Pharmacology Biochemistry & Behavior, Vol. 19, pp. 791–794, 1983.

Leucinal Inhibits Brain Aminopeptidase Activity and Potentiates Analgesia Induced by Leu-Enkephalin

KENNETH R. DAVIS,* DANIEL E. HERNANDEZ† AND RICHARD WOLFENDEN*

**Department of Biochemistry and Nutrition*
†Department of Psychiatry
University of North Carolina at Chapel Hill, Chapel Hill, NC 27514

Received 9 February 1983

DAVIS, K. R., D. E. HERNANDEZ AND R. WOLFENDEN. *Leucinal inhibits brain aminopeptidase activity and potentiates analgesia induced by leu-enkephalin.* PHARMACOL BIOCHEM BEHAV **19**(5) 791–794, 1983.—L-Leucinal, a transition state analog inhibitor of kidney leucine amino-peptidases, was found to serve as a competitive inhibitor of soluble aminopeptidase activity from rat brain (K_i=22 μM). Simultaneous intracisternal administration of leucinal and leu-enkephalin produced dose-dependent analgesia in the hot plate test in mice, in concentrations at which neither L-leucinal nor leu-enkephalin alone elicited a significant response. Simultaneous administration of leucinal and β-endorphin also resulted in potentiation of β-endorphin's analgesic activity. Leucinal did not enhance the antinociceptive response to neurotensin or to [D-ala^2]-met-enkephalinamide, which are structurally resistant to the action of brain aminopeptidases.

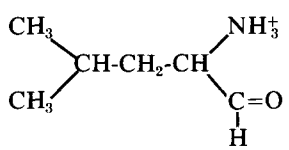
Leucine aldehyde Inhibitor Aminopeptidase Analgesia Enkephalin Neurotensin

THE lifetime of exogenously administered enkephalins in brain is short, and a considerable body of evidence suggests that brain aminopeptidases participate in their degradation [10, 12, 13, 16, 25]. Analgesic effects of enkephalins, enkephalin analogues, and morphine have been found to be enhanced by co-administration of protease inhibitors [1, 9, 18]. In some cases, analgesia has been observed when protease inhibitors were injected alone at very high doses [9, 15, 17, 18, 20]. Many of these compounds are related in structure to opiate peptides, and it remains to be established whether their effects result from protease inhibition, from direct combination with opiate receptors, or both.

Aldehydes, structurally related to peptide substrates, serve as transition state analog inhibitors of endopeptidases such as papain and elastase [19,26]. These inhibitors form complexes that resemble intermediates in substrate transformation, and are therefore bound with extraordinary affinity and specificity. Very recently, simple amino aldehydes such as leucinal have been found to act as reversible inhibitors of kidney, with affinities far exceeding those of inhibitors previously examined [2]. Accordingly we decided to examine the effects of leucinal (see below) on aminopeptidase activity from brain, and on the antinociceptive activity of exogenously administered leu-enkephalin, [D-ala^2]-met-enkephalinamide, β-endorphin and neurotensin in the hot-plate test in mice.

METHOD

Leucinal was prepared by the action of alcohol dehydrogenase on L-leucinol, as previously described [3]. The concentration of leucinal in stock solutions was determined spectrophotometrically by quantitative ninhydrin analysis [14]; the observed color yield of leucinal at 570 nm was equivalent to 86% of that obtained with leucine. Leu-enkephalin, [D-ala^2]-met-enkephalinamide, β-endorphin, puromycin, and L-leucine *p*-nitroanilide were purchased from Sigma (St. Louis, MO). Neurotensin was obtained from Bachem (Torrance, CA). [Tyrosyl-3, 5-^{3}H]enkephalin (5-L-leucine) (61 mCi/mg) was purchased from Amersham (Arlington Heights, IL) and was purified by Porapak Q column chromatography. Because of its known lability, tritiated enkephalin was purified within three days of use. Adult male Swiss-Webster mice were obtained from Flow Laboratories (Dublin, VA). Sprague-Dawley rat brains were provided by Dr. Greg King, Department of Pharmacology, University of North Carolina at Chapel Hill.



Leucinal

Soluble aminopeptidase activity, prepared by centrifugation of brain homogenates obtained from Sprague-Dawley rats as previously described [5], was monitored by following the breakdown of radioactive enkephalin [23] and by a continuous spectrophotometric assay based on the release of *p*-nitroaniline from the substrate L-leucine *p*-nitroanilide ($\Delta\epsilon^{405}$=9620) [24].

