Comparison of the Antinociceptive Effect between the Cyclic Dipeptide Cyclo [Tyr(Et)-Homoarginine] and the Linear Dipeptide Boc-Tyr(Et)-Homoarginine-OMe in Rats*

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Abstract—The antinociceptive effect of the cyclic dipeptide cyclo[Tyr(Et)-homoarginine] (C.TEHA) was examined utilizing the tail flick test and the digitus pinching test in rats in comparison with the linear dipeptide Boc-Tyr(Et)-homoarginine-OMe (B.TEHM). Though both dipeptides administered into the lateral, 3rd and 4th cerebroventricles produced antinociceptive effects equipotent to morphine, except for the 4th cerebroventricular administration of B.TEHM, the administration into the spinal subarachnoid space was without effect. The effect of B.TEHM was completely antagonized by the pretreatment of naloxone (i.p.) when administered into the 3rd cerebroventricle where its effect was demonstrated to be the most potent. However, naloxone had no significant effect on C.TEHA administered into the 3rd cerebroventricle in both tests. It was concluded that both dipeptides act on the upper brain stem, especially around the 3rd cerebroventricle. Moreover, it was also thought that the effect of B.TEHM may be involved in the brain opioid system, and that of C.TEHA can be produced via a naloxone-resistant opioid system or a non-opioid system.

Since the antinociceptive effects of the endogenous opioid dipeptide kyotorphin (L-tyrosyl-L-arginine) were firstly reported by Takagi et al. (1, 2), the antinociceptive profiles (3-5) and several behavioral effects of this dipeptide have been ascertained. Subsequent studies indicated that kyotorphin delayed the conditioned avoidance response in rats (6) and decreased the number of pecks in chicks (7), but did not affect food intake in sheep (8), body temperature in cats (9) and heart rate in dogs (10).

On the other hand, Hoffman et al. reported (11) that a cyclic dipeptide or diketopiperazine was expected to resist peptidases in vivo. After that, we have also started to examine the antinociceptive activities of cyclic dipeptide analogs of kyotorphin and recently reported that utilizing the tail pressure test in mice, an analog in which its side-chain hydroxy group was ethylated, cyclo[Tyr(Et)-Arg], or an analog in which Arg was replaced by homoarginine (Har), cyclo(Tyr-Har), showed more potent antinociceptive activity than cyclo(Tyr-Arg). Therefore, O-alkylation of the Tyr residue and the replacement of Arg by Har were found to contribute greatly to its higher activity. Cyclo[Tyr(Et)-Har] (Fig. 1A) which was synthesized on the basis of these results possessed, indeed, the most potent antinociceptive activity (12, 13).

The present study was undertaken to investigate the effective sites of C.TEHA and simultaneously with this study, its effect was compared to that of the linear dipeptide B.TEHM (Fig. 1B), obtained in the process of C.TEHA synthesis (13).
Materials and Methods

Animals

Male Wistar rats weighing 250–300 g were used in all experiments. They were housed in aluminum cages and were supplied with food and water ad libitum in a room that was illuminated from 9:00 to 21:00 and kept 22±2°C.

Intracerebroventricular and spinal cannula implantation

Each animal was anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a stainless steel guide cannula modified hypodermic needle (approx. 0.6 mm in diameter) with an inner obdulator (approx. 0.3 mm in diameter) was implanted stereotaxically into the left lateral, 3rd and 4th cerebroventricles (14) according to the atlas of Pellegrino et al. (15).

The techniques for spinal administration were essentially identical to those developed by Yaksh and Rudy (16). A polyethylene cannula was inserted through a slit in the cisternal membrane under pentobarbital sodium anesthesia. The tip of the cannula was placed at the level of L4 or L5 beyond the rostral aspect of the lumber enlargement. The cannula was fixed permanently to the skull with stainless steel screws and dental cement. Before use in the experiments, both the implanted animals were allowed to recover for at least 10 days and 2 weeks, respectively. They were used only once and killed for histological confirmation of infusion sites after experiments.

