Neuropharmacology Vol. 25, No. 4, pp. 351–359, 1986
Printed in Great Britain.

0028-3908/86 $3.00 + 0.00

INTERACTION BETWEEN [^{3}H]ETHYLKETOCYCLAZOCINE AND [^{3}H]ETORPHINE AND OPIOID RECEPTORS IN MEMBRANES FROM RAT BRAIN

A KINETIC ANALYSIS

S. V. FISCHEL* and F. MEDZIHRADSKY†
Departments of Biological Chemistry and Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

(*Accepted* 18 *July* 1985)

Summary—Scatchard analysis of the binding to opioid receptors of [^{3}H]ethylketocyclazocine ([^{3}H]EKC) and [^{3}H]etorphine at equilibrium yielded biphasic plots and computer fitting of the data resulted in a minimal model of two independent saturable binding sites. The K_D values for the high- and low-affinity sites were 0.58 and 38 nM for [^{3}H]EKC, and 0.13 and 22 nM for [^{3}H]etorphine. The corresponding density of binding sites was 157 and 418 fmol/mg protein for [^{3}H]EKC, and 220 and 289 fmol/mg protein for [^{3}H]etorphine. The K_D values calculated from the association and dissociation rate constants corresponded to those observed at equilibrium. In the course of equilibrium binding, various opioids competed with [^{3}H]EKC and [^{3}H]etorphine preferentially at the high-affinity opioid receptor sites. No difference between the competition patterns of putative μ and κ ligands was observed.

The kinetics of association and dissociation of [^{3}H]EKC and [^{3}H]etorphine revealed that the apparently homogeneous high-affinity binding site observed at equilibrium consisted of two components characterized by their fast and slow equilibrium times, respectively. While none of the μ and κ opiates investigated altered the rate of dissociation of [^{3}H]EKC or [^{3}H]etorphine, in the presence of sodium ions the rapidly dissociating binding component of [^{3}H]etorphine became refractory to inhibition by μ but not κ agonists. The results underline the advantages of evaluating both equilbrium binding and the kinetics of ligand-receptor interactions.

Key words: multiple opioid receptors, equilibrium binding, binding kinetics, ethylketocyclazocine, etorphine, rat brain, brain membranes.

In the research on multiple opioid receptors, the characterization of opioid alkaloids as ligands for the postulated μ and κ types of receptor has been hampered by the considerable cross-reactivity of these compounds, particularly pronounced in membranes of the rat brain (Kosterlitz and Paterson, 1980; Pfeiffer and Herz, 1981; Magnan, Paterson, Tavani and Kosterlitz, 1982). Although lately some novel alkaloids have been described which exhibited distinct κ activity in bioassay systems (Romer, Hill and Maurer, 1982; von Voigtlander, Lahti and Ludens, 1983; Lahti, Mickelson, McCall and von Voigtlander, 1985), ethylketocyclazocine (EKC) has widely been used as a κ agonist in studies of ligand binding to opioid receptors in membranes of the rat brain (Hiller and Simon, 1980; Pasternak, 1980; Snyder and Goodman, 1980; Chang, Hazum and Cuatrecasas, 1980). However, the shortcomings of this opiate and of the brain of the rat as the ligand and tissue of choice to characterize the κ opioid receptor have recently been emphasized (Goldstein and James, 1984). Indeed, apparently due to the lack of selectivity, the findings of early binding studies have failed to substantiate the existence of separate receptors for narcotic drugs of the κ type in the brain of the rat. Subsequently, partial support for discrete κ sites in rat brain was obtained from simultaneous computer analysis of the binding of labelled μ and κ opiates (Pfeiffer and Herz, 1982) and from competitive ligand displacement in brain membranes from the rat (Chang, Hazum and Cuatrecasas, 1981; Wolozin, Nishimura and Pasternak, 1982) and mouse (Garzon, Sanchez-Blazquez and Lee, 1984).

Recently, it has been possible to differentiate between putative μ and κ opiates on the basis of their responses to sodium in displacing [^{3}H]EKC and [^{3}H]etorphine in membranes from rat and pigeon brain (Medzihradsky, Dahlstrom, Woods, Fischel and Mitsos, 1984). The biphasic nature of the binding of opiate alkaloids in membranes from the brain of the rat has also been described and the strong influence of methodology on the outcome of the kinetic analysis of opioid receptor binding has been demonstrated (Fischel and Medzihradsky, 1981). As a follow-up to these studies, the present work has investigated the interaction between [^{3}H]EKC and

*Present address: Department of Neuroscience, Children's Hospital Medical Center, and Department of Neuropathology, Harvard Medical School, Boston, MA 02115, U.S.A.
†To whom correspondence should be addressed at the Department of Biological Chemistry.

opioid receptors in membranes from the brain of the rat under conditions of (a) equilibrium binding, (b) association and dissociation, and (c) competitive inhibition by different opioid alkaloids. The aim was to probe the resolution of the binding of κ opioid receptors in membranes of the rat brain, obtainable by the kinetic approach with EKC as the prototype ligand. For comparative purposes, the relevant properties of tritiated etorphine, an opiate with broad specificity in binding to opioid receptors, were also investigated.

