Biochemical Pharmacology, Vol. 28, pp. 3333–3337.

0006-2952/79/1115-3333 $02.00/0

OPIOID ACTIVITY OF SYNTHETIC AND NATURALLY OCCURRING ENKEPHALIN PEPTIDES

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(*Received* 21 *April* 1978; *accepted* 9 *May* 1979)

Abstract—Analogs of methionine–enkephalin were synthesized, with alterations made at the *N*-terminus or at the C-terminus. Alterations at only the *N*-terminus increased *in vitro* stability of peptides when they were incubated in homogenates. Injection of peptides intracerebrally indicated that an analog with the addition of a D-alanine at the C-terminus produced potent analgesia and had a longer duration of action than methionine–enkephalin, suggesting that peptidase hydrolysis at the *N*-terminus may not be the only important mechanism of inactivation.

The naturally occurring opioid peptides, the endorphins, have been demonstrated to produce analgesia *in vivo*, and exhibit selective distribution in various forebrain regions and in the dorsal horn of the spinal cord [1–6]. A long duration of action is well demonstrated for exogenously applied endorphins in several neuronal systems [7, 8]. However, reports of studies with the smaller enkephalin peptides characteristically indicate a brief duration of action and the requirement for high concentrations of oligopeptide to demonstrate physiological effects [9–11].

The activity of the opioid peptides appears to require an amino-terminal tyrosine residue that contains a free hydroxyl group [12]. Substitution of D-Ala for Gly in the second position of the methionine–enkephalin sequence provides longer lasting enkephalin activity with retention of affinity for the opiate receptor [13]. This (D-Ala^2) methionine–enkephalin also has been further protected by modification of the carboxyl-terminus to include an amide moiety [14].

We have synthesized several peptides containing the methionine–enkephalin sequence with modifications either at the amino- or the carboxyl-terminus (Table 1). Structure A is the naturally occurring sequence of methionine–enkephalin. Peptides B, C and D were synthesized to study resistance to peptidase degradation, and peptides C and D were specifically designed to assess the necessity for a free amino group on the *N*-terminal tyrosine for opioid activity. Peptide E contains an *N*-terminal tyrosine, but it is three residues removed from Tyr^1 (by a repetition of the first three amino acids) of the enkephalin sequence. These peptides were assayed for their affinity for the opiate receptor, their stability in rat brain homogenate, and their analgetic potency *in vivo*.

METHODS AND MATERIALS

Solid phase peptide synthesis was carried out using a typical manual procedure [15]. Boc-D-alanine was esterified to chloromethylated co-poly (styrene-1% divinylbenzene) using the cesium salt procedure [16]. Chloromethyl-resin was converted to hydroxymethyl resin using potassium acetate and potassium hydroxide [17]. Boc-Methionine was esterified to hydroxymethyl resin using carbonyldiimidazole [17]. This method was used to prevent sulfonium ion formation which is known to occur during esterification of Boc-methionine to chloromethyl-resin [18]. Trifluoroacetic acid (50%) in methylene chloride was used for neutralization. Coupling was done with three equivalents of Boc-amino acid and three equivalents of dicyclohexylcarbodiimide (DCC) in methylene chloride. The Boc-amino acid was incubated for 10 min with the resin before the addition of DCC. Coupling was allowed to proceed for 1 hr. Two couplings of each amino acid were done before the next cycle was started. N^{α}-Boc-*O*-(2,6-dichlorobenzyl)-tyrosine was used in each synthesis. Peptides were removed from the resin by treatment for 1 hr at 0° with hydrogen fluoride containing 10% anisole as a cation scavenger. Methionine–enkephalin (peptide A in Table 1) was purified as follows. It was loaded on a 3 × 15 cm carboxymethyl cellulose (Bio-Rad Cellex CM) column and was eluted with a 0.04–0.4 M pyridine acetate gradient (pH 5.2) Two symmetrical peaks were visualized by absorbance at 280 nm. Peak I eluted at 100 ml; peak II eluted at 125 ml in a ratio of 2:1. A sample of each peak was subjected to paper electrophoresis. The conditions were 1000 V for 1 hr in pyridine acetate buffer (pH 5.0) on Whatman No. 1 paper. Peptidic material moved toward the cathode. Peak I gave one spot 1.3 cm from the origin. Peak II gave two spots, one at 1.3 cm and another at 3.9 cm. The behaviour of peak II in electrophoresis and on the carboxymethyl cellulose column was taken to indicate the presence of a methionine sulfonium ion formed by alkylation of sulfur. The material from peak I was then chromatographed on a 0.9 × 60.5 cm Dowex 50X-4 (Bio-Rad AG50w-X4, 200–400 mesh) column attached to a Beckman 120B amino acid analyzer. The chromato-

