Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 38-44

DEMONSTRATION AND DISTRIBUTION OF AN OPIATE BINDING SITE IN RAT BRAIN WITH HIGH AFFINITY FOR ETHYLKETOCYCLAZOCINE AND SKF 10,047

Andreas Pfeiffer and Albert Herz

Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, Kraepelinstrasse 2, D-8000 München 40, F.R.G.

Received May 12, 1981

SUMMARY: The existence of various classes of opiate receptors has been proposed based on the results of pharmacological studies. The interaction of prototype opiate ligands with μ-, δ-, κ-, and σ-binding sites was studied in 3 areas of the rat brain. The experimental data were analysed by means of a computerized curve fitting technique. This indicated the existence of 3 classes of opiate binding sites. Two of these displayed characteristics of the previously described μ- and δ-binding sites with regard to affinities and distribution. The third site (R_3) was characterized by a high affinity for the prototype κ- and σ-receptor agonists ethylketocyclazocine and SKF 10,047 in contrast to a low affinity for the δ- and μ-receptor ligands D-Ala^2,D-Leu^5-enkephalin and dihydromorphine. The distribution of R_3 was distinct from that of δ-binding sites, and resembled that of μ-sites.

The observation that some benzomorphane narcotics which antagonize the actions of morphine, produce naloxone-reversible effects (1), led Martin (14) to postulate the existence of multiple opiate receptors. Further studies performed in the chronic spinal dog (15), in the guinea-pig ileum and mouse vas deferens bioassays (10,13,24) permitted the differentiation of 4 types of opiate receptors termed μ, κ, σ and δ, with the prototype agonists morphine, ethylketocyclazocine (EK), N-allyl-normetazocine (SKF 10,047) and the endogenous enkephalins or their stable analogue D-Ala^2,D-Leu^5-enkephalin (DADL), respectively. Radioligand binding studies succeeded in differentiating μ- and δ-binding sites with the prototype ligands dihydromorphine (DHM) and DADL (2,5,13) respectively, whereas attempts to demonstrate the presence of κ- (4,8,9,18) or σ-binding sites (21) in the rat brain have, as yet, proven unsuccessful. Kosterlitz and coll. (12) acquired evidence suggestive of the presence of κ-binding sites in the guinea-pig brain.

0006-291X/81/130038-07$01.00/0

The high affinity of SKF 10,047 and EK for both, δ- and μ-sites, on the other hand, complicates the demonstration of an additional site displaying selectivity for either of these opiates. Recently, computerized curve fitting techniques have proven of value in the identification of small populations of binding sites (7). In this study, a non-linear least squares curve fitting program, as developed by Munson and Rodbard (17), was applied in order to investigate the interaction of DHM, DADL, EK and SKF 10,047 with binding sites in homogenate obtained from three different areas of the rat brain.

METHODS AND MATERIALS

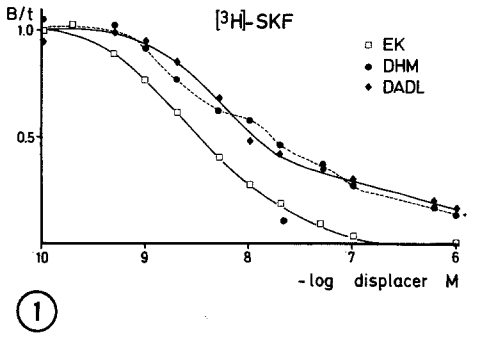
Rats were killed by decapitation, and brain dissection performed according to Glowinski and Iversen (6). Tissue from 20-40 rat brains was pooled for each experiment. Homogenization was performed in 5 mM Tris buffer, pH 7.4 with an Ultraturrax homogenizer at halfmaximal setting for 15 s followed by centrifugation at 40 000 g for 20 min. The pellet was resuspended, permitted to stand on ice for 30 min and recentrifuged. After repetition of this procedure, the pellet was resuspended 1:100 (w/v) in Tris Krebs-Ringer buffer of the following composition in mM: NaCl:118; KCl:4,75; $CaCl_2$:2,54; $MgSO_4$:1,2; Tris:50; pH 7.4. Two ml portions of the homogenate were incubated at 28°C for 35 min in the dark with 12 concentrations of unlabelled inhibitor ranging from 0.05-10000 nM and 0.5-0.9 nM of the tritiated ligand. Incubations were terminated by filtration through prewetted GFC glass-fiber discs (Whatman) under reduced pressure followed by a wash with 10 ml of ice cold buffer. Radioactivity retained on the filters was measured by liquid scintillation spectrometry 24 h after addition of 8 ml of Aqualuma Plus (Baker, North-Holland).

