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BINDING CHARACTERISTICS OF A POTENT ENKEPHALIN ANALOG

Richard M. Kream and R. Suzanne Zukin
Department of Biochemistry
Albert Einstein College of Medicine
Yeshiva University
Bronx, New York 10461

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SUMMARY

A radioiodinated form of the highly potent enkephalin analog FK 33-824 has been characterized with respect to its binding properties in vitro. ^{125}I-FK 33-824 is distinctive among the short opioid peptides in three ways. First, ^{125}I-FK 33-824 binds stereospecifically to rat brain homogenates with very high affinity (K_d = 0.42 nM). Secondly, dissociation of the ^{125}I-labelled peptide from membrane-bound opiate receptors occurs with a relatively long $\tau_{\frac{1}{2}}$ of 25 min at 4° in contrast to other enkephalins which dissociate more rapidly. Third, competitive binding analyses reveal that the ^{125}I-FK 33-824 binds equally well to both enkephalin (δ) and morphine (μ) classes of opiate receptors. These characteristics distinguish the ^{125}I-labelled peptide as a particularly suitable probe for molecular studies and purification of the opiate receptor.

INTRODUCTION

In 1975 Hughes and coworkers isolated the pentapeptides methionine and leucine enkephalin and showed them to be endogenous ligands of the mammalian opiate receptor (1). Studies including the regional distribution of enkephalins in the brain and spinal cord, their localization at nerve terminals, and their physiological mode of action implicate enkephalins as putative neurotransmitters or neuromodulators (2-4). Recent studies of Lord et al. (5) suggest that enkephalin actions are mediated primarily through binding to a pharmacologically distinct subclass of opiate receptors (δ receptors) in contrast to alkaloid opiates which exert their action preferentially through binding to μ receptors.

Experiments involving the naturally occurring enkephalins, however, have been difficult because these small peptides are rapidly degraded in most tissue preparations (6,7). FK 33-824, an unusually stable sulfoxide-

Abbreviations used: FK 33-824, D-Ala^2, N-Me-Phe^4, Met(O)^5-ol-enkephalin; DALA, D-Ala^2, Met^5-enkephalin; BSA, bovine serum albumin.

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carbinol derivative of methionine enkephalin first synthesized by Roemer and coworkers at Sandoz Ltd., produces prolonged analgesia even when administered orally (8). Because of its unique potency among enkephalins, we chose to radioidinate FK 33-824 in order to study its action in vitro, and to employ it as a probe to characterize the opiate receptor with respect to molecular weight, subunit composition and pharmacological subclasses.

MATERIALS AND METHODS

Materials

D-ala^2, N-Me-phe^4, met (O)^5-ol-enkephalin (Sandoz, FK 33-824) and all other peptides were obtained from Peninsula Laboratories. [^{3}H] DALA (37.3 Ci/mmol), [^{3}H] naloxone (15.2 Ci/mmol), [^{3}H] dihydromorphine (43.2 Ci/mmol and carrier-free Na^{125}I (17 Ci/mg in 0.1 M NaOH) were obtained from New England Nuclear Corp. Levorphanol and dextrorphan were gifts from Hoffman LaRoche. Naloxone was obtained from Endo Labs., while etorphine was a gift from Dr. M. Makman. Lactoperoxidase (purified grade) was obtained from Calbiochem, and 30% hydrogen peroxide (reagent grade) was from Fisher. Brij 36-T was from Sigma.