gram was developed in 0.5 M pyridine acetate buffer (pH 5.2) at 57°. The main peak, representing methionine–enkephalin, was symmetrical and eluted at 301 ml. This represented 93 per cent of the total ninhydrin positive material. The other 7 per cent was found in two peptide peaks which were shown to contain methionine sulfoxide or methionine sulfone in place of methionine. It appears that methionine sulfoxide and sulfone arise in the workup of the peptide because collection, multiple lyophilization and rechromatography of the homogenous methionine–enkephalin peak again showed the same proportions of the three peaks. The overall yield of methionine–enkephalin was 15.5 per cent.

Peptides B–D (Table 1) were subjected to the protocol described for methionine–enkephalin. The procedure on the Dowex 50 column was altered for peptide E. Since this peptide was retained longer or eluted over too broad a range in 0.5 M pyridine acetate, due to the additional tyrosine, it was eluted in 1.0 M pyridine acetate (pH 5.2).

The lyophilized dry samples were 94–98 per cent peptide according to amino acid analyses. The amino acid ratios were correct except for low methionine values, which controls showed to be the result of partial oxidation of methionine during hydrolysis.

Stereospecific binding to the opiate receptor was carried out in rat brain homogenate preparations as described elsewhere but with a slight modification [19]. Mature rat brain was homogenized in 4 vol. of 0.05 M Tris–HCl buffer (pH 7.4). Aliquots containing 2.0 mg protein were incubated with 10^{-9} M [^{3}H]naloxone (20 Ci/mmole), together with various concentrations of enkephalin, endorphin or an enkephalin analog, for 10 min at 25° followed by immersion in ice water for 10 min. This procedure yielded essentially identical binding activity as that produced with a 2-hr incubation at 0°. The aliquots were then washed on Whatman GF/B filters under suction. The filters were dried under infrared lamps, and radioactivity remaining with the protein on the filter was determined, in a toluene-based scintillation mixture (10 ml), on a scintillation spectrometer. Control samples of homogenate were incubated for the same time periods prior to binding with [^{3}H]naloxone in the presence and absence of 10^{-6} M levallorphan. The IC_{50} values were calculated from log-probit plots.

The stability of the peptides was determined by incubating each peptide sample in brain homogenate at 25° for 0, 30, 60 and 90 min, followed by binding with [^{3}H]naloxone as described above. Control samples of homogenate preparations were incubated for the same time periods prior to binding with [^{3}H]naloxone. Protein concentration was determined by the method of Lowry *et al.* [20].

