂ values for naloxone against mitragynine pseudoindoxyl, mitragynine, and morphine was observed. Their Schild slopes were not significantly different from a unity. In addition, naloxone abolished the inhibitory effect of morphine on the electrically stimulated contraction in the ileum, but did not show any effect on inhibition by clonidine and nicardipine. The effect of naloxone is specific to the opioid receptors. Taken together, mitragynine pseudoindoxyl is thought to act on opioid receptors.

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 (Goldstein and James, 1984; Martin, 1984; Dhawan et al., 1996). The guinea pig ileum contains functional populations of both μ- and κ-receptors (Hutchinson, et al., 1975; Chavkin and Goldstein, 1981; Traynor, 1994). Although the enteric nervous system of the ileum contains a number of opioid receptors, the actions of μ-agonist such as morphine is thought to be mediated through μ₂-receptors, a subtype of μ-class of opioid receptors (Goldstein and James, 1984; Martin, 1984; Dhawan et al., 1996). Naloxonazine is an irreversible opioid antagonist for μ₁-receptors, however it is known that naloxonazine also antagonizes the opioid effect in the guinea pig ileum in a reversible manner by acting on μ₂-receptors (Gintzler and Pasternak, 1983; Pasternak and Wood, 1986). Taking into account that naloxonazine alone had no effect on the contraction elicited by electrical stimulation in the ileum, and that naloxonazine antagonized the effects of mitragynine and mitragynine pseudoindoxyl, mitragynine and mitragynine pseudoindoxyl are considered to act on μ-receptors.

pD₂ value for DAMGO at μ-receptors is reported to be about 8.0 (Elliot and Traynor, 1995; Zadina et al., 1997). Mitragynine pseudoindoxyl showed to be as potent as DAMGO in functional experiments. These data are well correlated to results from binding assay, which demonstrated that mitragynine pseudoindoxyl has a similar affinity to DAMGO for μ-binding site. Mitragynine pseudoindoxyl potency was about 100-fold greater than that of mitragynine. Oxidation at 7 position of mitragynine led to the increase of opioid activities. This may mean that the oxyindole structure or the indole ring perpendicular to C and D rings is important for the opioid activities of this class of compounds.

Mitragynine pseudoindoxyl inhibited vas deferens contraction in a concentration-dependent manner, as did DPDPE and morphine. A shift to the right of concentration–response curves for mitragynine pseudoindoxyl in the presence of naltrindole or naloxone was observed. Moreover, the pA₂ values for naltrindole and

naloxone against mitragynine pseudoindoxyl were rather similar to those against DPDPE. These results suggest that mitragynine pseudoindoxyl interacts with δ -opioid receptors to inhibit the twitch response of mouse vas deferens preparation.

In contrast to the close similarity of the pA_2 values for naloxone against mitragynine pseudoindoxyl and morphine in the guinea pig ileum, their pA_2 values in the mouse vas deferens are quite different. However, approximately equal pA_2 values of naltrindole and naloxone against mitragynine pseudoindoxyl and DPDPE were observed in the mouse vas deferens. These observations demonstrate that mitragynine pseudoindoxyl acts preferentially on μ -receptors in the guinea pig ileum and on δ -receptors in the mouse vas deferens.

Mitragynine inhibited the vas deferens contraction only at high doses ($>10 \mu\text{M}$). Moreover, mitragynine pseudoindoxyl was sensitive to naltrindole and naloxone in mouse vas deferens, but the inhibitory effect of mitragynine is refractory to micromolar doses of naltrindole and naloxone. Taking into consideration that high doses of mitragynine show Ca^{2+} channel (L- and T-type) blocking action in neuroblastoma cells by patch clamp and fluorescent dye techniques (Horie et al., 1995), mechanisms other than opioid receptors may be involved in mitragynine action in this smooth muscle.

The receptor binding assay showed that affinities of mitragynine pseudoindoxyl at μ - and δ -receptors are similar to DAMGO and naltrindole, respectively. Because mitragynine did not display receptor preferences higher than 100-fold for μ - or δ -receptors (Yamamoto et al., 1997), the proposed criterion for opioid selectivity (Goldstein and James, 1984), it was suggested to be a non-selective ligand at these opioid receptors. Mitragynine pseudoindoxyl also showed no selectivity for either μ - or δ -receptors.