Enzymatic Iodination

FK 33-824 was enzymatically labelled by a modification of the procedure of Bolton (10). Na ^{125}I (2m Ci), FK 33-824 (1 µg), lactoperoxidase (50 ng), and hydrogen peroxide (300 ng) were reacted in 0.15 ml 0.5 M sodium acetate buffer, pH 5.6 at 22° C for 10 min. Two further additions of 300 ng hydrogen peroxide were made at 10 min intervals. Ten min after the final addition of peroxide, the reaction mixture was quenched with 0.3 ml of 0.125 M sodium phosphate buffer containing 0.05% BSA (Sigma) pH 7.4. The reaction mixture was immediately applied to a 1 x 15 cm Bio-Gel P-2 column (Bio-Rad), 200-400 mesh, and eluted with the same buffer at 4° C (Figure 1). Approximately 70% of the radioactivity applied to the Bio-Gel P-2 column eluted just behind the protein void peak; these fractions and no others exhibited opiate receptor binding activity. In addition, peaks of radioactivity coincident with the protein void volume and with the elution position of free Na ^{125}I were observed. The first of these probably represents self-iodinated lactoperoxidase or radiolabelled BSA.

Purification of ^{125}I-labelled peptide

The eluted fractions from the Bio-Gel P-2 column (18-23) which exhibited stereospecific binding in the in vitro opiate receptor binding assay were concentrated by lyophilization resuspended in 1/10 volume 0.1 M potassium chloride containing 0.01 M potassium phosphate, pH 9.0 and applied to a 1 x 10 cm DEAE-Sephadex column (acetate form, A-25, Pharmacia). The mixture was eluted in the same buffer. A single peak of radioactivity coincident with binding activity was observed (data not shown).

The peak fractions were combined and an aliquot spotted on thin layer plates. Thin layer chromatography of purified ^{125}I-

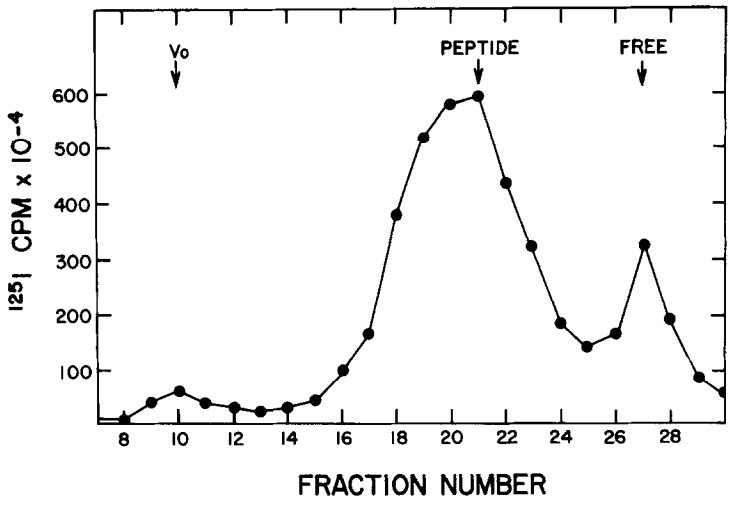

Figure 1. Gel filtration chromatography of ^{125}I-labelled FK 33-824 on a Bio-Gel P2 column (1 x 15 cm). The column was eluted with 0.125 M sodium phosphate and 0.05% BSA at 4°. Half ml fractions were collected and 10 µl aliquots counted in a gamma counter.

labelled FK 33-824 was performed on pre-coated cellulose plates (20 x 20 cm, 0.1 mm thick, Kodak) using the solvent system n-butanol: water:pyridine:acetic acid (15:12:10:3). Greater than 95% of the radioactivity applied to the plates chromatographed as a single band. The specific activity of purified ^{125}I-labelled FK 33-824 was calculated to be 1000 Ci/mmol based on 1:1 stoichiometry of iodine to recovered peptide.

Binding Studies

P2 (mitochondrial-synaptosomal)membranes (0.5 mg protein per ml) were prepared from whole brains minus cerebella of male Sprague-Dawley rats (150 grams) (11). 0.1% BSA was added to supplement membrane protein content. All incubations were done at 4° C for 90 minutes, and bound ligand was separated from free ligand by rapid filtration through glass fiber filters (12). The filters were washed two times with ice-cold buffer containing 0.05% BSA and 1% polyethylene glycol 6000 (Baker). Stereospecific binding represents the difference in total counts per minute of duplicate samples incubated either in the presence of 10 µM levorphanol or 10 µM dextrorphan (inactive enantiomer of levorphanol).