In vivo testing of analgetic potency of various peptides was done using intracerebral injection of the compounds. Adult male Sprague–Dawley rats, 70 days of age, were implanted with indwelling 30 gauge cannulae by stereotactic procedures under pentobarbital anesthesia. Each animal was implanted with an outer cannula to a depth of 1.5 mm above the cerebral aqueduct (P, −0.5 mm; L, 0.0 mm; V, 2.3 mm below the dura), fitted with an inner stylet and plastic protective housing, and allowed 7 days to recover. Each animal was tested for footshock threshold by the flinch–jump technique on two successive days without intracerebral injection [21]. On a third day, each animal was tested prior to microinjection. A 35 gauge injection cannula was then used to inject 1.0 μl of solution into the indwelling cannula to a depth of 2.0 mm below the outer cannula over a period of 70 sec in the unanesthetized animal. The volume delivered by infusion pump was metered in all cases. Following the first infusion of sterile saline, the animal was retested at 10, 30, 60, 90, 180 and 240 min post-injection. Each test consisted of 0.2-sec duration footshocks at 10-sec intervals at each level, with five consecutive repetitions at 0.03, 0.05, 0.075, 0.100, 0.150, 0.200, 0.250 and 0.300 mA successively. Animals were subjected to repeated injection-test series with saline or sample in 1.0 μl saline at no less than 48-hr intervals. Naloxone–HCl (courtesy of Endo Laboratories, Garden City, NY) was injected i.p. at 2 mg/kg, s.c. Each animal was used for no more than five microinjections after ensuring that he was analgetic to microinjection of 2.5 μg morphine sulfate. Brains

Table 1. Analytical data on synthetic methionine enkephalin and analogues

	Structure	Amino acid ratios* Ala	Tyr	Gly	Phe	Met	Dowex 50 ion exchange elution volume† (ml)
A	Tyr–Gly–Gly–Phe–Met		1.00	2.00	0.94	1.03	301
B	Tyr–Gly–Gly–Phe–Met–(D)-Ala	1.05	0.97	2.00	0.98	0.86	303
C	Ala–Tyr–Gly–Gly–Phe–Met	0.98	0.95	2.06	1.00	0.83	263
D	(D)-Ala–Tyr–Gly–Gly–Phe–Met	1.01	0.94	2.07	1.00	0.84	270
E‡	Tyr–Gly–Gly–Tyr–Gly–Gly–Phe–Met		1.89	4.16	0.95	0.82	268

* Samples were hydrolyzed under vacuum with 6 N HCl at 110° for 24 hr. Samples were flushed with N_2 and mercaptoethanol was added prior to evacuation. The value for each amino acid is the average of three determinations. Small amounts of Met O and Met O_2 were detected, but are not included in the Met values shown.

† Peptides were chromatographed on a 0.9 × 60.5 cm Dowex 50X-4 column attached to a Beckman 120B amino acid analyzer. The elution buffer was 0.5 M pyridine/acetate (pH 5.2); temperature, 57°. Peptides were visualized using the ninhydrin system of the analyzer.

‡ The additional tyrosine residue caused this peptide to elute in an extemely broad peak in 0.5 M pyridine/acetate; therefore, a 1.0 M pyridine/acetate (pH 5.2) buffer was used.

were perfused, fixed in buffered formalin, and a 32 μm section was stained with cresyl violet to verify cannula placement. All peptides were suspended in sterile isotonic saline immediately prior to injection.

RESULTS

Data on the competition between peptides and [^{3}H]naloxone for binding to receptors in rat brain particulate fraction and on the stability of the peptides in the particulate fraction are presented in Table 2. As reported by others, methionine–enkephalin (peptide A) is about as effective as morphine (on a molar basis) in inhibiting [^{3}H]naloxone binding [12]. Similarly, β-endorphin was about seven times more effective in this respect than morphine. The addition of D-alanine to the *C*-terminus of methionine–enkephalin (peptide B) did not appear to alter the receptor binding activity. Addition of D-alanine to the *N*-terminus (peptide D), however, divested the peptide of nearly all binding activity. Addition of L-alanine to the *N*-terminus (peptide C) allows the retention of about 10 per cent of the binding activity and suggests that aminopeptidase activity in brain homogenates may cleave the L-alanine to produce sufficient methionine–enkephalin pentapeptide to manifest the observed binding. However, some intrinsic affinity of peptide C cannot be ruled out. An additional Tyr–Gly–Gly sequence on the *N*-terminus of methionine–enkephalin (peptide E) also allowed the retention of measurable receptor binding activity. Again, it is not clear whether this represents hydrolysis to methionine–enkephalin or intrinsic activity of the intact octapeptide.

