BINDING OF THE OPIATE-LIKE PENTAPEPTIDE METHIONINE-ENKEPHALIN TO A PARTICULATE FRACTION FROM RAT BRAIN

O. MORIN, M.G. CARON, A. DE LEAN and F. LABRIE

Medical Research Council Group in Molecular Endocrinology, Le Centre Hospitalier de l'Université Laval, Québec G1V 4G2, Canada

Received October 22,1976

SUMMARY

The characteristics of stereospecific binding of [^{3}H] met-enkephalin (15 Ci/mmole) were studied in a particulate fraction from rat brain. The binding assay was performed for 70 min at 0°C and the bound radioactivity separated by filtration through glass fiber filters (Whatman, GF/C). In the absence of sodium, binding of [^{3}H] met-enkephalin could be described on the basis of two independent binding sites with apparent K_Ds of 2.1 and 53 nM, respectively. The data are also consistent with one class of binding sites showing negative cooperativity. In the presence of 100 mM NaCl, binding of [^{3}H] met-enkephalin was 90-95% reduced, thus indicating the agonist properties of the peptide. The highly stereospecific binding of [^{3}H] met-enkephalin was evidenced by the 10,000-fold greater potency of levorphanol than its analgesically inactive enantiomer dextrorphan to compete for [^{3}H] met-enkephalin binding. Similar conclusions could be reached using levallorphan, (+)-3-hydro-N-allyl-morphinan, (-) methadone and (+) methadone. The apparent affinity of various opiate agonists and antagonists for the binding sites was closely correlated with their known pharmacological activity.

INTRODUCTION

Following reports that the brain contains endogenous substances with opiate-like activity (1-3), the structure of two potent pentapeptides isolated from porcine brain has been elucidated (4). The peptide H-Tyr-Gly-Gly-Phe-Met-OH (met-enkephalin) was then shown to possess the highest biological activity.

The sequence of met-enkephalin is in fact the same as that of the N-terminus of the C fragment (named β-endorphin) of β-lipotropin purified from sheep (5), human (6) and pig (7) pituitaries. β-lipotropin might well be the precursor of β-endorphin, a peptide with high opioid activity (8) which could then be converted to met-enkephalin.

The brain opiate receptor has so far been studied using [^{3}H] naloxone, [^{3}H] dihydromorphine and other labelled opiate agonists and antagonists (9-12). This paper describes characteristics of the highly stereospecific binding of the endogenous opiate-like peptide [^{3}H] met-enkephalin to a particulate fraction from rat brain.

MATERIALS AND METHODS

Preparation of particulate fraction

Brains were immediately obtained after decapitation of adult male Sprague-Dawley rats obtained from Canadian Breeding Farms, St. Constant, Quebec. After removal of the cerebellum known to contain little opiate binding (9), the remainder of the brain was placed in ice-cold 0.32 M sucrose. The tissue was homogenized in 9 volumes (v/w) of the same medium and centrifuged at 1000 xg for 10 min. The centrifugation was repeated twice to wash the pelleted material and the combined supernatants were then centrifuged at 30,000 xg for 20 min. This pellet was washed once with 25 mM Tris-HCl (pH 7.4 at 4°C), 2 mM $MgCl_2$ and resuspended in a small volume of the same buffer before storage at -90°C. Prior to each experiment, the particulate fraction likely to correspond to the P_2 fraction of Gray and Whittaker (13) was washed once.

Binding of [^{3}H] met-enkephalin

Except when indicated, approximately 100-150 µg of particulate fraction protein and 10 nM (80,000 cpm) [^{3}H] met-enkephalin (New England Nuclear, 15.15 Ci/mmole) were incubated in a final volume of 0.5 ml in 25 mM Tris-HCl (pH 7.4 at 4°C), 2 mM $MgCl_2$ at 0-4°C for 70 min in the presence or absence of 10^{-5}M unlabelled met-enkephalin. Specific binding was 60-70% of total binding. The reaction was stopped by the addition of 2 ml of ice-cold buffer. The medium was immediately filtered through glass fiber filters (Whatman, GF/C) under reduced pressure with three successive washes of the retained material with 5 ml of the same buffer. Radioactivity was determined in a Beckman liquid scintillation spectrometer after addition of 10 ml of Aquasol (NEN). All assays were done in triplicate. Protein concentration was measured according to Lowry et al. (14) using bovine serum albumin as standard.

Calculations

Free hormone concentration was obtained by substraction of total bound from total cpm added to the tubes. The non-linear Scatchard plots (15) were analyzed according to models involving two classes of specific binding sites (16) or one site and negative cooperativity (17).