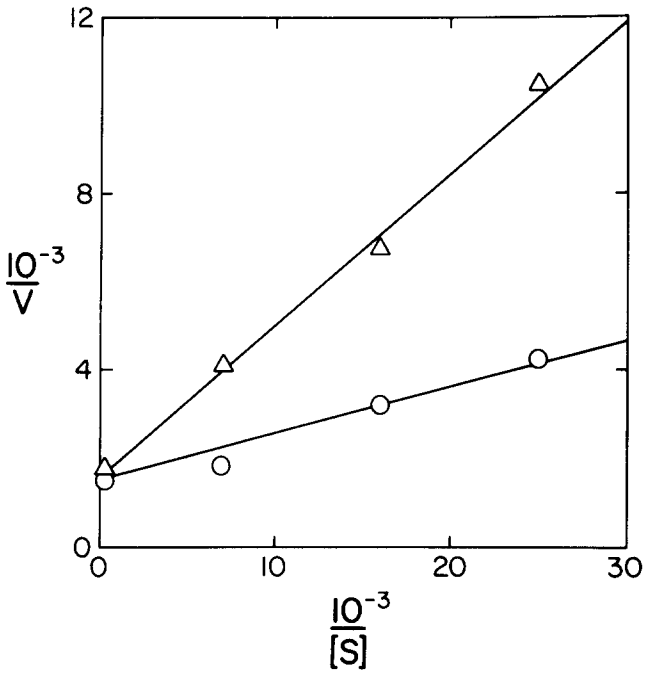


FIG. 1. Double reciprocal plot of the hydrolysis of L-leucine *p*-nitroanilide by rat brain extracts, in the presence (△) and absence (○) of L-leucinal (2×10^{-5} M), at 25° in Tris-HCl buffer (0.05 M, pH 7.4) containing dithiothreitol (1×10^{-3} M). Final protein concentration was 0.19 mg/ml; V is expressed as μmole min^{-1}.

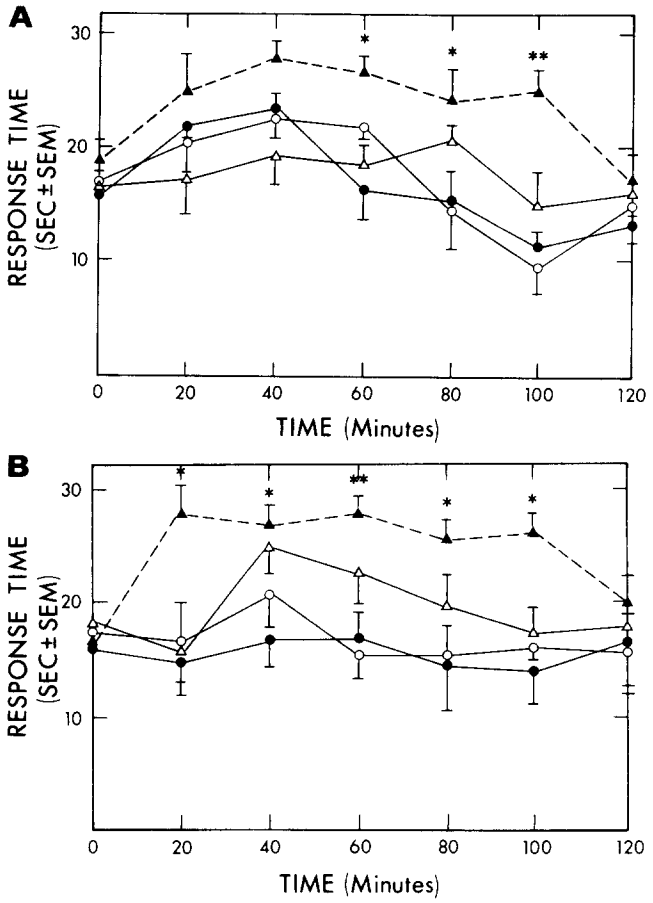


FIG. 2. The effect of intracisternally administered leu-enkephalin (180 nmole) (△), leucinal (●), or both (▲), on nociception in mice subjected to the hot-plate test. Responses were measured at times beginning 5 minutes after injection, when they had recovered from analgesia. Mice receiving saline only are marked (○). Doses of leucinal were 0.6 μmole (Panel A), and 0.95 μmole (Panel B). Means (±S.E.M.) of response time were calculated at each time point. The symbols (*) and (**) refer to differences from the saline control significant at the levels $p<0.05$ and $p<0.01$, respectively (see text).