Statistical analysis was performed by the runs-test and an F-test incorporated in the computer program. (For a detailed description see Munson and Rodbard (17).)

Materials: The tritiated ligands [^{3}H]-DHM (73.2 Ci/mmol) and [^{3}H]-DADL (42 Ci/mmol) were purchased from Amersham Buchler, Braunschweig, FRG, and [^{3}H]-SKF 10,047 (44 Ci/mmol) and [^{3}H]-EK (15 Ci/mmol) were obtained from New England Nuclear, Dreieich, FRG.

RESULTS AND DISCUSSION

In order to investigate the possible occurrence of multiple opiate binding sites in rat brain, heterologous displacement experiments were performed, employing the proposed prototype ligands at μ-, δ-, κ- and σ-binding sites DHM, DADL, EK and SKF 10,047, respectively. Experiments were undertaken with all possible cominations of tritiated and un-



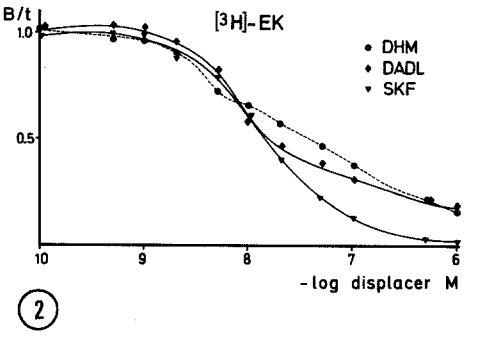


Figure 1

Displacement curve of ^{3}H-SKF by DADL, DHM and EK
The fraction of ^{3}H-ligand retained on the filters after substraction of nonspecific binding (= binding in the presence of 10^{-5} M SKF) is plotted on the ordinate. Data points are the average of duplicate determinations obtained in the frontal cortex.

Figure 2

Displacement curve of ^{3}H-EK by DADL, DHM and SKF
For details see under figure 1.

labelled ligands in 3 areas of the rat brain, which are known to differ in their content of δ- and μ-binding sites (3,21).

Displacement curves of DADL and DHM against [^{3}H]-EK and [^{3}H]-SKF 10,047 were multiphasic in all brain areas with an initial steeper slope and flattening of the curve at higher concentrations of the displacer (Fig. 1, 2). At 10 μM both, DHM and DADL displaced 95-100% of [^{3}H]-EK and [^{3}H]-SKF 10,047 in the striatum and frontal cortex, and 85-95% in the midbrain/thalamus. Displacement isotherms of EK and SKF 10,047 against [^{3}H]-EK and [^{3}H]-SKF 10,047 were monophasic in appearance, and displacement was complete at a concentration of 1 μM (Fig. 1, 2).

The computerized curve fitting program used, simultaneously analyses the binding data derived from a number of experiments which may be performed with various labelled and unlabelled ligands. The mathematical procedure for quantitative analysis of binding parameters is based on the law of mass action for interaction of multiple ligands with

Table 1: Parameter estimates of binding capacity and dissociation constant (K_d) for DHM, DADL, EK and SKF in the frontal cortex, striatum and midbrain/thalamus.
The root mean square error, a measure of the goodness of fit was 8.4 in the frontal cortex and 9.4 in the striatum and midbrain/thalamus.