Competition curves of [^{3}H] met-enkephalin binding were analyzed using the four parameter logistic model (18) for estimation of ED_{50} values. Data are expressed as mean ± SEM of triplicate determinations.

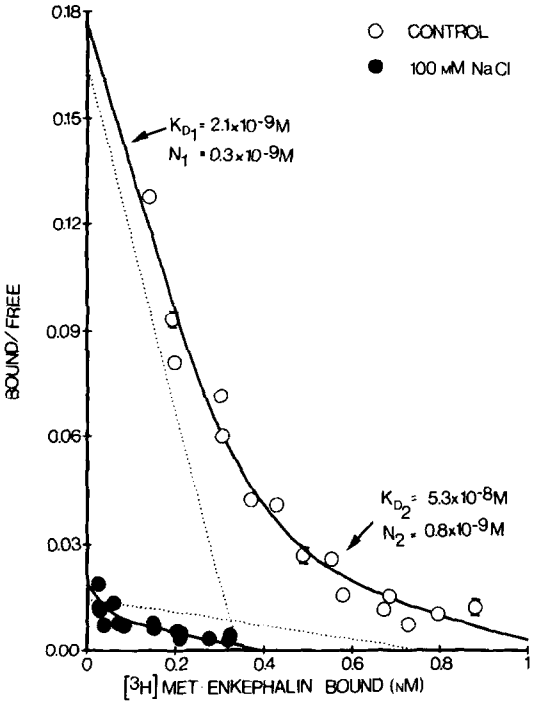

Fig. 1 Scatchard plot of [^{3}H] met-enkephalin stereospecific binding to a particulate fraction of rat brain. 150 µg of protein were incubated with increasing concentrations of [^{3}H] met-enkephalin om the presence or absence of 100 mM NaCl as described under "Materials and Methods". Results shown here are means of triplicate determinations Brackets represent the standard errors of the means.

RESULTS

Since maximal binding of [^{3}H] met-enkephalin was observed after 60-70 min of incubation and a plateau was maintained for at least 120 min at 0-4^{o}C, an incubation time of 70 min was adopted for the following experiments. Although the binding assay could also be performed at 25^{o}C or 37^{o}C, the incubation at 0-4^{o}C was chosen in order to minimize proteolytic degradation of [^{3}H] met-enkephalin.

As illustrated by the Scatchard plot of Fig. 1, the binding of [^{3}H] met-enkephalin can be described by two binding components

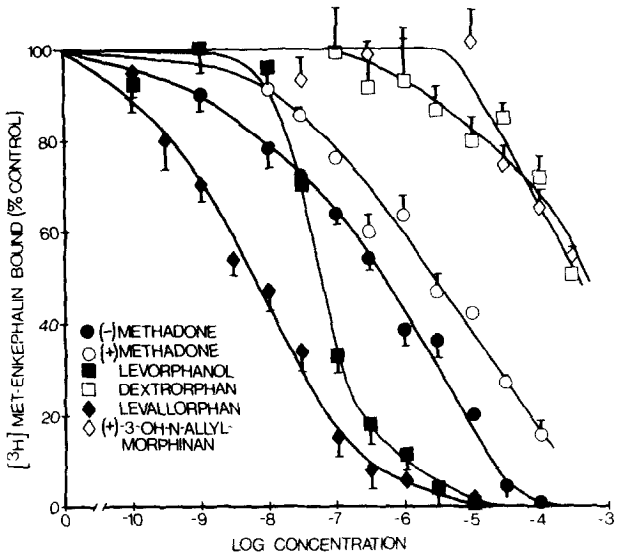

Fig. 2 Competition of [^{3}H] met-enkephalin binding by various opiate agonists and antagonists. Stereospecific binding was determined as described under "Materials and Methods" in the presence of increasing concentrations of the various agents.

with dissociation constants of 2.1 and 53 nM, respectively. With the protein concentration used, the levels of binding sites of high and low affinity were 0.34 and 0.76 x 10^{-9}M, respectively. In the presence of 100 mM NaCl, there was a marked reduction of [^{3}H] met-enkephalin binding with almost complete disappearance of binding to the high affinity sites while the low affinity binding was only slightly affected.

The stereospecificity of [^{3}H] met-enkephalin binding is illustrated in Fig. 2. As determined by their ability to compete with [^{3}H] met-enkephalin, levorphanol, a potent opiate, was approximately 10,000 times more potent than dextorphan, its analgesically inactive enantiomer. Similarly, the antagonist levallorphan was found to be about 2000 times more active than its enantiomer (+)-3-hydroxy-N-allyl-morphinan. (-) methadone, on the

other hand, showed an approximately 10-fold higher binding activity than (+) methadone.