Antinociceptive activity was assessed using the hot-plate test in adult mice (25–30 g), that had been given laboratory chow and water and kept on a light-dark cycle of 12 hours for at least one week. Groups of 8 mice were injected intracisternally (IC) under light anesthesia [11]; substances injected in vehicle (10 μl 0.16 mol/L NaCl, pH 7.5) included: neurotensin (18 nmol), leu-enkephalin (180 nmol), [D-ala^2]-met-enkephalinamide (17 nmol), β-endorphin (18 nmol), and L-leucinal at various concentrations. To examine the combined effects of leucinal and one of the peptides, they were administered in separate injections. After 5 minutes (time zero in Figs. 2 and 4), mice had recovered from anesthesia and were placed with all four paws on a heated copper plate (50–52°). This temperature had been reported to be effective in screening substances with potential analgesic activity [4, 8, 18]. A trained observer, unaware of the treatment regimen, recorded the time elapsed until the mouse licked its paws or jumped. To avoid injury, animals not responding within 30 sec were removed from the stimulus. Each animal was tested every 20 min over a period of 2 hr. A significant ($p<0.05$) increase in response time for experimental as compared with control animals, assessed by Dunnett's test for multiple comparisons [7], was defined as antinociception. The dose-response curve was analyzed by the method of Wilkinson [27].

RESULTS

Figure 1 shows that inhibition of the soluble brain aminopeptidase by leucinal was apparently competitive, with $K_m=9.4\ (\pm1.1)\times10^{-5}$ mol/L for leucine-*p*-nitroanilide, and apparent $K_i=2.2\ (\pm0.4)\times10^{-5}$ mol/L for leucinal. Of the total aldehyde in solution, 97.5% is present as the gem-diol in neutral aqueous solution [2]. If it is assumed that the free aldehyde is the true inhibitor, then the true K_i value is 5.5×10^{-7} mol/L. Similar results were obtained using radioactive leu-enkephalin: at substrate concentrations ranging from 5×10^{-6} to 2.5×10^{-4} mol/L, at 32° in Tris-HCl buffer (0.1 mol/L, pH 7.5), the concentration of inhibitor that was required for 50% inhibition ranged from 5×10^{-6} to 1.5×10^{-5} mol/L. Only a single aminopeptidase, sensitive to puromycin, appears to be present in the soluble portion of rat brain extracts [12, 19, 25]. Both activities examined above were strongly inhibited by puromycin at a concentration of 10^{-5} mol/L.

Leucinal or leu-enkephalin, when injected intracisternally into mice, exhibited no significant analgesic activity as measured by the hot-plate test. However simultaneous administration of leucinal and leu-enkephalin produced a rapid and prolonged antinociceptive response that was dose-dependent. Figure 2 illustrates the effect of co-administration of leu-enkephalin (180 nmol) and leucinal at two different dose levels (60 nmol in Fig. 2A; 95 nmol in Fig.

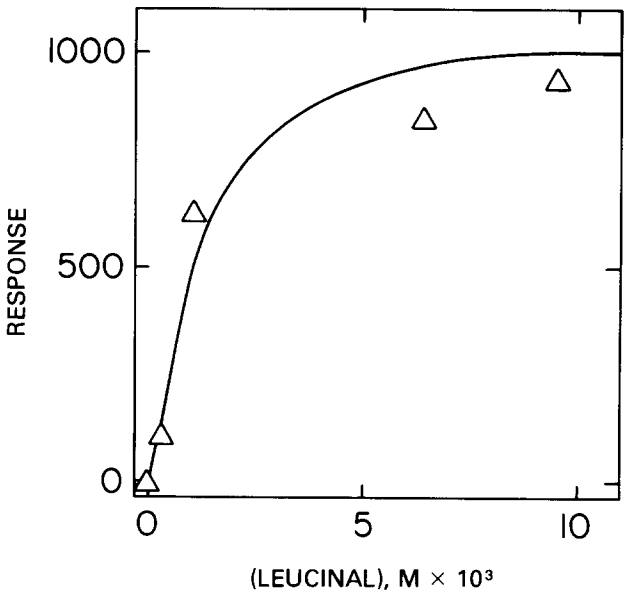


FIG. 3. Effect of variable leucinal on the antinociceptive activity of leu-enkephalin; conditions as in Fig. 2. The ordinate, in units of (sec)×(min), was calculated by determining the area separating dosed animals from controls. The abscissa represents the concentrations of leucinal present in a fixed volume (10 μl) of vehicle injected into each animal. The line is a theoretical curve generated by an unweighted least-squares fit of the data to a rectangular hyperbola, using the method of Wilkinson [27].