Peptides A and B were used to compete against [^{3}H]naloxone binding in rat brain homogenates in the presence and absence of 100 mM Na^+ to determine if the peptides exhibit a sodium shift [22, 23]. Peptide A (Met^5–enkephalin) exhibited a 25-fold decrease in binding in the presence of sodium, and peptide B an 83-fold decrease in binding, indicating that these peptides have agonist-like properties.

The stability of the various peptides incubated with homogenates indicated similar stability of methionine–enkephalin from our synthetic and commercial sources. Met^5–enkephalin showed a 50 per cent loss of binding activity at 60 min when incubated at 3.5×10^{-7} M in a brain particulate preparation at 25°. Peptide B exhibited a "half-life" of 67 min at a similar concentration. Peptide C showed a slight prolongation of activity that could result from aminopeptidase removal of the l-alanine *N*-terminus to yield Met^5–enkephalin, which is subsequently degraded with the time course characteristic of this peptide. Peptide E showed a substantial increase in stability that may also result from a process similar to that for peptide C. Peptide D showed no substantial binding activity and no change in activity with incubation. This confirms the necessity of the tyrosine amino group for activity. α-Endorphin evidenced no measurable loss of activity for up to 90 min of incubation.

In vivo, 2.5 μg of morphine sulfate injected intracerebrally produced an 88 per cent increase in footshock at peak analgesia that persisted for a total duration of about 45 min (Fig. 1). Ten μg of morphine sulfate produced a proportionately greater peak analgesia and a longer duration of detectable analgesia. Methionine–enkephalin (peptide A) at 2.5 μg produced a 44 per cent increase in footshock threshold ($P < 0.05$) with a duration similar to that produced by the same weight of morphine sulfate. Naloxone was injected (2 mg/kg, i.p.) in some animals immediately following the 10 min Met–enkephalin test time. Twenty min later the analgesia normally evident with Met–enkephalin was completely attenuated in naloxone-treated animals. On a molar basis, methionine–enkephalin was thus 2- to 3-fold less effective than morphine as an analgetic. At 10 μg, methionine–enkephalin evidenced a greater peak effect than with 2.5 μg, but the duration of this analgesia was considerably shorter and was detectable for only a few minutes. This effect is puzzling and is under further investigation. Peptide B is an effective analgetic at very low doses; 0.5 μg produced a 40 per cent increase in footshock threshold with a duration of 30 min. Increased doses at 2.5 μg and 10 μg produced dose-related threshold increases. There was a marked dose-related increase in the duration of analgesia as well. Analgesia was clearly evident at 10 min following microinjection but appeared to increase later, at both 2.5 μg and 10 μg, to reach peak effect at 60–90 min. It is apparent that protection of opioid peptides at the carboxyl-terminus is sufficient to produce long-lasting effects when these peptides are injected into the cerebral aqueduct.

Table 2. Structure–activity relationships in receptor binding and relative stability of synthetic and naturally occurring opioid peptides

	Structure	IC_{50}* (nM)	Stability† (min)
A	Tyr–Gly–Gly–Phe–Met	20	60
B	Tyr–Gly–Gly–Phe–Met–(D)-Ala	30	67
C	Ala–Tyr–Gly–Gly–Phe–Met	185	87
D	(D)-Ala–Tyr–Gly–Gly–Phe–Met	8000	No activity loss
E	Tyr–Gly–Gly–Tyr–Gly–Gly–Phe–Met	230	116
	α-Endorphin	35	No activity loss
	β-Endorphin	3	
	Morphine	21	

* IC_{50} is the concentration that effects a 50 per cent reduction in the binding of [^{3}H]naloxone (10^{-9} M).

† Stability was determined by incubation with brain homogenate prior to binding assay. Times are approximate incubation times (min) to loss 50 per cent activity, when preincubated at 25° at concentrations from 0.3 to 10 μM.

