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D-ARG^2-DERMORPHIN TETRAPEPTIDE ANALOGS : A POTENT AND LONG-LASTING ANALGESIC ACTIVITY AFTER SUBCUTANEOUS ADMINISTRATION

Yusuke Sasaki, Michiko Matsui, Masumi Taguchi, Kenji Suzuki, Shinobu Sakurada, Takumi Sato, Tsukasa Sakurada and Kensuke Kisara

Tohoku College of Pharmacy, Komatsushima, Sendai 983, Japan

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SUMMARY : To examine pharmacological properties of D-Arg^2-dermorphin tetrapeptides, six tetrapeptide analogs based on the following formulas, H-Tyr-D-Arg-Phe-Gly-OX (X = H, ethyl, n-propyl), H-Tyr-D-Arg-Phe-Sar-OX (Sar = sarcosine; X = H, methyl, ethyl), were prepared. All these analogs exhibited highly potent and long-lasting analgesia as compared with that of morphine after subcutaneous administration in mice. Among analogs tested, H-Tyr-D-Arg-Phe-Sar-OH showed the highest activities, which were 21, 30 and 58 times more active than morphine in the tail pressure, tail flick and phenylbenzoquinone writhing tests, respectively, on a molar basis.

The very potent heptapeptide, dermorphin, with opioid activity has the following sequence of amino acid :

$$H\text{-}Tyr^1\text{-}D\text{-}Ala^2\text{-}Phe^3\text{-}Gly^4\text{-}Tyr^5\text{-}Pro^6\text{-}Ser^7\text{-}NH_2$$

(1,2). Studies on the structure-activity relationship have shown that the N-terminal tetrapeptide is the minimum sequence for the opioid activity (2-5). On the other hand, enkephalin analogs having Tyr^1-D-Arg^2 sequence have shown to possess potent analgesic activities after intracisternal administration (6, 7).

In the present work, we synthesized six dermorphin tetrapeptide analogs having Tyr^1-D-Arg^2 sequence (Table I) and it was found that those analogs showed very potent and long-lasting analgesic activities after subcutaneous (s.c.) administration in mice.

MATERIALS and METHODS

Synthesis of peptide : Syntheses of peptides were performed by the conventional solution method. Protected tetrapeptide, Boc-Tyr-D-Arg(Tos)-Phe-X-OEt (VII), where X is Gly or Sar, was constructed by the stepwise addition of each amino acid starting from H-X-OEt using dicyclohexylcarbodiimide — 1-hydroxybenztriazole method (8). Saponification of VII gave Boc-Tyr-D-Arg(Tos)-Phe-X-

Abbreviations : Sar = sarcosine, Boc = t-butoxycarbonyl, Me = methyl, Et = ethyl, Pr = n-propyl, Tos = tosyl.

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OH (VIII), which was esterified by methyliodide or n-propylbromide in the presence of KF (9) to give Boc-Tyr-D-Arg(Tos)-Phe-X-OR (IX), where R is methyl or n-propyl. Deprotection of all intermediary tetrapeptides (VII, VIII and IX) was carried out by treatment with a mixture of trifluoromethanesulfonic acid — thioanisole — trifluoroacetic acid in the presence of o-cresole (10), and followed by purifications using column chromatography on carboxymethyl cellulose, Toyopearl HW-40 and/or partition column chromatography on Sephadex G-25. Homogeneity of each peptide was checked by thin-layer chromatography, amino acid analysis and elementary analysis. Details in the synthesis will be reported in a separate paper.

The values of optical rotation and Rf on silicagel plates (GF_{254}, Merck) were as follows, I : $[\alpha]_D$ +36.0°; Rf_1 0.28, Rf_2 0.57. II : $[\alpha]_D$ +31.9°; Rf_1 0.52, Rf_2 0.74. III : $[\alpha]_D$ +26.2°; Rf_1 0.57, Rf_2 0.74. IV : $[\alpha]_D$ +45.2°; Rf_1 0.30, Rf_2 0.59. V : $[\alpha]_D$ +38.2°; Rf_1 0.41, Rf_2 0.72. VI : $[\alpha]_D$ +41.0°; Rf_1 0.48, Rf_2 0.73. Optical rotation was measured in H_2O (c=1) at 24°C. Rf values were determined using following solvent systems, Rf_1 : 1-butanol-acetic acid-H_2O (4:1:5, upper phase); Rf_2 : 1-butanol-acetic acid-pyridine-H_2O (15:3:10:12). H-Tyr-D-Ala-Phe-Gly-OH (4) was also synthesized as a reference peptide by the method essentially the same manner as described above.

