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The Opioid Receptor Selectivity for Trimebutine in Isolated Tissues Experiments and Receptor Binding Studies

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Differences of affinity to and selectivity for trimebutine between peripheral and central opioid receptors have been investigated. Trimebutine inhibited electrically induced contraction of guinea-pig ileum (GPI) and mouse vas deferens (MVD) but not of rabbit vas deferens, and the inhibition was antagonized by naloxone and, to lesser extent, by nor-binaltorphimine (nor-BNI). The pA_2 values for morphine and trimebutine with naloxone were higher than the values for these compounds with nor-BNI in both GPI and MVD preparations. GPI preparations incubated with a high concentration of morphine or trimebutine developed tolerance; however, there was no cross-tolerance between them, suggesting difference in the underlying mechanisms. In mouse and guinea-pig brain homogenate trimebutine was about 1/13 as potent as morphine to displace the [^{3}H]naloxone binding, while it has no appreciable affinity for κ-opioid receptors in [^{3}H]U-69593, a selective κ-receptor agonist. These results suggest that trimebutine, showing its low affinity to opioid receptors, possesses μ-receptor selective properties rather than those of κ-opioid receptor in the peripheral tissues and in the central brain homogenate.

Keywords — trimebutine; opioid receptor selectivity; mouse vas deferens; guinea-pig ileum; receptor binding assay

Trimebutine maleate (2-dimethylamino-2-phenylbutyl 3,4,5-trimethoxybenzoate hydrogen maleate) has been used in the treatment of various digestive tract disorders including dyspepsia, abdominal cramping and irritable bowel syndrome.[1,2] Previous studies[3] have suggested that peripheral opioid receptors are involved in the actions of trimebutine on small intestine, since the effect was suppressed by pretreatment with intraperitoneal but not intracerebroventricular naloxone. However, there were some discrepancies among the reports in the relative involvement of opioid receptor subtypes in the actions of trimebutine.[4,5] The selectivity has not been definitely determined.

It is the aim of the present study to characterize the selectivity of trimebutine for the opioid receptor subtypes in peripheral and central tissues, and to compare the mode of actions between trimebutine and morphine.

Materials and Methods

Drugs — Trimebutine maleate and U-50488H (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene-acetamide methansulfonate hydrate) were generous gifts from Tanabe and Upjohn, respectively. Nor-binaltorphimine (nor-BNI) was provided by Dr. H. Nagase, Toray. All the other compounds were obtained from the following companies: morphine (Takeda), naloxone (Sigma), leucine-enkephalin (Protein Research Foundation), [^{3}H]naloxone and [^{3}H]U-69593 (New England Nuclear).

Preparation of Iongitudinal Muscles of Guinea-pig Ileum, Mouse vas Deferens and Rabbit vas Deferens — Male Hartley guinea-pigs weighing 300—350 g were sacrificed by a blow to the head. Segments (about 4 cm) of the ileum (GPI) 10 to 15 cm from the ileo-cecal valve were isolated and then, the longitudinal muscle with myenteric plexus was prepared as described by Paton.[6] Mouse vas deferens (MVD) and rabbit vas deferens (RVD) were rapidly isolated from male ddY mice weighing 30 to 35 g and albino rabbit weighing 2.5 to 3.0 kg, respectively. These preparations were mounted in a 10 ml organ bath filled with Krebs–Hensleit solution (118 mM

NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4, 2.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose) for GPI or Mg^{2+} free Krebs–Hensleit solution for MVD and RVD, respectively, kept at 37 °C. These muscle strips were stimulated transmurally with square-wave electrical pulses of 80 V, duration of 0.8 ms for GPI or 1 ms for MVD and RVD, at a frequency of 0.1 Hz through platinum ring electrodes. The contractions were recorded through an isotonic transducer.

Development of Tolerance to Morphine and to Trimebutine — After 30 min equilibration, the tissues were left in contact with 10 μM morphine or 10 μM trimebutine for 90 min at 37 °C. After incubation, the tissues were washed several times with corresponding medium at 10 min interval until the electrically stimulated contractions returned to the preincubation level. The inhibitory effect of trimebutine on electrically evoked contractions between morphine- and trimebutine-tolerated GPI preparations was compared to examine the development of tolerance and cross-tolearance between them.

