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# Selectivity for Opioid Receptor Subtypes of Enkephalin Analogues in Isolated Smooth Muscle and in the Analgesic Effect in Mice

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Selectivity for opioid receptor subtypes of enkephalin analogues (KK-1, -2, -3 and -4) of Tyr moiety on the N-terminal, and Phe-ol group on the C-terminal, connected with the methylene group ($n = 1-4$) were examined in isolated smooth muscle preparations and in the analgesic effect in mice. In the longitudinal muscle preparations of guinea pig ileum (GPI), morphine, U-50488H and all the enkephalin analogues inhibited electrically evoked contractions, and the inhibitory effects of morphine, KK-1, KK-2 and KK-3 were antagonized by naloxone with relatively high $pA_2$ values, while that of U-50488H and KK-3 were preferentially antagonized by norbinaltorphimine. In the rabbit vas deferens preparations (RVD), on the other hand, U-50488H, KK-3 and KK-4 showed weak inhibitory effects and the inhibition of U-50488H and KK-3 were antagonized by norbinaltorphimine. By intracerebroventricularly (i.c.v.) injection, enkephalin analogues produced analgesia in the acetic acid (AcOH) writhing test, and the effect of KK-1 and KK-2 as well as morphine was antagonized by 1 mg/kg naloxone, while those of U-50488H and KK-3 were sensitive to 1 mg/kg Mr2266.

In conclusion, enkephalin analogues with a short methylene chain between the functional groups, KK-1 and KK-2, mainly exert their effect through opioid $\mu$-receptors, while those of longer chain, KK-3 and KK-4, act through $\kappa$-receptors preferentially, and KK-3 is situated in the alternating point of the selectivity for $\mu$- and $\kappa$-receptors.

**Keywords** — enkephalin analogues; opioid receptor; guinea-pig ileum; rabbit vas deferens; analgesic effect

## Introduction

We have developed enkephalin analogues, Tyr moiety on the N-terminal and Phe-ol on the C-terminal connected with various length of methylene chain, and with opioid activities in the *in vitro* and *in vivo* experiments.[1,2] We have also demonstrated that minor alterations in chemical structure of these compounds resulted in the changes of their selectivity for opioid receptor subtypes and pharmacological potencies.[2] In order to investigate the selectivity for receptor subtypes more precisely, in the present study, we compared the effect of the enkephalin analogues with morphine and U-50488H, a typical $\kappa$-agonist, in isolated smooth muscle preparations and in the analgesic effect after intracerebroventricularly (i.c.v.) injection in mice.

## Materials and Methods

**Drugs** — Synthesis and purification of the enkephalin analogues*** has been reported previously.[1] Morphine-HCl (Takeda), naloxone-HCl (Sigma), U-50488H*4 ($\kappa$-agonist, Upjohn), norbinaltorphimine*5 ($\kappa$-antagonist, gift from Dr. Nagase, Toray), Mr2266*6 ($\kappa$-antagonist, Boehringer Ingelheim) were all obtained from these sources.

**Smooth Muscle Preparations** — The preparation of longitudinal muscle strips of guinea pig ileum (GPI) was followed as described by Rang.[3] Rabbit vas deferens (RVD)

***, Tyr-N($CH_3$)-($CH_2$)$n$-Phe-ol: KK-1, $n$= 1; KK-2, $n$= 2; KK-3, $n$= 3; KK-4, $n$= 4.

*4, *trans*-(+)-3,4-dichloro-*N*-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide.

*5, 17,17′-bis(cyclopropylmethyl)-6,6′,7,7′-tetradehydro-4,5:4′,5′-diepoxy-6,6′-(imino)[7,7′-bimorphinan]-3,3′,14,14′-tetrol.

*6, (−)-(1*R*, 5*R*, 9*R*)-5,9-diethyl-2-(3-furyl-methyl)-2′-hydroxy-6,7-benzomorphan.