RESULTS

The purified ^{125}I-labelled enkephalin exhibited a high degree of stereospecific binding to rat brain homogenates (Figure 2a). Saturation occurred at about 5 nM ^{125}I-labelled FK 33-824. Specific binding was greater than non-specific binding at each ligand concentration over the tested range. At 1.0 nM ^{125}I-labelled peptide 80% stereospecific binding

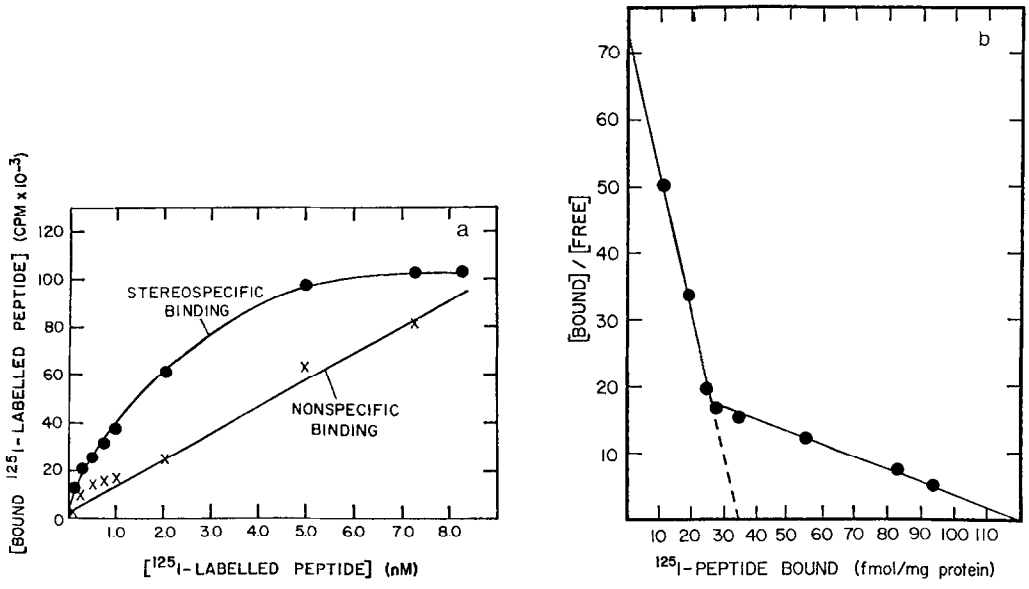

Figure 2a. Saturation analysis of ^{125}I-labelled FK 33-824 binding to rat brain homogenates. Details of binding assays and definition of stereospecific binding are described in Materials and Methods. Each point represents the mean of triplicate analyses.

Figure 2b. Scatchard plot from data in Figure 2a.

was observed (Figure 2a). When these binding data were replotted as a Scatchard analysis (13) (Figure 2b), two classes of binding sites were observed with apparent K_d's of 0.42 nM and 3.70 nM. The ratio of high to low affinity sites was approximately 1:3. The total number of sites occupied at saturation was 120 fmol per mg protein. When the identical experiment was performed in the presence of buffered 150 mM KCl, pH 7.4, the K_d and receptor number were unchanged for the high affinity site (data not shown). However, increasing amounts of Na^+ or Li^+ ion in buffered 150 mM KCl, pH 7.4, produced a linear reduction in the K_a associated with the high affinity binding site (40 mM Na^+ or Li^+ ion reduced the apparent affinity for these sites by 50%). Sodium effects on opiate agonist receptor binding are well documented (14). In our study, lithium and sodium are equipotent in reducing high affinity binding in the presence of 150 mM KCl (physiological ionic strength).