2B). Each point in the dose-response curve of Fig. 3 represents one of a series of experiments like the two shown in Figs. 2A and 2B. Each response, expressed as the change in area under the 2 hr antinociception curve (combination minus saline control) is plotted as a function of the concentration of leucinal used in that experiment.

In an additional set of experiments, we examined the effect of leucinal on the antinociceptive activity of β-endorphin (18 nmol). This dose produced antinociception that was significantly enhanced by co-administration of leucinal (80 nmol) (Fig. 4). However similar experiments performed with neurotensin, even at a dose (18 nmol) that was sufficient by itself to produce significant antinociceptive activity, showed no enhancement of this activity by leucinal (80 nmol). Finally, in a similar manner, IC administration of leucinal (80 nmol) did not significantly enhance the analgesic response to [D-ala²]-met-enkephalinamide (17 nmol) (data not shown).

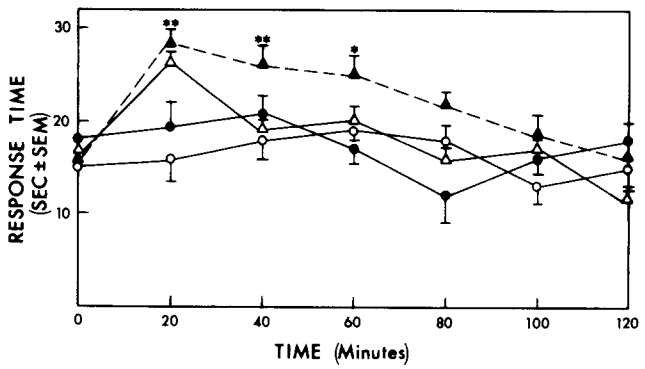


FIG. 4. The effect of intracisternally administered β-endorphin (18 nmol) (△), leucinal (80 nmol) (●), or both (▲) on nociception. Mice receiving saline only are marked (○). For other symbols, see Fig. 2.

DISCUSSION

The N-terminus of enkephalin condenses reversibly with acetaldehyde in concentrated solution, to yield adducts with diminished opiate activity [21]. Concentrations of leucinal and leu-enkephalin present in the injections used in the present experiments were far below those that produced detectable condensation *in vitro*, so that adducts of this kind are unlikely to have been present in more than trace quantitites.

In earlier work it was demonstrated that enkephalins, injected into the cerebrospinal fluid at doses up to 360 nmol, did not produce any antinociceptive response in rats [6]. This lack of effect of enkephalins was believed to be due to rapid degradation by aminopeptidases. In view of the powerful inhibition of soluble and microsomal kidney aminopeptidases by leucinal [2], the physiological site of action of leucinal in the present experiments seems likely to be a proteolytic enzyme or group of enzymes. Its identification as an aminopeptidase is consistent with the failure of leucinal to potentiate the analgesic effects of exogenous neurotensin or [D-ala²]-enkephalinamide. Neurotensin lacks a free amino terminus, and [D-ala²]-enkephalinamide is structurally resistant to the action of brain aminopeptidase [16].

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Health (GM-18325, MH-32316, MH-34121, MH-33127, MH-22536 and HD-03110). We thank Donald A. Stanley for excellent technical assistance.

REFERENCES

1. Algeri, S., M. Altstein, G. M. De Simone and V. Guardabasso. In vivo potentiation of [D-ala²]met-enkephalinamide central effects after administration of an enkephalinase inhibitor. *Eur J Pharmacol* **74:** 261–262, 1981.
2. Andersson, L., T. C. Isley and R. Wolfenden. α-Aminoaldehydes: transition state analog inhibitors of leucine aminopeptidase. *Biochemistry* **21:** 4177–4180, 1982.
3. Andersson, L. and R. Wolfenden. A general method of α-aminoaldehyde synthesis using alcohol dehydrogenase. *Anal Biochem* **124:** 150–157, 1982.
4. Ankier, S. I. New hot plate tests to quantify antinociceptive and narcotic antagonist activities. *Eur J Pharmacol* **27:** 1–4, 1974.
5. Barclay, R. K. and M. A. Phillips. Inhibition of enkephalin-degrading aminopeptidase activity by certain peptides. *Biochem Biophys Res Commun* **96:** 1732–1738, 1980.
6. Belluzzi, J. D., N. Grant, V. Garsky, J. Sarantakis, C. D. Wise and L. Stein. Analgesia induced *in vivo* by central administration of enkephalin in rat. *Nature* **260:** 625–626, 1976.
7. Dunnett, C. W. New tables for multiple comparisons with a control. *Biometrics* **20:** 284–491, 1964.
8. Frederickson, R. C. A., E. L. Smithwick, R. Shuman and K. G. Bernis. Metenkephalinamide, a systemically active analog of methionine, enkephalin with potent opioid δ-receptor activity. *Science* **211:** 603–605, 1981.