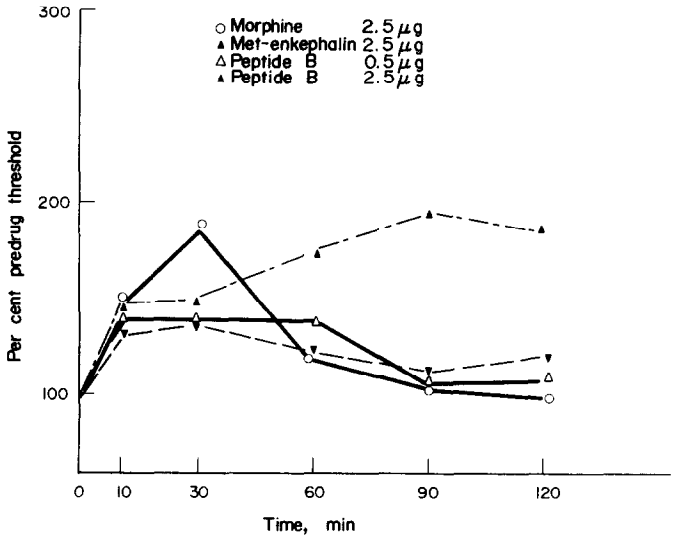

Fig. 1. *In vivo* analgetic effects of various opioids. Each compound was injected in a 1.0 μl volume into indwelling cannulae in the cerebral aqueduct of rats. Animals were tested for footshock thresholds before and at various intervals after the microinjection, as described in the text.

Microinjection of peptides into the aqueduct resulted in demonstrable analgesia. However, injection of Met^5–enkephalin into the floor of the aqueduct at the level of the boundary of the dorsal raphe nucleus resulted in the appearance of no analgetic effects.

DISCUSSION

Injection into the cerebral aqueduct is a very sensitive route by which to measure analgesia. Injection of peptides with opioid activity into periaqueductal tissue does not permit detection of analgesia, in our hands, at the low doses used. We postulate that this is due to the tearing of tissue in the vicinity of the injection site, thereby releasing lysosomal degradative enzymes that attack the injected peptide. Takagi *et al.* [24] have also reported reliable analgesia production by microinjection of very low doses of Met–enkephalin at the level of the brainstem. From analysis of our data it is apparent that protection of enkephalin peptides at the carboxyl-terminus is sufficient to convey longer-lasting analgetic effectiveness when they are injected into the cerebral aqueduct.

The stability of two peptides with *N*-terminal amino acid additions was somewhat greater than that of methionine–enkephalin itself. This we interpret as indicating aminopeptidase cleavage to produce methionine–enkephalin that is subsequently degraded at the rate observed for methionine–enkephalin in homogenate. This interpretation is supported by the hexapeptide in which D-alanine is added to the *N*-terminus. The binding activity of this peptide was extremely low, yet it remained completely stable when incubated in the brain homogenate for up to 90 min. Addition of D-alanine to the carboxyl-terminus had virtually no effect on the rate of loss of binding activity. This high affinity opioid (peptide B) was not distinguishable from methionine–enkephalin itself with respect to *in vitro* stability and receptor affinity. The activity of peptide B could be thought to reside in the methionine–enkephalin moiety subsequent to carboxy terminal degradation of the D-Ala^6 residue. However, the binding would require that 100 per cent of peptide B be degraded rapidly to render complete conversion to methionine–enkephalin early in the binding assay. This is highly unlikely under the assay conditions used in these studies. However, upon microinjection into the rat brain, this peptide exhibited greater potency and longer duration of analgesia than methionine–enkephalin. The reason for this increased analgetic activity is not clear. The possibility is entertained that degradative enzyme at or near the carboxy-terminal may play a greater role in peptide hydrolysis in whole brain or CSF than it does in brain homogenate [25]. Protection of the carboxy-terminus would then be of greater importance for *in vivo* potency than that reflected by studies of stability of the peptide during incubation with brain homogenate *in vitro*.