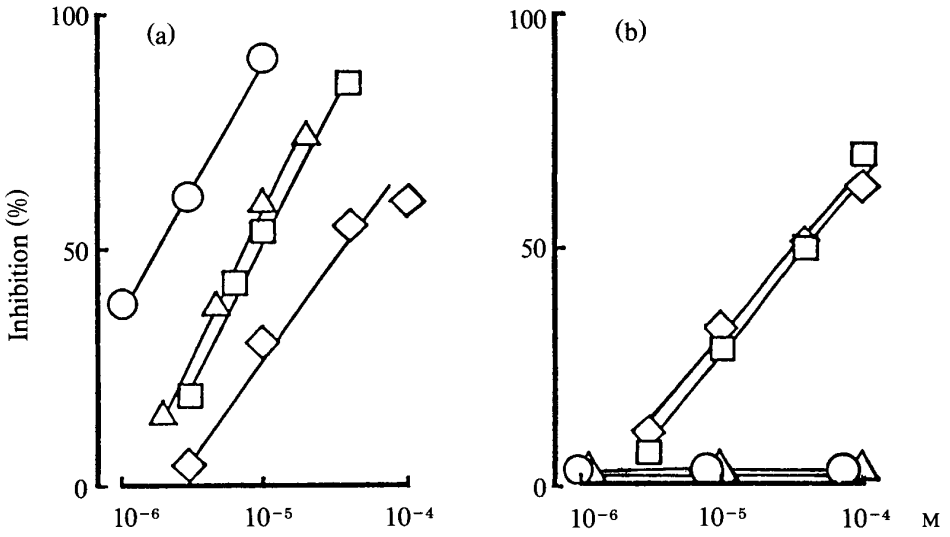


Fig. 1. Inhibitory Effects of Enkephalin Analogues on the Electrically Evoked Contractions of Isolated Smooth Muscle Preparations

(a) guinea-pig ileum and (b) rabbit vas deferens. KK-1 (○), KK-2 (△), KK-3 (□) and KK-4 (◇). Each point is the mean of the results obtained from 3—10 experiments.

was isolated according to the method of Oka *et al.*[4] Each preparation was mounted in a 10 ml organ bath filled with Krebs-Hensleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.4 mM $MgSO_4$, 1.1 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 11 mM glucose) or $Mg^{2+}$-free Krebs-Hensleit solution, kept at 37 °C, for GPI and RVD, respectively. The GPI preparation was stimulated transmurally by electrical pulses of 0.8 ms duration of 100 V, at a frequency of 0.1 Hz. The contractions were recorded isotonically and the $IC_{50}$ of the test compound was estimated from the concentration-response curve and $pA_2$ values for antagonist were calculated according to the method of Schild.[5]

**Analgesic Effect** — Using male ddY mice weighing 20—30 g, each group consisted of 7—15 animals, the analgesic effect was measured by the AcOH writhing test. Drugs were given i.c.v. 5 min prior to the i.p. administration of 0.6% AcOH, and control animals were treated with saline instead of drugs. Writhes observed during 5 min period were counted 3 times at 10 min intervals after administration of AcOH, and the $ED_{50}$ for each drug was estimated according to the methods of Litchfield and Wilcoxon.[6]

## Results

### Isolated Smooth Muscle Preparations

TABLE I. Inhibitory Effect on the Electrically-Induced Contractions of Isolated Smooth Muscle Preparations

| Drug | GPI | | | RVD | | |
|---|---|---|---|---|---|---|
| | *N* | $IC_{50}$ | Relative activity | *N* | $IC_{50}$ | Relative activity |
| Morphine | 6 | 65 ±6 nM | 7.69 | 5 | >100 μM | — |
| U-50488H | 5 | 5 ±0.3 | 100 | 5 | 208±26 nM | 100 |
| KK-1 | 15 | 1.6±0.2 μM | 0.31 | 5 | >100 μM | — |
| KK-2 | 6 | 6.2±0.5 | 0.08 | 5 | >100 | — |
| KK-3 | 10 | 8.0±0.9 | 0.06 | 9 | 39±5 | 0.53 |
| KK-4 | 6 | 34.5±5.7 | 0.01 | 7 | 32±9 | 0.65 |

GPI, guinea-pig ileum; RVD, rabbit vas deferens; N, number of experiments. Concentration of each drug producing 50% inhibition ($IC_{50}$) of the electrically-induced contractions was estimated from the concentration-inhibition curve.

TABLE II. Antagonism by Naloxone and Norbinaltorphimine to the Inhibitory Effect of Test Compounds on the ES-Induced Contractions of Isolated Smooth Muscle Preparations

| Drug | GPI | | RVD | |
|---|---|---|---|---|
| | $pA_2$ | | $pA_2$ | |
| | Naloxone | Norbinaltorphimine | Naloxone | Norbinaltorphimine |
| Morphine | 8.61±0.05 | 7.38±0.11 | – | – |
| U-50488H | 7.36±0.02 | 10.10±0.07 | 7.25±0.03 | 9.44±0.11 |
| KK-1 | 8.54±0.15 | 7.58±0.06 | – | – |
| KK-2 | 8.55±0.11 | 7.47±0.08 | – | – |
| KK-3 | 8.21±0.02 | 8.84±0.04 | <7 | 9.01±0.11 |
| KK-4 | 7.20±0.13 | <7 | <7 | <7 |

The $pA_2$ values for antagonists were calculated according to the method of Schild and expressed as the mean ± S.E.M. –, inactive in RVD.