Binding Assay Procedure — The binding assay was carried out as described by Terenius[7] with modifications. Male ddY mice weighing 20—24 g or guinea-pigs weighing 200—300 g were decapitated and their brains were rapidly removed. The mouse brain except cerebellum was homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) for opioid μ-receptor assay, or the guinea-pig brain in ice-cold 50 mM potassium phosphate (pH 8.0) for κ-receptor study. The homogenate which was incubated for 20 min at 37 °C to inactivate endogenous opioid peptides was centrifuged twice and re-suspended with respective buffer. The reaction mixture contained the following reagents in the final volume of 0.5 ml; 2 nM [^{3}H]naloxone (41.4 Ci/mmol), 100 mM NaCl, 40 μM bacitracin and the tissue preparation (0.6 mg as protein) in 0.5 ml of Tris-HCl buffer for μ-receptor assay; or 1 nM [^{3}H]U-69593 (75.0 Ci/mmol), 40 μM bacitracin and the tissue preparation (0.6 mg as protein) in 0.5 ml of potassium phosphate buffer for κ-receptor assay. Stereospecific binding was defined as the difference between binding in the presence or absence of excess (1 μM) unlabeled drug. The mixture was incubated at 25 °C for 20 min and the reaction terminated by centrifugation. For counting the radioactivity, the pellet was digested using 0.2 ml Protosol. The protein was estimated according to the method of Lowry *et al.*[8] using bovine serum albumin (BSA) as the standard.

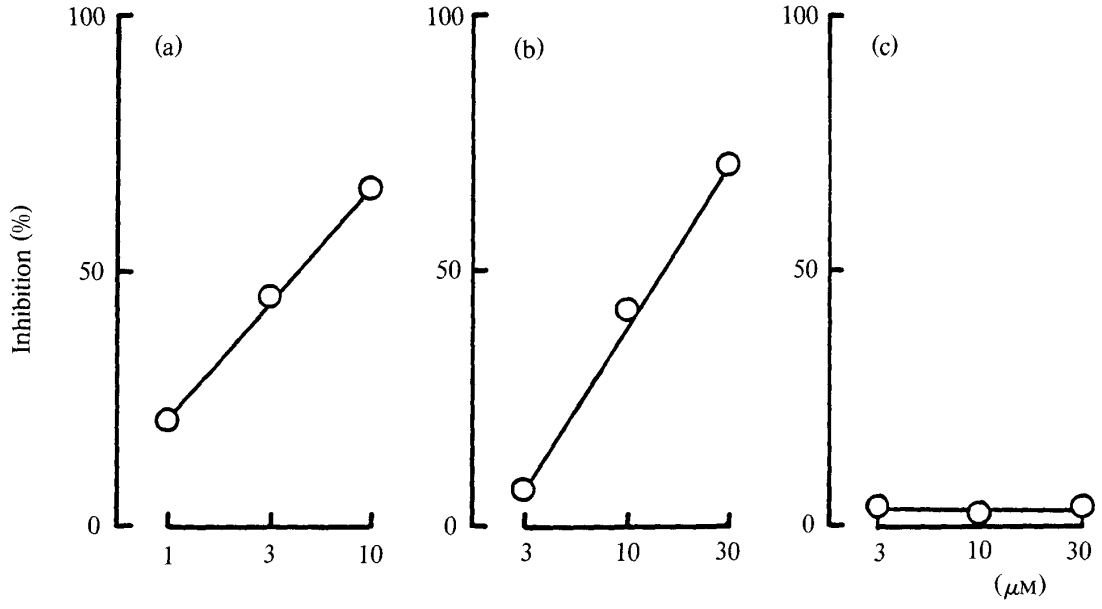

Fig. 1. Inhibition by Trimebutine of the Electrically Evoked Contractions of Guinea-pig Ileum (a), Mouse vas Deferens (b) and Rabbit vas Deferens (c) Preparations

Results

Inhibition by Trimebutine of the Contractions in Isolated Tissues

Trimebutine inhibited electrically induced contractions of GPI and MVD preparations in a concentration dependent fashion. The IC_{50} value for the drug was 4.7 ± 0.2 μM and 21 ± 2 μM for GPI and MVD, respectively.