9. Hachisu, M., T. Nakamura, H. Kawashima, K. Shitoh, S. Fukotsu, T. Koeda, Y. Sekizawa, M. Munakata, K. Kenji, H. Umezawa, T. Takeuchi and T. Aoyagi. Relationship between enhancement of morphine analgesia and inhibition of enkephalinase by (2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoic acid derivatives. *Life Sci* **30:** 1739–1746, 1982.
10. Hambrook, J. M., B. A. Morgan, M. J. Rance and C. F. C. Smith. Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. *Nature* **262:** 782–783, 1976.
11. Hernandez, D. E., C. B. Nemeroff and A. J. Prange, Jr. Ontogene of the hypothermic response to centrally administered neurotensin in rats. *Dev Brain Res* **3:** 497–501, 1982.
12. Hersh, L. B. and J. F. McKelvey. An aminopeptidase from bovine brain which catalyzes the hydrolysis of enkephalin. *J Neurochem* **36:** 171–178, 1981.
13. Knight, M. and W. A. Klee. The relationship between enkephalin degradation and opiate receptor occupancy. *J Biol Chem* **253:** 3843–3847, 1978.
14. Moore, S. and W. H. Stein. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J Biol Chem* **211:** 907–913, 1954.
15. Patthy, A., L. Gráf, A. Kenessey, J. I. Székely and S. Bajusz. Effect of bacitracin on the biodegradation of β-endorphin. *Biochem Biophys Res Commun* **79:** 254–259, 1971.
16. Pert, C. B., A. Pert, J.-K. Chang and B. T. W. Fong. [D-Ala^2]-met-enkephalinamide: a potent, long-lasting synthetic pentapeptide analgesic. *Science* **194:** 330–332, 1976.
17. Pinsky, C., A. K. Dua and F. S. LaBella. Phenylmethylsulfonyl Fluoride (PMSF) given systemically produces naloxone-reversible analgesia and potentiates effects of beta-endorphin given centrally. *Life Sci* **31:** 1193–1196, 1982.
18. Roques, B. P., M. C. Fournié-Zaluski, E. Soroca, J. M. Lecomte, B. Malfroy, C. Llorens and J. C. Schwartz. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* **288:** 286–288, 1980.
19. Schnebli, H. P., M. A. Phillips and R. K. Barclay. Isolation and characterization of an enkephalin-degrading aminopeptidase from rat brain. *Biochim Biophys Acta* **569:** 89–98, 1979.
20. Simmons, W. H. and R. F. Ritzmann. An inhibitor of opioid peptide degradation produces analgesia in mice. *Pharmacol Biochem Behav* **13:** 715–718, 1980.
21. Summers, M. C. and S. L. Lightman. A reaction of acetaldehyde with enkephalins and related peptides. *Biochem Pharmacol* **30:** 1621–1627, 1981.
22. Thompson, R. C. Binding of peptides to elastase: implications for the mechanisms of substrate hydrolysis. *Biochemistry* **13:** 5495–5501, 1974.
23. Vogel, Z. and M. Altstein. The adsorption of enkephalin to porous polystyrene beads: a simple assay for enkephalin hydrolysis. *FEBS Lett* **80:** 332–336, 1977.
24. Wachsmuth, E. G., I. Fritze and G. Pfleiderer. An aminopeptidase occuring in pig kidney. I. An improved method of preparation, physical and enzymic properties. *Biochemistry* **5:** 169–174, 1966.
25. Wagner, G. W., M. A. Tavianini, K. M. Hermann and J. E. Dixon. Purification and characterization of an enkephalin aminopeptidase from rat brain. *Biochemistry* **20:** 3884–3890, 1981.
26. Westerik, J. O. and R. Wolfenden. Aldehydes as inhibitors of papain. *J Biol Chem* **247:** 8195–8197, 1972.
27. Wilkinson, G. N. Statistical estimations in enzyme kinetics. *Biochem J* **80:** 324–332, 1961.