If peptide E, Tyr–Gly–Gly–Tyr–Gly–Gly–Phe–Met, remains intact during the binding assay it might be concluded that the methionine–enkephalin moiety is responsible for activity and, therefore, a free amino group on the tyrosine need not be in position 1 relative to methionine at position 5. However, all previous data on enkephalin analogs have indicated that a free amino group on tyrosine is essential for activity and, in addition, Tyr–Gly–Gly–Tyr–Met is not active [12]. Therefore, it is more probable that the observed binding activity was the result of peptidase activity yielding free methionine–enkephalin. The data suggest several structural analogs that may clarify these questions.

Acknowledgements—This research was supported by grants to Dr. E. J. Simon (DA 00017) and to Dr. K. A. Bonnet (DA 01113) from the National Institute of Drug Abuse.

REFERENCES

1. J. D. Beluzzi, N. Grant, V. Garsky, D. Saratakis, C. D. Wise and L. Stein, *Nature, Lond.* **258**, 577 (1975).
2. F. Bloom, D. Segal, N. Ling and R. Guillemin, *Science* **194**, 330 (1976).
3. Y. F. Jacquet and N. Marks, *Science* **194**, 632 (1976).
4. R. Elde, T. Hokfeldt, O. Johansson and L. Terenius, *Neuroscience* **1**, 347 (1976).
5. S. G. Atweh and M. J. Kuhar, *Brain Res.* **129**, 1 (1977).
6. R. Simantov, A. Snowman and S. H. Snyder, *Brain Res.* **107**, 650 (1976).
7. W. Feldberg and D. G. Smyth, *Br. J. Pharmac.* **60**, 445 (1977).
8. H. H. Loh, L. F. Tseng, E. Wei and C. H. Li, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2895 (1976).
9. H. H. Büchser, R. C. Hill, D. Römer, F. Cardinaux, A. Closse, D. Hauser and J. Pless, *Nature, Lond.* **261**, 423 (1976).
10. R. C. A. Frederickson, R. Nickander, E. L. Smithwick, R. Shuman and F. H. Norris, in *Opiates and the Endogenous Opioid Peptides* (Ed. H. Kosterlitz), p. 239. Elsevier/North Holland, Amsterdam (1967).
11. L. Graf, J. I. Szekeley, A. Z. Ronai, Z. Dunai-Kovacks and S. Bajusz, *Nature, Lond.* **263**, 240 (1976).
12. J-K. Chang, B. T. W. Fong, A. Pert and C. B. Pert, *Life Sci.* **18**, 1973 (1976).
13. C. B. Pert, A. Pert, J-K. Chang and B. T. W. Fong, *Science* **194**, 330 (1976).
14. M. M. Puig, P. Gascon, G. L. Graviso, R. A. Bjur, G. Matsueda, J. M. Steward and J. M. Musacchio, *Archs. int. Pharmacodyn. Thér.* **226**, 69 (1977).
15. B. W. Erickson and R. B. Merrifield, in *The Proteins* (Eds. H. Neurath and R. L. Hill), Vol. II, 3rd Edn., p. 312. Academic Press, New York (1976).
16. B. F. Gisin, *Helv. chim. Acta* **56**, 1476 (1973).
17. J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*. W. H. Freeman, San Francisco (1969).
18. P. Sieber and B. Iselin, *Helv. chim. Acta* **51**, 622 (1968).
19. E. J. Simon, J. M. Hiller and I. Edelman, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1947 (1973).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. K. A. Bonnet and K. Peterson, *Pharm. Biochem. Behav.* **3**, 47 (1975).
22. E. J. Simon, J. M. Hiller, J. Groth and I. Edelman, *J. Pharmac. exp. Ther.* **192**, 531 (1975).
23. C. B. Pert and S. H. Snyder, *Molec. Pharmac.* **10**, 868 (1974).
24. H. Takagi, M. Satoh and A. Akaike, *Eur. J. Pharmac.* **45**, 91 (1977).
25. N. Marks, in *Peptides in Neurobiology* (Ed. H. Gainer), p. 221. Plenum Press, New York (1977).