Morphine, a typical μ-agonist, also suppressed the contractions of GPI and MVD preparations with 59 ± 6 nM and 1.5 ± 0.1 μM, respectively, of IC_{50} values. On the other hand, neither morphine nor trimebutine had any effect on the contractions of the RVD preparation at concentrations up to 100 μ M. Leu-enkephalin blocked electrically evoked contractions of GPI and MVD preparations, U-50488H, a selective κ-receptor agonist, likewise inhibited the contractions of not only these preparations but also RVD preparation (Fig. 1 and Table I).

TABLE I. Inhibitory Effect of Trimebutine and Opioid Agonists on the Electrically Evoked Contractions of GPI, MVD and RVD Preparations

Drug	GPI IC_{50}	MVD IC_{50}	RVD IC_{50}
Trimebutine	4.7±0.2 μM	21 ±2 μM	>100 μM
Morphine	59 ±6 nM	1.5±0.1	>100
U-50488H	2.2±0.3	11 ±1 nM	170±17 nM
Leu-Enk	150 ±12	17 ±2	>100 μM

Concentration of each drug giving 50% inhibition (IC_{50}) of the electrically evoked contractions was determined by interpolating by log-linear regression analysis; the IC_{50} values were expressed as the mean ± S.E.M. of 3 separate determinations.

Antagonism of Naloxone and Nor-BNI on the Inhibition of the Contraction by Trimebutine and Opioid Agonists

The inhibitory effect of both morphine and trimebutine on electrically evoked contractions of GPI and MVD was antagonized by naloxone and nor-BNI.

In the GPI preparation, the pA_2 values for the interaction of trimebutine and morphine with naloxone were similar, and these values were higher than the values for morphine and trimebutine with nor-BNI. On the contrary, the pA_2 value for U-50488H with nor-BNI was extremely high, but the value for U-50488H-naloxone was low. Similar results were obtained from the MVD preparation.

Meanwhile, the pA_2 value for the interaction of δ-agonist leu-enkephalin with naloxone was lower than the value for trimebutine with naloxone in the MVD preparation (Table II). The curves of the pA_2 plot obtained here were straight lines with slopes of 0.95—1.05 (data not shown).

The Development of Tolerance and no Cross-tolerance between Trimebutine and Morphine in GPI Preparation

The concentration-response curve for morphine (a) shifted to the right after incubation with 10 μM morphine for 90 min, indicating the development of tolerance. Similarly, the curve for trimebutine (b) shifted to the right after incubation with trimebutine. In contrast, the identical activities of trimebutine in control and morphine-tolerant GPI, and of morphine in control and

TABLE II. The pA_2 Values for Naloxone and Nor-BNI with Trimebutine and Opioid Agonists in GPI, MVD and RVD Preparations

Drug	GPI		MVD		RVD	
	pA_2		pA_2		pA_2	
	Naloxone	Norbinal-torphimine	Naloxone	Norbinal-torphimine	Naloxone	Norbinal-torphimine
Trimebutine	8.27±0.07	7.57±0.06	8.50±0.01	7.66±0.05	—	—
Morphine	8.61±0.05	7.38±0.11	8.57±0.04	7.88±0.24	—	—
U-50488 H	7.36±0.02	10.33±0.15	7.32±0.10	10.38±0.06	7.25±0.03	9.44±0.11
Leu-Enk	8.56±0.03	7.59±0.06	7.45±0.03	7.77±0.05	—	—

The pA_2 values were determined by plotting according to Schild[12] and expressed as the mean ± S.E.M. of 3 separate determinations.