Enkephalin analogues inhibited electrical stimulation (ES) induced contractions of the GPI preparations in a concentration-dependent manner, and the order of the potencies was KK-1 > KK-2 = KK-3 > KK-4 (Fig. 1a). In the RVD preparation, on the other hand, KK-3 and KK-4 produced nearly equipotent inhibitory effect but KK-1 and KK-2 were ineffective up to $10^{-4}$ M (Fig. 1b).

The inhibitory effect of the enkephalin analogues on the ES-induced contractions of GPI and RVD in comparison with those of morphine and U-50488H are summarized in Table I. U-50488H was more effective in both GPI and RVD preparations than other drugs. Morphine was effective in GPI but was ineffective in RVD. The inhibitory effect of KK-1 was highest in enkephalin analogues but the activity was about 25 times less than that of morphine, and the activities of enkephalin analogues decreased by increasing the number of methylene groups between Tyr and Phe-ol. KK-3 and KK-4 were effective in RVD but KK-1 and KK-2 were devoid of the inhibitory activities in this preparation (Table I).

The $pA_2$ values for naloxone and norbinaltorphimine on the ES-induced contractions of isolated smooth muscle preparations of morphine, U-50488H and enkephalin analogues are summarized in Table II. In the GPI preparation, the effect of morphine, KK-1, KK-2 and KK-3 were antagonized by naloxone with relatively high $pA_2$ values, but U-50488H and KK-4 were rather resistant against naloxone showing one order less $pA_2$ values. On the other hand, U-50488H was highly sensitive against norbinaltorphimine antagonism, the $pA_2$ was more than 10, and the effect of KK-3 was also antagonized by norbinaltorphimine rather selectively in the enkephalin analogues. Morphine, KK-1 and KK-2 were also sensitive to norbinaltorphimine but the $pA_2$ values were one order less than

TABLE III. Analgesic Effects and Its Antagonism by Naloxone and Mr-2266 in Mice

| Drug | $ED_{50}$ [a] (95% confidence limits) | Antagonism [b] | |
|---|---|---|---|
| | | Naloxone | Mr-2266 |
| Morphine | 0.2 (0.1– 0.3) | + | – |
| U-50488H | 0.5 (0.2– 1.1) | – | + |
| KK-1 | 8.5 (3.3–22.1) | + | – |
| KK-2 | 10.1 (4.6–22.2) | + | – |
| KK-3 | 4.3 (1.9– 9.9) | – | + |
| KK-4 | 8.4 (3.5–20.2) | – | – |

*a)* AcOH writhing test after i.c.v. injection. *b)* Significant antagonism by 1 mg/kg.

KK-3. KK-4 was resistant against norbinaltorphimine, the $pA_2$ was <7. In the RVD preparation, U-50488H and KK-3 were highly sensitive to norbinaltorphimine, and the effect of U-50488H was also antagonized by naloxone with low $pA_2$.

**Analgesic Effects in Mice**

All compounds showed dose-dependent analgesic effect after i.c.v. injection, and the $ED_{50}$ values are summarized in Table III. The potency was in a higher order of morphine > U-50488H > KK-3 > KK-4 = KK-1 ≧ KK-2. The analgesic effect of morphine, KK-1 and KK-2 were significantly antagonized by 1 mg/kg naloxone, and by a higher dose of naloxone, the effect of U-50488H and KK-3 were partially antagonized. On the other hand, the analgesic effect of U-50488H and KK-3 were significantly antagonized by 1 mg/kg Mr2266, and Mr2266 2 mg/kg partially antagonized the effect of morphine, KK-1 and KK-2. The analgesic effect of KK-4 was resistant to naloxone and Mr2266.

## Discussion

Although the opioid activity of a series of enkephalin analogues (KK-1, KK-2, KK-3 and KK-4) were very weak compared with those of morphine and U-50488H, simple but systematic structures of these compounds were suitable for clarifing the difference on the selectivity for opioid receptor subtypes.

GPI contain $\mu$- and $\kappa$-opioid receptors but most of them are the former.[7] On the other hand, RVD possesses only $\kappa$-receptors and lack of other receptor subtypes.[4] These facts are compatible with our present data that morphine, a typical $\mu$-agonist, was only active in GPI and U-50488H, a selective $\kappa$-agonist, active in both GPI and RVD. All enkephalin analogues exert their inhibitory effect in GPI preparations but only KK-3 and KK-4 were effective in RVD, though the activities were extremely low compared with those of morphine and U-50488H. These data are indicative of the selectivity for opioid receptor subtypes, namely, that the inhibitory effect of KK-1 and KK-2 is mediated through $\mu$-receptors and those of KK-3 and KK-4 is mediated through $\kappa$-receptors rather than $\mu$-receptors.