TABLE 1

DISPLACEMENT OF ^{125}I-LABELLED FK 33-824 BINDING TO RAT BRAIN HOMOGENATES BY OPIATE PEPTIDES AND NON-PEPTIDES

Opiate	IC_{50} (nM) ‡ S.E.M.
(Peptides)	
FK 33-824	1.80 ± 0.12
DALA	1.78 ± 0.01
D-Ala^2, Leu^5-enkephalin	1.60 ± 0.10
β_C-endorphin	1.05 ± 0.05
D-ala^2,β_H-endorphin	16.7 ± 2.00
(Non-Peptides)	
Naloxone	1.55 ± 0.01
Etorphine	1.33 ± 0.25
Levorphanol	2.80 ± 0.30
Dextrorphan	> 1000

P_2 membranes (0.5 mg of protein/ml) were incubated with 1 nM ^{2125}I-labelled FK 33-824 plus displacer at eight concentrations for 90 minutes at 4° C. Incubation details are described and stereospecific binding is defined in Materials and Methods. An IC_{50} represents the concentration of displacer required for 50% inhibition of bound ^{125}I-labelled FK 33-824. The mean ± S.E.M. for at least three independent experiments is reported for each compound tested.

The time course of ^{125}I-labelled FK 33-825 dissociation from rat brain homogenates was examined. Homogenates were incubated with ^{125}I-labelled peptide (1.0 nM) for 1 hr at 4° C, and then non-radioactive FK 33-824 (1.0 nM) was added. Bound ^{125}I-labelled ligand was determined as a function of time at 22° C. Binding declined in an exponential manner with a $\tau_{1/2}$ of approximately 25 minutes. A very similar $\tau_{1/2}$ (34 min at 37°) was reported for dissociation of [^{3}H] etorphine (1 nM) from rat brain homogenates (14).

Table 1 shows the displacement of ^{125}I-labelled FK 33-824 by various opiates. Nonradioactive FK 33-824 displaces the ^{125}I-labelled

TABLE 2

RELATIVE POTENCIES OF DRUGS FOR DISPLACEMENT OF RADIOLABELLED OPIATES FROM RAT BRAIN RECEPTOR SITES *

Compound	[^{3}H] Naloxone	[^{3}H] Dihydromorphine	^{125}I-labelled FK 33-824	[^{3}H] DALA	^{125}I-labelled FK 33-824/ [^{3}H] DALA
DALA	1.00	1.00	1.00	1.00	1.00
FK 33-824	1.87 ± .05	1.33 ± 0.15	0.99 ± .06	0.54 ± .05	1.83
Naloxone	2.13 ± .08	1.00 ± 0.20	1.15 ± .01	0.04 ± .01	27.4
Levorphanol	3.0[a]	1.72 ± 0.20	0.64 ± .09	0.12 ± .02	5.33
Etorphine	2.8	1.80 ± 0.25	1.33 ±0.16	1.60 ± .15	

* Incubation conditions and definition of specific binding are the same as described in Table 1. In each analysis, 1 nM radiolabelled opiate was displaced by nonradioactive ligand at a minimum of eight concentrations. An IC_{50} was calculated for each compound; relative potencies are reported as ratios of IC_{50}'s relative to DALA = 1.00. Each value listed represents the average of three separate experiments ± S.E.M.

[a] The value reported was calculated from the ratio of the IC_{50} for displacement of [^{3}H] naloxone by etorphine or levorphanol (20) and that determined by us for [^{3}H] naloxone displacement by DALA.

peptide with an apparent IC_{50} of 1.8 nM. When a variety of other opiate displacers were tested, both opioid peptides and morphine analogs displaced ^{125}I-labelled FK 33-824 with very similar potencies; apparent IC_{50}'s range from 1.6 nM for D-ala^2, leucine^5 enkephalin to 2.8 nM for levorphanol. The only exception is D-ala^2, β_H-endorphin which exhibited an IC_{50} of 17 nM.