Antinociceptive evaluations

Tail flick test: A mechanized tail flick apparatus (model-DS20, Ugo bacile) was used to determine the reflex latency according to the method of D’Amour and Smith (17). The distance of the heat-source bulb and reflector from the rat tail was adjusted so that the untreated rats had tail flick latencies of around 5 sec. The latencies to thermal stimulation were determined in seconds at 5, 10, 15, 30, 45, 60, 75, 90 and 120 min after the drug administration. To avoid tail tissue damage, a cut-off of 20 sec was imposed on animals failing to remove their tail from the light beam. The antinociceptive responses for each animal were represented as % of the maximum possible effect (% MPE) and calculated by the following formula:

\[
\text{% MPE} = \frac{T_t - T_o}{T_c - T_o} \times 100
\]

where \(T_o\) is the pre-drug tail flick reaction time, \(T_t\) is the reaction time at \(t\) time after drug administration, and \(T_c\) is the cut-off time.

Digitus pinching test: For another antinociceptive effect assay, the method to nip the hind-paw digitus was used as mechanical nociceptive stimulation. After the hind-paw digitus was nipped by arterial clamp, the antinociceptive effect was expressed behaviorally by the following criteria: 0: quick biting with vocalization, 1: vocalization without biting for 20 sec, 2: vocalization without biting for 5 sec, 3: vocalization without
bating in a moment, 4: no reaction.

Drugs

Drugs used were pentobarbital sodium (Dainippon), cyclo [Tyr(Et)-Har] and Boc-Tyr(Et)-Har-OMe (13), morphine hydrochloride (Takeda Chemicals, Ltd) and naloxone hydrochloride (Endo Laboratories Inc.). Each drug was dissolved in sterile Ringer's solution and administered in a volume of 5 ml. In the case of intrathecal administration, drugs were administered in a volume of 5 ml followed by a 15 ml administration of Ringer's solution to wash the cannula. Only naloxone was administered intraperitoneally (i.p.) in a volume of 0.1 ml/100 g.

Statistics

Statistical significance of the data was estimated using Student's t-test. The values of ED50 and the 95% confidence limits were determined by the method of Litchfield and Wilcoxon (18).

Results

The antinociceptive effect of C.TEHA in the tail flick response to thermal stimulation: C.TEHA administered into the lateral, 3rd and 4th cerebroventricles resulted in a dose-related antinociceptive effect. This antinociceptive activity at the three sites peaked at 5 min and almost lasted from 45 to 60 min. No remarkable behavioral change was observed except that an abnormal behavior such as rolling occurred when high doses of C.TEHA were administered into the 4th cerebroventricle.

When administered into the lateral cerebroventricle, 20 nmole of C.TEHA produced a complete inhibition of the tail flick response. Though even 10 nmole of C.TEHA showed about 50% inhibition, at doses below 20 nmole, their values of % MPE fell under one half within 15 min (Fig. 2A).

Twenty nmole of C.TEHA into the 3rd cerebroventricle completely inhibited the tail flick reaction. Its time-course or intensity was almost the same as when it was administered into the lateral cerebroventricle. (Fig. 3A).

Despite of approx. 100% inhibition by 20 nmole of C.TEHA, 10 nmole of C.TEHA produced only 22% inhibition when administered into the 4th cerebroventricle (Fig. 4A).

Fig. 2. The antinociceptive effect of C.TEHA administered into the lateral cerebroventricle in rats. A: tail flick test, B: digitus pinching test. The definition of criteria in the digitus pinching test is shown in Materials and Methods. Asterisks indicate significant difference from the Ringer control. (*P<0.05, **P<0.01 and ***P<0.001, Student's t-test)

The administration into the spinal subarachnoid space was without effect at doses of 25, 50 and 100 nmole (6 rats in each group).

In the tail flick response to thermal stimulation, the ED50 values when administered into the lateral, 3rd and 4th cerebroventricles were 9.9, 9.2 and 12.5 nmole, respectively (Table 1).

The antinociceptive effect of C.TEHA in the digitus pinching test: C.TEHA administered into the three sites was found to have dose dependent antinociceptive activities which peaked at 5 min and then decreased until about 30 min. Only by the administration into the lateral cerebroventricle was a perfect inhibition in the digitus pinching test observed with 20 nmole of C.TEHA (Figs. 2B, 3B and 4B).

No effect was observed by the administration into the spinal subarachnoid space.
Fig. 3. The antinociceptive effect of C.TEHA administered into the 3rd cerebroventricle in rats. A: tail flick test, B: digitus pinching test. For other details, see Fig. 2.

Fig. 4. The antinociceptive effect of C.TEHA administered into the 4th cerebroventricle in rats. A: tail flick test, B: digitus pinching test. For other details, see Fig. 2.