SITE	CAPACITY pmoles/g	K_d(nM) DHM	DADL	SKF	EK
R_1	6.9±0.6	2.5± 0.3	5.9 ± 0.5	3.7±0.3	1.7±0.2
R_2	5.5±1.2	174 ± 22	1.21± 0.4	18.5±2.4	6.5±0.9
R_3	7.1±1.7	539 ±112	2650 ±907	11.8±3.9	7.7±2.2
	Striatum				
R_1	6.9±1.1	2.3± 0.4	4.8 ± 0.7	4.0±0.5	1.6±0.2
R_2	8.5±2.6	132 ± 20	1.9 ± 0.9	20.8±3.9	6.3±1.3
R_3	6.1±1.9	57 ± 35	1100 ±354	4.8±2.2	4.6±1.8
	Midbrain/thalamus				
R_1	7.8±1.0	3.6± 0.6	8.3 ± 1.1	5.0±0.5	1.9±0.3
R_2	1.7±1.2	114 ± 77	1.9 ± 1.8	7.3±4.4	11.3±7.3
R_3	4.8±2.3	143 ± 73	2170 ±863	6.2±4.2	4.4±2.8

multiple binding sites. In each of the brain areas, 24 displacement curves with 12 heterologous combinations of the 4 opiate ligands investigated were simultaneously analysed. The results obtained in the three brain areas (Table 1) were most adequately represented by a model assuming the existence of 3 types of binding sites (R_1-R_3) which fitted the experimental data significantly better than a 2 or 1 site model. The binding site R_1 was the only site displaying high affinity for DHM and also exhibited high affinities fot the other ligands with K_d's ranging between 1.5 and 8 nM. R_1 thus corresponds to the characteristics of the μ-binding site (2,13,21). To R_2, only DADL exhibited a high affinity, whereas EK and SKF 10,047 had intermediate and DHM low affinities. R_2 thus conforms to the characteristics of the δ-binding site (2,5,13,21). To R_3 only EK and SKF 10,047 had affinities in the nM range, whereas DHM and DADL had low affinities.

Displacement of [^{3}H]-EK and [^{3}H]-SKF 10,047 by DHM and DADL was not complete at 10 μM concentrations in the midbrain/thalamus. This might indicate the presence of a binding site to which DADL and DHM display negligible affinity. A separate analysis of the curves obtained with combinations of EK and SKF 10,047 was therefore performed. Data thus obtained were best represented by assuming additional binding to a low affinity component of about 170 pmol/g tissue to which EK and SKF 10,047 had affinities (K_d) of 230 and 830 nM, respectively. Low affinity binding, however, was poorly characterized with a large standard error. In the striatum and frontal cortex no low affinity binding of EK and SKF 10,047 could be detected by computer analysis.

The affinities of the 4 ligands to μ- and δ-sites varied little between the three brain areas (Table 1). To R_3, the affinities of EK and SKF 10,047 varied 2-fold, being highest in the striatum and lowest in the frontal cortex. In view of the low affinity component of EK and SKF 10,047 binding observed in the midbrain/thalamus, a possible explanation for the variation in affinity to R_3 is that binding in the frontal cortex also occurred to an additional low affinity component too small to be detected. This could result in the apparently lower affinity of the interaction of EK and SKF 10,047 with R_3. The affinity of DHM to R_3 varied by a factor of 10 in the three areas, however, which may indicate further heterogeneity of this site.

A very homogeneous distribution pattern was observed for μ-binding sites in all three brain areas. In contrast, δ-binding sites showed 5-fold variation with highest levels in the striatum and very low levels in the midbrain/thalamus. Due to the low binding capacity in this latter area, the δ-binding site was determined rather inaccurately. The distribution pattern of δ- and μ-sites observed in this study is in good agreement with published data (3,21). The binding capacity

of R_3 was rather homogeneous in the three brain areas, and approximately parallelled the distribution of μ-sites.

Previous attempts have not succeeded in demonstrating the occurrence of a binding site possessing a selective high affinity for EK and SKF 10,047 (4,8,9,18,21). Considering the high affinity interaction of EK and SKF 10,047 with δ- and μ-sites, only about 30% of the high affinity binding of these ligands occurred to R_3, rendering it difficult to demonstrate this site by use of conventional techniques. The power of the computerized analysis lies in its ability to characterize binding sites by quantitative analysis of heterologous displacement experiments. This approach permits characterization of 3 opiate binding sites despite the present lack of a selective ligand at R_3 by exploiting the large differences in relative affinities of the four opiates investigated to the 3 sites.