In addition, the displacement of [^3H] naloxone, [^3H] dihydromorphine, and [^3H] DALA binding by DALA, FK 33-824, and morphine-like compounds was examined (Table 2). DALA displaces [^3H] DALA with 10 times the potency of levorphanol and 25 times that of naloxone. Conversely, naloxone displaces [^3H] naloxone with twice the potency of DALA, while levorphanol and etorphine displace [^3H] dihydromorphine two times better than does DALA. FK 33-824 displaces [^3H] naloxone with a relative potency very similar to that of naloxone itself; in the displacement of all other radiolabelled ligands, the sulfoxide-carbinol peptide exhibits an intermediate rank order of potency.

DISCUSSION

The highly potent sulfoxide-carbinol enkephalin analog FK 33-824 has been prepared in radioactive form and has been characterized with respect to its binding properties and receptor site. ^{125}I-labelled FK 33-824 bound with very high affinity to rat brain homogenates; Scatchard analysis revealed two classes of sites with K_d's of 0.42 nM and 3.7 nM. These affinities are among the tightest observed for opioid peptides. In addition, the $\tau_{1/2}$ (= 25 min at 4°) for dissociation of FK 33-824 from membrane-bound receptor sites is considerably longer than those which we observed for D-Ala^2-Met^5- and D-Ala^2-Leu^5-enkephalin ($\tau_{1/2}$'s = 12 min and 16 min, respectively (Zukin, unpublished)) under the same conditions. Thus, the advantageous clinical properties of FK 33-824 as an analgesic agent (long-lasting action and high potency

even when administered orally) may be related to a high binding affinity and slow dissociation from the receptor in addition to enhanced stability in the presence of proteolytic agents.

Nonradioactive FK 33-824 displaced tritiated opiates and opioid peptides with relatively similar IC_{50}'s. In addition, the ^{125}I-labelled peptide is displaced equally well by both opiate alkaloids and enkephalin derivatives. Together these findings indicate that the sulfoxide-carbinol derivative of enkephalin interacts equally well with μ and δ opiate receptors. Of the opiates which have been tested, β-endorphin is the only other ligand which binds equally well to these receptor classes (5). Roemer et al. (8,15) showed in a variety of bioassays that the pharmacological actions of FK 33-824 most closely resemble those of β-endorphin. These findings suggest that some or all of the synthetic structural features of the Sandoz FK 33-824 peptide confer in vivo and in vitro characteristics of β-endorphin to the shorter peptide.

Radioiodination was performed using soluble lactoperoxidase in the first application of this enzymatic labelling technique to opioid peptides. The radiolabelling technique of choice in the case of FK 33-824 is lactoperoxidase-catalyzed iodination because this enzymatic procedure obviates the problem encountered in Chloramine T reactions of oxidation of methionine side-chains to the sulfone derivatives (10). Previously, ^{125}I-labelled D-ala^2, leucine enkephalin was prepared using the chemically-catalyzed technique (16). Our study is the first case in which a radioiodinated methionine-containing opioid peptide with receptor binding activity has been prepared. The purified ^{125}I-labelled FK 33-824 has been shown to be homogeneous by both DEAE-Sephadex ion-exchange chromatography and thin layer chromatography. That the peptide is monoiodinated can be inferred from studies involving ^{125}I-labelled D-ala^2, leucine enkephalin (16), in which it is shown that the monoiodinated, but not the diiodinated enkephalin, is active in the opiate receptor binding assay. Monoiodination of FK 33-824

would be consistent with the stoichiometry of Na ^{125}I to peptide (1:1.5) in the labelling reaction.