Table 1. The antinociceptive activities of C. TEHA, B. TEHM and morphine administered into the lateral, 3rd, 4th cerebroventricles and the spinal subarachnoid space measured by the tail flick test and digitus pinching test in rats. Relative potency is on a molar basis (morphine=1.00).

| Sites and drugs | Tail flick test | Digitus pinching test |
|-----------------|----------------|----------------------|
|                 | ED50* (nmole) | Relative potency | ED50* (nmole) | Relative potency |
| L.V.            |                |                     |                |                     |
| C.TEHA          | 9.9 (7.6-12.9) | 1.36                | 12.5 (10.3-15.1)| 1.76                |
| B.TEHM          | 12.0 (9.5-15.1)| 1.13                | 21.0 (16.8-26.3)| 1.05                |
| Morphine        | 13.5 (10.6-17.5)| 1.00               | 22.0 (17.3-27.9)| 1.00                |
| III.V.          |                |                     |                |                     |
| C.TEHA          | 9.2 (7.5-11.2) | 1.09                | 11.0 (9.6-12.6) | 1.45                |
| B.TEHM          | 8.2 (6.5-10.3) | 1.22                | 19.0 (13.6-26.8)| 0.84                |
| Morphine        | 10.0 (6.4-15.5)| 1.00               | 16.0 (10.7-24.0)| 1.00                |
| IV.V.           |                |                     |                |                     |
| C.TEHA          | 12.5 (9.1-17.1)| 0.56                | 14.5 (10.8-19.4)| 0.79                |
| B.TEHM          | 36.0 (22.5-57.6)| 0.19               | 43.5 (32.2-58.7)| 0.26                |
| Morphine        | 7.0 (4.5-10.6) | 1.00                | 11.5 (7.1-18.6) | 1.00                |
| Spinal          |                |                     |                |                     |
| C.TEHA          |                |                     |                |                     |
| B.TEHM          |                |                     |                |                     |
| Morphine        | 8.4 (5.6-12.6) | 1.00                | 10.5 (6.6-16.8) | 1.00                |

C.TEHA: Cyclo [Tyr(Et)-Har]. B.TEHM: Boc-Tyr(Et)-Har-OMe. *ED50 values were calculated from the values obtained at the time of peak effect. 95% confidence limits are given in parentheses.
The ED50 values for the administration into the three sites described above were 12.5, 11.0 and 14.5 nmole, respectively (Table 1).

The antinociceptive effect of B.TEHM in the tail flick response to thermal stimulation: As shown in Figs. 5A, 6A and 7A, B.TEHM produced a dose-related antinociceptive effect in the tail flick response. These effects peaked at 5 min post-administration and by 45 min were almost absent. The tail flick response was completely inhibited by 20 nmole of B.TEHM administered into the lateral and 3rd cerebroventricles, while even 40 nmole of B.TEHM administered into the 4th cerebroventricle produced only 73% inhibition. In general, the duration of B.TEHM tended to be shorter than that of C.TEHA, and the marked tendency was seen especially when administered into the 3rd and 4th cerebroventricles.

The spinal subarachnoid space administration of B.TEHM was without effect.

The ED50 values when administered into the three sites were 12.0, 8.2 and 36.0 nmole, respectively (Table 1).

The antinociceptive effect of B.TEHM in the digitus pinching test: The antinociceptive activities of B.TEHM were rather weaker than that of C.TEHA. In fact, even 30 nmole of B.TEHM was incapable of producing a complete inhibition, and especially when administered into the 4th cerebroventricle, only 38% inhibition was obtained by 40 nmole of B.TEHM (Figs. 5B, 6B and 7B).

No effect was observed by the administration into the spinal subarachnoid space.

The ED50 values in the digitus pinching test when administered into the three sites were 21.0, 19.0 and 43.5 nmole, respectively (Table 1).

Antagonism to the antinociceptive effect of both dipeptides by naloxone: Naloxone was administered i.p. 10 min prior to 15 nmole of B.TEHM into the 3rd cerebroventricle where its effect was demonstrated to be the
most potent. The antinociceptive effect of B.TEHM was antagonized about 75% by 2 mg/kg of naloxone and was completely antagonized by 8 mg/kg of naloxone compared with B.TEHM alone at 5 min in the tail flick test (Fig. 8A). In the digitus pinching test, the effect of B.TEHM was completely blocked by 2 mg/kg of naloxone (Fig. 8B). Naloxone hardly had any effect on C.TEHA administration in both tests. On the other hand, the pretreatment of 2 mg/kg of naloxone completely reversed the antinociceptive effect produced by high doses of morphine through all routes of administration (data not shown).