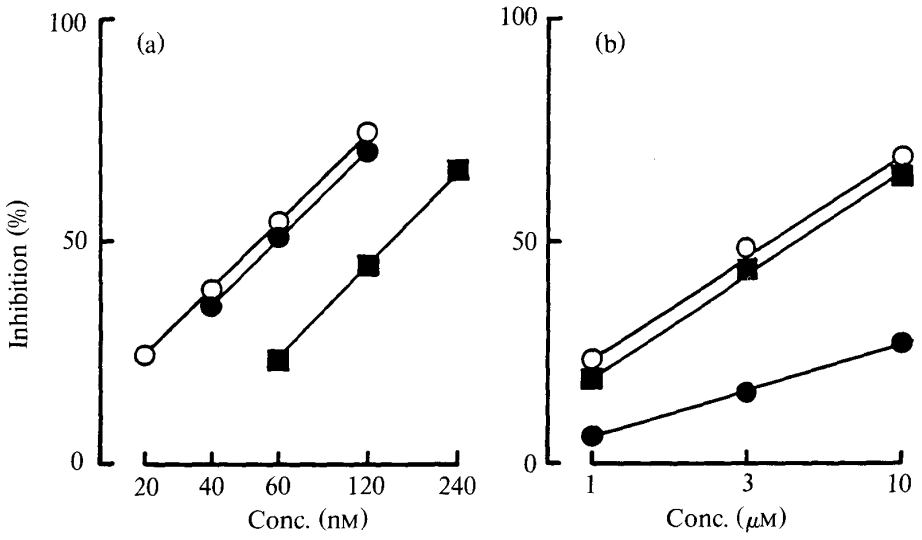

Fig. 2. The Development of Tolerance to the Inhibitory Effect of Morphine (a) and Trimebutine (b) on the Electrically Evoked Contractions of Guinea-pig Ileum and the Development of Cross-tolerance to the Effect between Them
Data are for preparations from naive (○), and from those with exposure to 10 μM morphine (■) or trimebutine (●) for 90 min.

trimebutine-tolerant GPI, were shown, which indicated no cross-tolerance between both drugs (Fig. 2).

The Opioid Receptor Binding Studies

Morphine was most effective in competition with [^{3}H]naloxone binding to mouse brain homogenate, and was about 13 times more potent than trimebutine. It also can be seen that U-50488H is 1/3 as effective as trimebutine in reducing the [^{3}H]naloxone binding. Meanwhile, U50488H was most effective in competition with [^{3}H]U-69593, a selective κ-opioid receptor agonist, binding to guinea-pig brain homogenate, whereas competitive binding of trimebutine against [^{3}H]U-69593 was comparably low and was virtually less than that of morphine (Fig. 3). The IC_{50} values of the drugs to displace 2

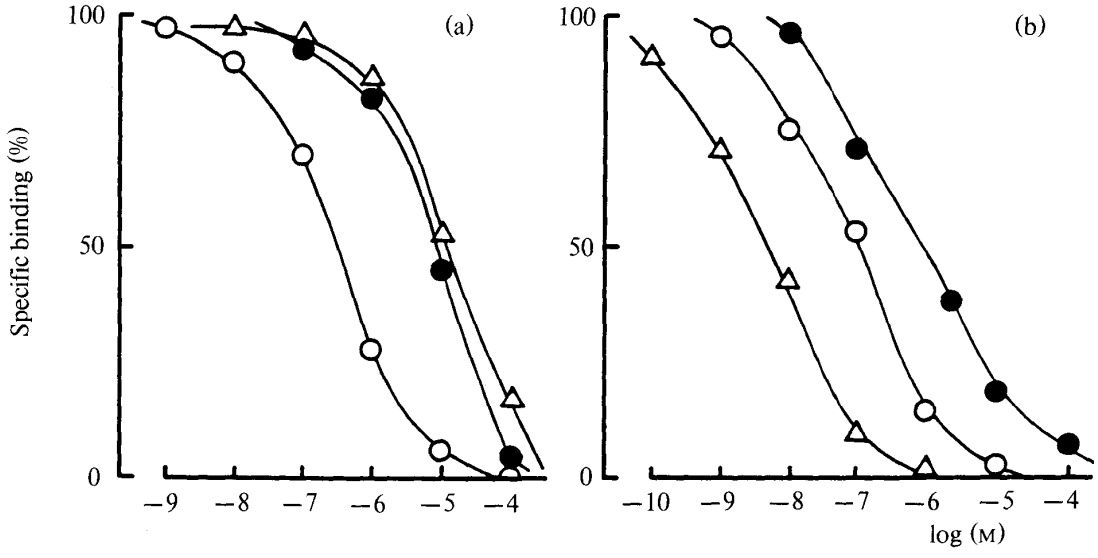

Fig. 3. Inhibition by Various Compounds of Specific Binding of [^{3}H]Naloxone (a) to Mouse Brain Homogenates and [^{3}H]U-69593 (b) to Guinea-pig Brain Homogenates
Morphine (○), trimebutine (●) and U-50488H (△).