A good correlation between both functional and binding assays was observed with mitragynine pseudoindoxyl. Surprisingly, the potency ratio estimated by comparing the potency of mitragynine pseudoindoxyl in the ileal preparation with that of morphine showed that it coincided with that estimated in the binding assay. Although a detailed study on mitragynine pseudoindoxyl action on μ -receptors with selective antagonists was not carried out in the ileal experiments, restoration by naloxonazine of the inhibitory effect of mitragynine pseudoindoxyl on the ileum contraction and its binding affinity support its action on μ -receptors. The action of mitragynine pseudoindoxyl on δ -receptors in the mouse vas deferens was also supported in this binding assay. Mitragynine pseudoindoxyl potency was one or two orders of magnitude greater than that of morphine in both assays, nevertheless this compound is structurally unrelated to opioids. In addition, its low selectivity for μ - and δ -opioid receptors compared with morphine was also evident in both assays.

A close correlation between the pharmacological potency of opiates and their affinity for receptor binding has been established for a large number of opioid analgesic drugs (Pert and Snyder, 1973; Lord et al., 1977). Moreover, agonists with high affinity at both μ - and δ -receptors have the potential to attenuate acute and chronic pain (Lazarus et al., 1996). Although in vivo experiments are necessary to study analgesic effects of mitragynine pseudoindoxyl, the present results in part explain the antinociceptive property (Zarembo et al., 1974). In agreement with reported analgesic potency, mitragynine pseudoindoxyl also showed to be 100-fold more potent than mitragynine in inhibiting electrically stimulated ileum contraction. Hence, it raises the possibility of mitragynine pseudoindoxyl to be a potent opioid analgesic drug.

In summary, the present study is, to our knowledge, the first demonstration that mitragynine pseudoindoxyl, a novel alkaloid structurally different from other opioid agonists, acts on opioid receptors, leading to a potent inhibition of electrically stimulated contraction in guinea pig ileum and in mouse vas deferens. In addition, mitragynine pseudoindoxyl showed to be very potent μ - and δ -opioid agonists in both functional and binding assays.

4. Summary

Mitragynine pseudoindoxyl is a compound related to mitragynine, which is structurally unrelated to morphine and has opiate-like antinociceptive activity (Zarembo et al., 1974). Mitragynine is a main alkaloid isolated from leaves of *M. speciosa*, a Thai folk medicine with opiate-like medicinal use. Effects of mitragynine pseudoindoxyl on electrically stimulated contraction in guinea pig ileum and mouse vas deferens, and its binding affinity in the guinea pig brain membranes were studied in order to elucidate opioid effect of mitragynine pseudoindoxyl. Mitragynine pseudoindoxyl (100 pM–30 nM) inhibited the electrically stimulated ileum contraction in a concentration-dependent manner, and its pD_2 value was 8.96 ± 0.09 . This potency was about 100- and 20-fold greater than that of mitragynine ($pD_2 = 6.92 \pm 0.05$) and morphine ($pD_2 = 7.67 \pm 0.06$), respectively. On the other hand, it had no effect on the contraction elicited by direct stimulation of the cholinergic receptors on the smooth muscle. Moreover, mitragynine pseudoindoxyl (1 nM–1 μM) also inhibited the electrically stimulated vas deferens contraction in a concentration-dependent manner and afforded a pD_2 value of 7.40 ± 0.11 . This was 35-fold greater than that of morphine ($pD_2 = 5.85 \pm 0.08$). The inhibitory effects of mitragynine pseudoindoxyl on electrically stimulated ileum and vas deferens contractions were completely reversed by naloxone and naltrindole, respectively. The pA_2 value of naloxone (8.73 ± 0.15) against mitragynine pseudoindoxyl is almost the same that against morphine

(8.77 ± 0.12) in the ileum preparation. The pA_2 value of naltrindole against mitragynine pseudoindoxyl (9.93 ± 0.20) is very similar to that against DPDPE (9.48 ± 0.16). In the receptor binding assay, mitragynine pseudoindoxyl showed similar affinities to DAMGO and DPDPE at μ - and δ -receptors, respectively. However, it showed a negligible affinity at κ -receptors. The results suggest that mitragynine pseudoindoxyl inhibits electrically stimulated ileum contraction through the action on μ -opioid receptors and inhibits mouse vas deferens through the action on δ -receptors. The present study is, to our knowledge, the first demonstration that mitragynine pseudoindoxyl, a novel alkaloid structurally different from other opioid agonists, acts on opioid receptors.

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