Finally, the ^{125}I-labelled FK 33-824 of high specific activity has been used as a marker of its receptor in solubilization experiments (17,18). In order to determine whether the high affinity binding component is associated with a membrane-bound receptor capable of being recovered in soluble form, P_2 membranes were pre-incubated with ^{125}I-labelled FK 33-824 in the presence or absence of a 1000-fold excess of unlabelled FK 33-824. The labelled membranes were solublized with a 1% solution of the non-ionic detergent Brij 36-T in modification of the procedure of Simon et al. (19). The solubilized peptide-macromolecular complex was shown to have a Stokes radius of 48 Å by gel filtration chromatography; this size corresponds to a molecular weight of 380,000 daltons for a spherical molecule (19). These values agree very closely with those reported by Simon et al. (19) for a solubilized [^{3}H]etorphine-macromolecular complex. Together these results are consistent with binding of the enkephalin analog FK 33-824 and etorphine to the same receptor protein complex. In preliminary experiments, electrophoresis of the ^{125}I-FK 33-824 complex on NaDodSO$_4$ / 10% polyacrylamide gels (under conditions where the gel is maximally loaded) resulted in three radioactive bands corresponding to 27,000, 35,000, and 42,000 daltons (18). By contrast, ^{125}I-D-ala^2-leu^5-lys^6-enkephalin, a "pure" δ agonist labels a single constituent of approximately 35,000 daltons.

The finding that the ^{125}I-FK 33-824 peptide has labelled three constituents may indicate that this ligand interacts with heterogeneous receptors or receptor subunits. This finding is consistent with our finding here that FK 33-824 interacts equally well with multiple opiate receptor subclasses. Such characterization studies of ^{125}I-peptide-macromolecular complexes by gel electrophoresis may provide the first direct evidence at the molecular level for the heterogeneity of the receptor protein.

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REFERENCES

1. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1975) Nature 258, 577-579.

2. Johansson, O., Hokfelt, T., Elde, R.P., Schultzberg, M. and Terenius, L. (1978) Adv. Biochem. Psychopharmacol. 18, 51-70.

3. Yang, H.-Y., Hong, J.S., Fratta, W. and Costa, E. (1978) Adv. Biochem. Psychopharmacol. 18, 149-159.

4. Zieglgansberger, W., Fry, J.P., Herz, A., Moroder, L. and Wunsch, E. (1976) Brain Research 115, 160-164.

5. Lord, J.A.H., Waterfield, A.A., Hughes, J. and Kosterlitz, H.W. (1977) Nature 267, 495-499.

6. Hambrook, J.M., Morgan, B.A., Rance, J.M. and Smith, C.F.C. (1976) Nature 262, 782-783.

7. Erdos, E.G., Johnson, A.R. and Boyden, N.T. (1978) Adv. Biochem. Psychopharmacol. 18, 45-50.

8. Roemer, D., Buescher, H.H., Hill, R.C., Pless, J., Bauer, W., Cardinaux, F., Closse, A., Hauser, D. and Huguenin, R. (1977) Nature, 268, 547-549.

9. Morrison, M., and Schonbaum, G.R. (1976) Ann. Rev. Biochem. 45, 861-888.

10. Bolton, A.E. (1978) Radioiodination Techniques Amersham Corporation, Illinois, 55-57.

11. Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 78-86.

12. Pert, C.B. and Snyder, S.H. (1974) Science, 179, 1011-1013.

13. Scatchard, G., Coleman, J.S. and Shen, A.L. (1957) J. Am. Chem. Soc. 79, 12-20.

14. Simon, E.J., Hiller, J.M., Groth, J. and Edelman, I. (1975) J. Pharmacol. Exp. Ther. 192, 531-537.

15. Roemer, D. and Pless, J. (1979) Life Sciences 24, 621-624.

16. Miller, R.J., Chang, K.-J., Leighton, J. and Cuatrecasas, P. (1978) Life Sciences 22, 379-388.

17. Zukin, R.S. and Kream, R.M. (1979) Proc. N atl. Acad. Sci. USA 76, 1593-1597.

18. Zukin, R.S., Federoff, G.D., and Kream, R.M. (1979) Life Sciences, in press.

19. Simon, E.J., Hiller, J.M. and Edelman, I. (1975) Science 190, 389-396.

20. Miller, R.J. and Cuatrecasas, P. (1978) Vitamins and Hormones (eds. P.L. Munson, J. Glover, E. Diczfalusy, and R.E. Olsen) Vol. 36, pp. 297-375, Academic Press, New York.