Analgesic assay : Male Std-ddy strain mice (20-25g) were used in the present study. The mice were housed under an automatically lighting regimen turned on at 0090 hr and off at 2100 hr, and supplied with free access to food and water. All experiments were conducted in an ambient temperature of 23 ± 2.0°C. Mice were injected s.c. with peptides or morphine dissolved in Ringer solution.

To assess the analgesic potencies of various peptides, the tail pressure, tail flick and phenylbenzoquinone (PBQ)-induced writhing tests were utilized. The procedure of the tail pressure and tail flick tests were described previously (11). Changes in responsive tail pressure were expressed as a percentage of maximum possible effects (% MPE) as follows : % MPE = (Pt-Po/100-Po) where Po is pre-drug responsive pressure (mmHg) and Pt is responsive pressure (mmHg) at t time after drug administration.

PBQ-induced writhing test was originally described by Siegmund et al.(12). Mice were given i.p. 0.02 % PBQ in 0.5 % ethanol and saline in a volume of 0.2 ml/20 g 30 min after peptides or morphine administration. The frequency of abdominal constrictions was then counted for 20 min. The ED_{50} value was defined as the dose of drug required to reduce the number of abdominal constrictions to 50 % of the number observed in Ringer-treated mice. The ED_{50} values and 95 % confidence limits were determined by the method of Litchfield and Wilcoxone (13). The data was analysed employing the Student's *t*-test, and a significant difference between groups were taken as $p < 0.05$.

RESULTS and DISCUSSION

Analgesic potency of each analog after s.c. administration was assessed by the tail pressure, tail flick and PBQ-induced writhing tests in mice and was compared with that of morphine on a molar basis (Table I). D-Arg^2-dermorphin tetrapeptide, I, showed remarkably increased activity; 4.8 times as potent as morphine and 12 times more potent than the parent tetrapeptide which had four-tenth the potency of morphine as measured by the tail pressure assay. Greatly improved activities of analog I were also observed in the other tests. Although, in the dermorphin molecule, D-Ala^2 has been considered to be of a crucial importance for the opioid activity (2,14), these results demonstrated

Table I. Analgesic Activities of D-Arg^2-Dermorphin Tetrapeptide Analogs after Subcutaneous Administration in Mice

Compound	Tail pressure test	
	ED_{50} [mg/Kg][a]	Relative potency[b]
Morphine	6.20 (4.08-9.42)	1
H-Tyr-D-Ala-Phe-Gly-OH	21.00 (13.73-31.46)	0.4
H-Tyr-D-Arg-Phe-Gly-OH (I)	2.40 (1.46-3.96)	4.8
H-Tyr-D-Arg-Phe-Gly-OEt (II)	2.40 (1.20-4.80)	4.9
H-Tyr-D-Arg-Phe-Gly-OPr (III)	1.60 (0.98-2.62)	7.5
H-Tyr-D-Arg-Phe-Sar-OH (IV)	0.55 (0.33-0.92)	21.4
H-Tyr-D-Arg-Phe-Sar-OMe (V)	1.15 (0.59-2.23)	10.1
H-Tyr-D-Arg-Phe-Sar-OEt (VI)	1.30 (0.94-1.81)	9.3

Compound	Tail flick test		PBQ writhing test	
	ED_{50} [mg/Kg][a]	Relative potency[b]	ED_{50} [mg/Kg][a]	Relative potency[b]
Morphine	2.15 (1.14-4.04)	1	0.52 (0.33-0.84)	1
I	0.72 (0.34-1.51)	5.6	0.13 (0.08-0.22)	7.4
II	0.64 (0.42-0.97)	6.4	0.092 (0.064-0.132)	10.6
III	0.41 (0.24-0.70)	10.1	0.088 (0.050-0.154)	11.3
IV	0.137 (0.074-0.253)	29.8	0.017 (0.009-0.034)	58.0
V	0.178 (0.110-0.288)	22.9	0.056 (0.019-0.164)	17.5
VI	0.175 (0.083-0.368)	23.9	0.049 (0.031-0.078)	20.7

a) ED_{50} values were calculated from the values obtained at the time of peak effect. 95 % confidence limits are given in parentheses.
b) Relative potency is on a molar basis (morphine = 1).