As regards KK-1, 2 and 3, the analgesic effects in mice after i.c.v. injection corresponded well to the data in isolated smooth muscle preparations. Namely, the effect of KK-1 and KK-2 was antagonized by naloxone but not by Mr2266, and that of KK-3 was reversed by Mr2266. Although KK-4 as well as KK-3 was active in RVD, the inhibitory effects of KK-4 on GPI and RVD were insensitive to norbinaltorphimine, a selective $\kappa$-antagonist,[8] and the analgesia of KK-4 was insensitive to both naloxone and Mr2266. These facts may be due to the differences in the selectivity between KK-3 and KK-4 for $\kappa$-receptor subtypes, $\kappa_1$ and $\kappa_2$.[9-11]

A series of enkephalin analogues used in the present experiment are derived from Leu-enkephalin, a $\delta$-receptor agonist, however, their effect on mouse vas deferens, with abundant $\delta$-receptors, were very low suggesting no appreciable selectivity for $\delta$-receptors (data not shown).

Thus, KK-3 of these enkephalin analogues possesses pharmacological properties mediated through $\mu$- and $\kappa$-receptors, preferentially $\kappa$-receptors and the compound seems to be situated in a turning point for the selectivity of opioid receptors. Actually, our recent findings[12] that only KK-3 of these enkephalin analogues given intrathecaly produced a unique pharmacological behavior such as scratching, biting and licking may support the importance of the lengh with 3 methylene chains.

Our present data not only confirms the importance of chain length between two functional groups in the enkephalin analogues for opioid activity, as reported previously,[2] but also extend the findings that the number of methylene bonds is of critical importance for the selectivity of opioid receptor subtypes.

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### References

1) M. Maeda, S. Okusada, and K. Kawasaki: Synthesis and analgesic effects of Tyr-containing dipeptide phenetylamides, *Chem. Pharm. Bull.*, **32**, 4157—4160 (1984).

2) J. Watanabe, H. Kaneto, M. Maeda, T. Tsuji and K. Kawasaki: Involvement of different receptor subtype for the production of *in vitro* and *in vivo* effects in a series of synthetic enkephalin analogues, *J. Pharmacobio-Dyn.*, **8**, 975—980 (1985).
3) H. P. Rang: Stimulant actions of volatile anaesthetics on smooth muscle, *Br. J. Pharmacol.*, **22**, 356—365 (1964).
4) T. Oka, K. Negishi, M. Suda, T. Matsumiya, T. Inazu, and M. Ueki: Rabbit vas deferens: a specific bioassay for opioid κ-receptor agonists, *Eur. J. Pharmacol.*, **73**, 235—236 (1980).
5) H. O. Schild: pA, a new scale for the measurement of drug antagonism, *Br. J. Pharmacol.*, **2**, 189—206 (1947).
6) J. T. Jr. Litchfield and F. Wilcoxon: A simplified method of evaluating dose-effect experiments, *J. Pharmacol. Exp. Ther.*, **96**, 99—113 (1949).
7) J. A. H. Lord, A. A. Waterfield, J. Hughes and H. W. Kosterlitz: Endogenous opioid peptides: multiple agonists and receptors, *Nature* (London), **267**, 495—499 (1977).
8) P. S. Portoghese, H. Nagase, A. W. Lipkowski, D. L. Larson and A. E. Takemori: Binaltorphimine-related bivalent ligands and their κ opioid receptor antagonist selectivity, *J. Med. Chem.*, **31**, 841—847 (1988).
9) E. Castanas, N. Bourhim, P. Giraud, F. Boudouresque, P. Cantau and C. Oliver: Interaction of opiates with opioid binding sites in the bovine adrenal medulla: Interaction with κ sites, *J. Neurochem.*, **45**, 688—699 (1985).
10) S. Iyenger, H. S. Kim, and P. L. Wood: Effects of kappa opiate agonists on neuroendocrine indices: Evidence for kappa receptor subtypes, *Life Sci.*, **39**, 637—644 (1986).
11) R. S. Zukin, M. Eghbali, D. Olive, E. M. Unterwald and A. Tempel: Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for $\kappa_1$ and $\kappa_2$ opioid receptors, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4061—4065 (1988).
12) M. Takahashi, S. Tokuyama and H. Kaneto: Release of substance P by intrathecal KK-3, a newly synthesized Leu-enkephalin derivative, *Pharmacol. Biochem. Behav.*, **33**, (4) 1—4 (1989).