TABLE III. Inhibitory Potencies of Various Compounds on the Specific Binding of [^{3}H]Naloxone to Mouse Brain Homogenates and [^{3}H]U-69593 to Guinea-pig Brain Homogenates

Drug	μ-Site ([^{3}H]Naloxone, 2 nM)		κ-Site ([^{3}H]U-69593, 1 nM)	
	IC_{50} (M)	Relative affinity	IC_{50}(M)	Relative affinity
Trimebutine	3.6×10^{-6}	7.7	6.0×10^{-7}	0.7
Morphine	2.8×10^{-7}	100	1.2×10^{-7}	3.9
U-50488H	1.3×10^{-5}	2.1	4.7×10^{-9}	100

These values indicate the concentration of the tested compound that has reduced the binding of [^{3}H]naloxone and [^{3}H]U-69593 by 50% as appeared in Fig. 3.

nM [^{3}H]naloxone and 1 nM [^{3}H]U-69593 binding to the brain homogenate is summarized in Table III.

Discussion

It is well established that the electrically evoked contractions of GPI, MVD[9] and RVD[10] are rather preferentially inhibited by μ-, δ-, and κ-opioids, respectively, and the inhibition is mostly reversed by their selective antagonists. However, these preparations also contain other opioid receptor(s) such as κ in GPI and μ and κ in MVD, hence, models are available for the characterization or assessment of the receptor selectivity for the compounds to be tested only in their combinations.

The inhibitory effect of trimebutine on the electrically evoked contractions of GPI preparations was 80 times less effective than that of morphine, and was antagonized by naloxone with a relatively high pA_2 value. In this preparation, the pA_2 values for the interaction of trimebutine and morphine with naloxone were similar to one another, and were different from the value for U-50488H with naloxone. Furthermore, the pA_2 value for trimebutine-naloxone was higher than that for trimebutine with nor-BNI. These findings suggest that trimebutine which was comparably less effective than morphine may act on μ-receptors. However, the possibility of the existence of another μ-like receptor on which trimebutine acts somewhat differently from the typical μ-receptor for morphine, could not be excluded. Actually, Pasternak *et al.*[11] reported the subdivision of opioid μ-receptor into μ_1 and μ_2, and morphine produces analgesic effect mediated through central μ_1 but inhibits the contractions of GPI through μ_2-receptor peripherally.

Inasmuch as cross-tolerance is generally supposed to develop between drugs which share the common mechanisms with each other, the fact that the development of cross-tolerance was not observed between morphine and trimebutine in the GPI preparation may suggest that the action mechanism of trimebutine is different from that of morphine.

The facts that the concentration-response curve for morphine in morphine tolerant GPI preparation shifted to the right parallel to that in the naive control preparation, and the curve for trimebutine in its tolerant preparation shifted but not in parallel at high concentration may also indicate a difference between morphine and trimebutine.

In MVD preparation, trimebutine showed significantly higher IC_{50} values than any other compounds tested, indicating low affinity of trimebutine to opioid receptors, especially δ-subtype. On the other hand, trimebutine had no effect on the contractions of RVD preparations. These findings may simply indicate that trimebutine acts weakly but selectively on peripheral μ-receptor rather than on κ-receptor.

To characterize the receptor selectivity for trimebutine in mouse and guinea-pig brain, we performed a series of competition binding experiments with [^{3}H]naloxone and [^{3}H]U-69593 as primary ligands. Morphine was approximately 13 and 47 times more effective than trimebutine and U-50488H, respectively, in μ-receptor binding assay. In addition, the fact that trimebutine showed comparably low affinity to κ-receptor by

the substitution for U-69593, almost as much as morphine did, clearly indicates that trimebutine preferentially binds to μ-receptor than κ-receptor in the brain homogenate.

Gue *et al.*[5] reported that the effect of trimebutine on the gastric motor disturbances, produced probably by acting selectively on peripheral κ-receptors. On the other hand, Roman *et al.*[4] reported that trimebutine showed a relative higher affinity for the μ-receptor subtype using guinea-pig brain membrane. Likewise, our data from these experiments show that trimebutine has a selectivity for μ-receptor rather than κ-receptor in the central although the affinity for both subtypes is substantially low. Thus, the possibility that trimebutine possesses central actions to some extent could not be simply excluded.

In the light of our studies that trimebutine has low affinity for the opioid receptors and of the report[3] that trimebutine had a naloxone insensitive inhibitory effect on colonic motility, it is possible that trimebutine contributes not only colonic motility but other actions, to some extent, through non-opioid properties.

In conclusion, we could indicate that trimebutine possesses μ-receptor selectivity rather than κ-receptor in the peripheral tissue and in the brain membrane preparation.

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