that the D-Ala^2 could be replaced by D-Arg with a great success as reported in the enkephalin analogs (6,7), indicating that the D-Ala residue as a D-amino acid at the second position is not always necessary for the opioid activity. Analog II or III formed by C-terminal esterification of I resulted in an equal or slightly increased activities in all assay systems. This might be attributed to an enhanced lipophilicity of the C-terminal portion in accord with observations that the opioid activities of dermorphin tetrapeptide increase with

the lipophilic character of the C-terminal substituents (15). Compared with these mono-substituted analogs, higher activities were obtained with double-substituted analogs (IV, V and VI), in which Gly^4 was also replaced by Sar to stabilize the C-terminus against carboxypeptidases. Thus, analog IV showed the highest activities among analogs tested in this study; the potencies relative to morphine were 21, 30 and 58 times more active in the tail pressure, tail flick and PBQ-induced writhing tests, respectively. Analog V or VI formed by C-terminal esterification of IV, however, resulted in a tendency to decrease activities in the tail pressure and PBQ-induced writhing tests in contrast to the results observed in mono-substituted analogs. It should be noted that such simple tetrapeptide free acid, IV, can elicite potent analgesia superior to its C-terminal ester compounds even after s.c. administration despite the fact that the lipophilic character of IV is apparently lower than those of the ester compounds.

As shown in Fig. 1, following s.c. administration, analog IV produced analgesia lasting 180 min with a maximum effect at 45 min, while analgesic action of morphine lasted 60 min with a maximum effect at 30 min when assessed

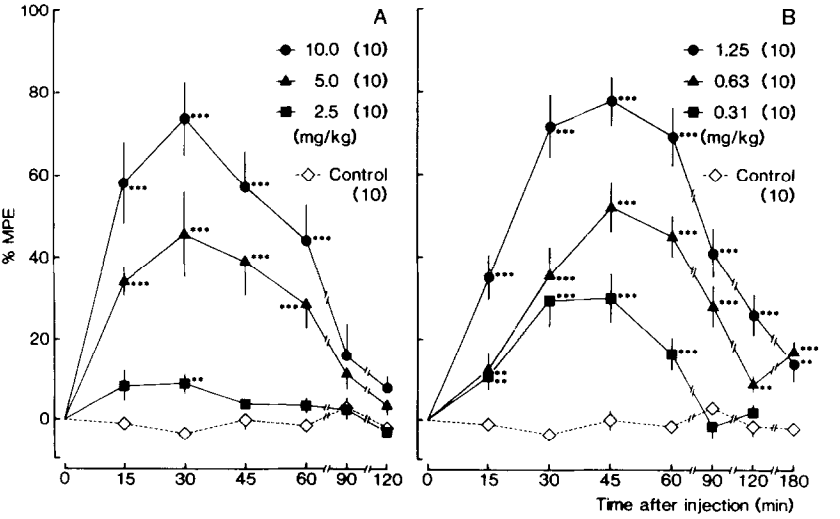

Fig. 1. Comparison of analgesic effects of morphine (A) and H-Tyr-D-Arg-Phe-Sar-OH (B) administered subcutaneously as measured by the tail pressure assay in mice.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with Ringer control.

by the tail pressure test. These results indicate that this analog has a long duration of action superior to that of morphine. Such prolonged effects exceeding that of morphine were seen in the rest of D-Arg^2-analogs as well.

Analog IV seems to be the most potent analgesic following parenteral administration among dermorphin-related peptides reported in the literature and to bear a very favorable comparison with excellent analgesics in the enkephalin series such as FK-33-824 (16), H-Tyr-D-Met(O)-Gly-Phe-NHNH-COEt (17) and SD-25 (18). In view of its outstanding potency and prolonged effect, D-Arg^2-dermorphin tetrapeptide analogs appear to have the profile of a new type of powerful analgesics.

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