DISCUSSION

The present data clearly demonstrate the stereospecific binding of the endogenous peptide [^{3}H] met-enkephalin to a particulate fraction from rat brain. In the absence of sodium, the binding can be adequately described on the basis of two different binding sites with respective K_Ds of 2.1 and 53 nM (Fig. 1). It should, however, be mentioned that the present data are also consistent with one class of binding sites showing negative cooperativity. Studies in progress should be able to discriminate between these two possibilities.

In initial studies using low specific activity [^{3}H] naloxone (19), a single class of binding sites with affinity constants of 20 to 60 nM could be observed. However, more recently, it was found (12) that [^{3}H] naloxone binding curves could be resolved into two linear components with K_D values of 0.4 and 30 nM, respectively. Using [^{3}H] dihydromorphine, dissociation constants of 3 and 30 nM were found. In our system at 0-4^{o}C, the binding of [^{3}H] naloxone could be described by the presence of two classes of binding sites with apparent K_Ds of 1 and 20 nM, respectively.

Much information using [^{3}H] naloxone and [^{3}H] dihydromorphine has been obtained about the stereospecificity of opiate binding and the correlation between affinity for the receptor sites and pharmacological activity of opiate agonists and antagonists (19). The data of Figure 2 indicating the high degree of stereospecific binding of [^{3}H] met-enkephalin are very similar to those previously obtained using [^{3}H] naloxone (19) and [^{3}H] etorphine (11).

An extremely useful observation has been that the presence of sodium ion increases the binding of antagonists while that of agonists is decreased (20). The agonist activity of met-enkephalin is clearly suggested by the 90-95% reduction of its binding in the presence of 100 mM NaCl (Fig. 1). These data are in agreement with the potent agonist activity of the peptide already observed in the guinea pig ileum (4) and its ability to induce the release of growth hormone in the rat (A. Dupont, L. Cusan and F. Labrie, unpublished observations).

The availability of a binding assay using the endogenous opiate-like peptide [^{3}H] met-enkephalin should help our understanding of the large variety of physiological functions of endogenous "opiates" and facilitate the design of synthetic analogs with potential use as agonists and antagonists.

ACKNOWLEDGEMENTS

O.M. is recipient of a studentship from the Medical Research Council of Canada (MRC) while M.G.C., A.D.L. and F.L. are respectively Scholar, Fellow and Associate of the MRC.

REFERENCES

1. Terenius, L. and Wahlström, A. (1975) Acta Physiol. Scand. 94, 74-81.
2. Hughes, J., Smith, T.W., Morgan, B. and Fothergill, L.A. (1975) Life Sci. 16, 1753-1758.
3. Pasternak, G.W., Goodman, R. and Snyder, S.H. (1975) Life Sci. 16, 1765-1769.
4. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1975) Nature 258, 577-579.
5. Li, C.H., Barnafi, L., Chretien, M. and Chung, D. (1965) Nature, 208, 1093-1094.
6. Cseh, G., Barat, E., Patthy, A. and Graf, L. (1972) FEBS Letters, 21, 344-346.
7. Graf, L., Barat, E., Cseh, G. and Sajgo, M. (1971) Biochim. Biophys. Acta, 229, 276-278.
8. Cox, B.W., Goldstein, A. and Li, C.H. (1976) Proc. Natl. Acad. Sci. USA, 73, 1821-1823.
9. Pert, C.B., and Snyder, S.H. (1973) Science, 179, 1011-1014.
10. Terenius, L. (1973) Acta Pharmacol. Toxicol. 32, 317-320.

11. Simon, E.J. (1973) Am. J. Med. Sci. 266, 160-168.
12. Pasternak, G.W., and Snyder, S.H. (1975) Nature, 253, 563-565
13. Gray, E.G. and Whittaker, V.P. (1962) J. Anat., 96, 79-96.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265.
15. Scatchard, G. (1949) N.Y. Acad. Sci., 51, 660-672.
16. Rodbard, D. (1973) Adv. Exp. Med. Biol., 36, 289-326.
17. De Meyts, P., and Roth, J. (1975) Biochem. Biophys. Res. Commun., 66, 1118-1126.
18. Rodbard, D. and Frazier, G.R. (1975) Methods in Enzymol., 37, 3-22.
19. Pert, C.B. and Snyder, S.H. (1973) Proc. Natl. Acad. Sci. USA, 70, 2243-2247.