METHODS

Materials

[^{3}H]Ethylketocyclazocine (15 Ci/mmol) and [^{3}H]-etorphine (39 Ci/mmol) were purchased from New England Nuclear Co., Boston, Massachusetts, U.S.A. and Amersham Corp., Arlington Heights, Illinois, U.S.A., respectively. The radiochemical purity of these compounds was 98%, as ascertained by thin-layer chromatography on silica gel G plates (Eastman Kodak Corp., Rochester, New York, U.S.A.) in two different solvent systems recommended by the manufacturers.

The unlabelled opiates were obtained through the Drug Abuse Basic Research Center at The University of Michigan. The enantiomers (1R, 5R, 9R, 2′R)-5,9-dimethyl-2″-tetrahydrofurfuryl-6,7-benzomorphan (UM1071-R) and the all-S isomer of UM1071-R (UM1071-S) are *N*-furyl-substituted benzomorphans with widely diverging κ agonist activity (Medzihradsky *et al.*, 1984; Woods, Smith, Medzihradsky and Swain, 1979), and were used in this study as the pharmacologically active and inactive isomer, respectively. 5,9-Diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan (MR2266) is a *N*-furyl-substituted benzomorphan antagonist that was classified as a κ opiate (Woods *et al.*, 1979), but in different studies had varying potency toward μ and κ agonists (Lord, Waterfield, Hughes and Kosterlitz, 1977; Barchfeld, Maassen and Medzihradsky, 1982). Biochemical agents were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A. Other compounds were of reagent grade, and were obtained from commercial distributors of chemicals.

Isolation and pretreatment of membranes

Parts of this procedure have been published previously (Fischel and Medzihradsky, 1981). Male, Sprague–Dawley rats weighing 200 g were decapitated and the cerebra pooled in 100 vol of ice-cold 50 mM Tris–HCl buffer, pH 7.4. After disrupting the tissue using a Polytron homogenizer, the resulting homogenate was centrifuged at 20,000 *g* for 15 min at 4°C. The pellet was resuspended in the same volume of the Tris buffer using a Dounce homogenizer. Aliquots of this suspension, sufficient for one experiment, were frozen at −70°C. The concentration of protein in these samples was approx. 0.6 mg/ml and opiate receptor binding was shown to be stable under such conditions for at least 6 months.

Upon thawing, the membranes were suspended in a Dounce homogenizer and used as such, or were subjected to an initial incubation. In the latter case, the membranes were centrifuged at 20,000 *g* for 15 min, the pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4 at 37°C, and the suspension then incubated at 37°C for 40 min. After a repeated centrifugation at 20,000 *g* for 15 min at 4°C, the pellet was resuspended in the appropriate buffer medium for the assay of receptor binding. Unless otherwise specified, the buffer was 50 mM Tris–HCl, adjusted to pH 7.4 at the assay temperature.

Analysis of receptor binding at equilibrium

To 8 ml polypropylene tubes in an ice–water bath, were added 400 μl membrane suspension (approx. 200 μg protein), 50 μl distilled water or 50 μl 1.575 M NaCl (final concentration in assay 150 mM) and 50 μl distilled water or solution of unlabelled drug. Unless specified otherwise, the assay mixture was incubated in a shaking water bath for 15 min at 25°C. After the addition of 25 μl aliquots of the radiolabelled ligand, the assay tubes were incubated to reach equilibrium in ligand binding and then either placed in an ice–water bath for 10 min or filtered directly. Prior to use, the Whatman GF/C filters employed were soaked in distilled water and washed on the filter assembly three times with 5 ml aliquots of distilled water saturated with amyl alcohol (Medzihradsky, 1976). The tube and the filtered material were rinsed with three 4 ml aliquots of ice-cold 50 mM Tris–HCl, pH 7.4. The filters were transferred into scintillation vials, 1 ml of absolute ethanol and 10 ml of a dioxane-xylene-napththalene scintillation mixture were added and the samples subjected to liquid scintillation counting. Average counting efficiency was 36% as determined by the use of ^3H_2O.