In this study, binding sites of EK and SKF 10,047 could not be differentiated, as would be expected according to Martin and coll. (15). The behavioural effects of EK and SKF 10,047 differ greatly (11,17,25). Naloxone-antagonizable agonistic effects of EK have been shown in the rat (11,20,23), whereas the agonistic effects of SKF 10,047 usually were not reversible by naloxone (11,23) in rats. In other species, naloxone-reversible agonistic effects of SKF 10,047 have been observed (14,15). Moreover, SKF 10,047 has been shown to antagonize the actions of morphine (15,22,23) and of EK (11,20). Thus, provided it is assumed that actions of EK are mediated by the R_3-site, SKF 10,047 may represent an antagonist at the R_3 and the μ-site. It remains to be explained, however, why EK exerts neither agonistic (10), nor antagonistic effects (15,16,22) at μ-receptors despite its high affinity for the μ-site.

The finding that DADL and, very probably the endogenous enkephalins also, displayed very low affinity to R_3 raises the question as to the endogenous ligand of this site. Recent experiments (19) indicate that

dynorphin$_{1-13}$ has a high affinity to R_3, suggestive that dynorphin may be a candidate for an endogenous ligand at this site.

Acknowledgement

The authors gratefully acknowledge P.J. Munson and D. Rodbard for providing the curve fitting program and are grateful to M.J. Millan for correction of the manuscript.

REFERENCES

1. Blumberg, H., Dayton, H.B., and Wolf, P.S. (1966) Proc. Soc. Exp. Biol. Med. 123, 755-758.
2. Chang, K.-J., and Cuatrecasas, P. (1979) J. Biol. Chem. 254, 2610-2618.
3. Chang, K.-J., Cooper, B.R., Hazum, E., and Cuatrecasas, P. (1979) Molec. Pharmacol. 16, 91-104.
4. Chang, K.-J., Hazum, E., and Cuatrecasas, P. (1980), Proc. Nat. Acad. Sci. USA 77, 4469-4473.
5. Childers, S.R., Creese, J., Snowman, A.M., and Snyder, S.H. (1978) Europ. J. Pharmacol. 55, 11-18.
6. Glowinski, J., and Iversen, L.L. (1966) J. Neurochem. 13, 655-669.
7. Hancock, A.A., DeLean, A.L., and Lefkowitz, R.J. (1979) Molec. Pharmacol. 16, 1-9.
8. Harris, D.W., and Sethy, V.W. (1980) Europ. J. Pharmacol. 66, 121-123.
9. Hiller, J.M., and Simon, E.J. (1979) Europ. J. Pharmacol. 60, 389-390.
10. Hutchinson, M., Kosterlitz, H.W., Leslie, F.M., Waterfield, A.A., and Terenius, L. (1975) Br. J. Pharmacol. 55, 541-546.
11. Iwamoto, E.T. (1980) J. Pharmacol. Exp. Ther., in press.
12. Kosterlitz, H.W., F.R.S., and Paterson, S.J. (1980) Proc. R. Soc. Lond., B. 210, 113-122.
13. Lord, J.A.H., Waterfield, A.A., Hughes, J., and Kosterlitz, H.W. (1977) Nature (Lond.) 267, 495-499.
14. Martin, W.R. (1967) Pharmacol. Rev. 19, 463-521.
15. Martin, W.R., Eades, G.C., Thompson, J.A., Huppler, R.E., and Gilbert, P.E. (1976) J. Pharmacol. Exp. Ther. 197, 517-522.
16. Michne, W.F., Pierson, A.K., and Albertson, N.F. (1974) Bull. Prob. Drug Dependence 36, Addendum, 524-532.
17. Munson, P.J., and Rodbard, D. (1980) Analytical Biochem. 107, 220-239.
18. Pasternak, G.W. (1980) Proc. Nat. Acad. Sci. USA 77, 3691-3694.
19. Pfeiffer, A., and Herz, A. (1981) Neurosci. Lett., in press.
20. Shearman, G.T., and Herz, A. (1981) Neurosci. Lett., in press.
21. Snyder, S.H., and Goodman, R.R. (1980) J. Neurochem. 35, 5-15.
22. Swain, H.H., and Seevers, M.H. (1974) Bull. Problems Drug Dependence 36, Addendum 1168-1195.
23. Ward, S.J., Metcalf, G., and Rees, J.M.H. (1978) Characteristics and Functions of Opioids, pp. 497-498, Elsevier/North-Holland Biomedical Press, Amsterdam.
24. Wüster, M., and Schulz, R. (1981) Biochem. Pharmacol. 30, in press.