Discussion

C.TEHA possesses the most potent activity of all the cyclic dipeptides we have synthesized until now (12, 13). However, C.TEHA had no effect when administered into the spinal subarachnoid space and was not reversed by naloxone at doses which completely antagonized the antinociceptive effect of morphine. We previously reported that the antinociceptive effect of cyclo(N-methyl-L-Tyr-L-Arg) was incompletely reversed by 2 mg/kg or 8 mg/kg of naloxone in the mouse tail pressure test (19, 20). In addition, since the effect of C.TEHA was incompletely reversed by high dose of naloxone, it may be certain that C.TEHA-induced antinociception is mediated through opioid receptors other than mu receptors or non-opioid receptors. This speculation is further supported by the report that the antinociception of intrathecally administered D-Ala²-D-Leu⁵-enkephalin was not influenced by 2 mg/kg of naloxone and was incompletely antagonized by 6 mg/kg of naloxone (21). These results suggest the difference of mechanism among C.TEHA, B.TEHM and morphine.

Hoffman et al. described (11) that cyclic
dipeptides were resistant to enzymatic degradation both in vivo and in vitro. Furthermore, after intravenous application in cats, they easily penetrated into the cerebrospinal fluid. Judging from the above, since C.TEHA possesses a diketopiperazine structure, it is conceivable that it can resist peptidases in vivo and consequently has a longer-acting effect than former linear dipeptides. The linear dipeptides quickly lose their activity because of their rapid enzymatic degradation, but a cyclic ring structure can contribute to the enzymatic stability and the production of naloxone-resistant antinociceptive activity. Our present data indicate that B.TEHM seems to be more rapidly degraded than C.TEHA, as the antinociceptive activity of B.TEHM disappears within 30–45 min.

Takagi et al. demonstrated that kyotorphin produced antinociception when administered into the cisterna magna of mice (2) or into the nucleus reticularis gigantocellularis of rats (22). This compound did not bind opioid receptors and in addition was ineffective on the contraction of the longitudinal muscle of guinea pig ileum induced by electrical field stimulation (1). In contrast, kyotorphin induced a release of Met-enkephalin from slices of guinea pig striatum and spinal cord (23). The antinociceptive mechanism of kyotorphin has been explained to be due to its depolarizing effect against the enkephalin-containing neurons and its inhibitory effect on enkephalinase A, B or aminopeptidase activity. Consequently, it released Met-enkephalin from its storage sites and inhibited its degradation. However, there are some contradictory opinions. Vaught and Chipkin previously reported (4) a lack of Met-enkephalin releasing effect of kyotorphin, and it has been definitely shown that kyotorphin had no effect on basal Met- or Leu-enkephalin in vitro (24, 25). Moreover, according to Kilpatrick et al. (26), it is presumed that kyotorphin conceivably acts via release of β-endorphin or perhaps one of the pharmacologically active enkephalin precursor peptides. On the other hand, an inhibitory effect on enzymes involved in the catabolism of enkephalins seems unlikely because kyotorphin inhibited only weakly these enzymes in the homogenate of mice brain (1). As Fournie-Zaluski et al. also reported (27), since kyotorphin has a low inhibitory potency on enkephalinase activity, it is unable to demonstrate any inhibitory effects of kyotorphin on these enzymes.

The antinociceptive effect of B.TEHM was reversed about 75% compared with B.TEHM alone at 5 min by the pretreatment with 2 mg/kg of naloxone and was completely blocked by the higher dose of 8 mg/kg of naloxone. Though this phenomenon of reversal by naloxone is weaker than morphine, B.TEHM is distinctly considered to be an opioid, and its effect may be induced via opioid receptor interaction.

From these points of view, it is highly probable that B.TEHM acts in the same way as kyotorphin or in a manner that resembles the kyotorphinergic neurons proposed by Ueda et al. (28).

Thus, kyotorphin is a naturally occurring opioid dipeptide with narcotic-like antinociceptive effect, and it has an unknown mechanism of action which is quite different from those of other identified opioid peptides. The similarity of the antinociceptive effect of B.TEHM with that of kyotorphin is considered to be due its linear-chain structure which more closely resembles kyotorphin. However, whether B.TEHM possesses the ability to bind opioid receptors, to release enkephalin or to inhibit their analytic enzyme activities remains to be proven.

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