The actual concentration of tritiated drug present in the assay medium was determined by counting aliquots of the corresponding stock solution prepared for that particular experiment. Specific, i.e. receptor-related, interaction of the radiolabelled ligand was determined either with a stereospecific "binding window" (Fischel and Medzihradsky, 1981) established for [^{3}H]EKC with the active and inactive isomers UM1071-R/UM1071-S and for [^{3}H]etorphine with levorphanol/dextrorphan, or was defined as the difference in binding obtained in the absence and presence of 10 μM unlabelled ligand.

Analysis of receptor binding kinetics

These experiments were performed as described above with the following exceptions. To determine the rates of ligand-receptor association, the assay was terminated at various times before and after binding

Table 1. Equations for computer modeling of nonlinear binding data

Model	Equation
One binding site + one linear component	$b = \frac{n_1[L]}{K_{D_1} + [L]} + k_L[L]$
Two binding sites	$b = \frac{n_1[L]}{K_{D_1} + [L]} + \frac{n_2[L]}{K_{D_2} + [L]}$
Two binding sites + one linear component	$b = \frac{n_1[L]}{K_{D_1} + [L]} + \frac{n_2[L]}{K_{D_2} + [L]} + k_L[L]$
One co-operative binding site	$b = \frac{n_1[L]^{nH}}{K_{D_1} + [L]^{nH}}$
Multiple dissociation rates	$b = \Sigma_i^j = 1^{A_i e^{-k_{-i}t}}$
Multiple association rates	$b = b_{eq}(1 - \Sigma_i^j) = 1^{A_i e^{-(k_i[L] + k_{-i})t}}$

b = amount of ligand bound at concentration, [L], or time, t; b_{eq} = amount of ligand bound at equilibrium; n_i = number of binding sites for site i; K_{D_i} = equilibrium dissociation constant for site i; k_L = linear binding constant; n_H = Hill coefficient; k_i = association rate constant; k_{-i} = dissociation rate constant; A_i = fractional contribution of site$_i$ to the total number of sites; j = number of different types of sites bound at equilibrium.

equilibrium. The kinetics of ligand-receptor dissociation were assessed by the method of "infinite dilution" (DeMeyts, Bianco and Roth, 1976), i.e. the reassociation of the radiolabelled ligand with the receptor was prevented by dilution in the presence of excess unlabelled ligand. Membrane suspensions were incubated with a tritiated opiate to equilibrium, centrifuged at 20,000 *g* at 4°C for 15 min, and rapidly suspended in 2 ml of ice-cold 50 mM Tris–HCl, pH 7.4 at 25°C. Ligand dissociation was initiated by the addition of 50 μl aliquots of this suspension to tubes containing 5 ml of prewarmed (25°C) 50 mM Tris–HCl buffer, pH 7.4, with or without 1 μM unlabelled ligand. Samples were filtered at the time of this addition (defined as 100% binding) and at various times thereafter.

Determination of protein

The method of Lowry *et al.* was applied (Lowry, Rosebrough, Farr and Randall, 1951). Standards of crystalized, lyophilized bovine serum albumin were prepared using the same buffer medium present in the biological samples being tested, unless it was demonstrated experimentally that the components of the medium did not interfere with the protein assay (Peterson, 1979).

Methods of computer analysis and statistical evaluation

The results from both equilibrium binding and kinetic experiments were evaluated using NONLIN, a weighted nonlinear least-squares regression analysis program (Metzler, 1969). The data were weighted according to the inverse of the observed experimental values (Boxenbaum, Riegelman and Elasoff, 1981) and were fitted to one or more of the appropriate equations listed in Table 1. Initial estimates for binding parameters were obtained from the respective double reciprocal plots (Neal, 1972), or by using CSTRIP, a computerized feathering technique for obtaining estimates from multiphasic exponential curves (Sedman and Wagner, 1976). The appropriateness of a binding model was assessed by the distribution of the weighted residuals about the theoretical curve and by the extent to which the sum of the weighted squared deviations was minimized. The significance of differences between the binding models was evaluated statistically using the *F*-ratio test (Table 2). Statistical differences between the calculated binding constants or between the means of various experiments were evaluted using a two-tailed Student's *t*-test (Boxenbaum *et al.*, 1981).

RESULTS

Equilibrium binding

Specific binding of [^{3}H]EKC, determined within the linear range of membrane protein dependence, reached equilibrium after 40 min at 25°C, both in the absence and presence of sodium. In agreement with the results of previous studies (Simon, Hiller and Edelman, 1973; Tolkovsky, 1982), steady-state in the receptor binding of [^{3}H]etorphine was obtained after 20 min incubation at 25°C. Scatchard analysis of specific binding of [^{3}H]EKC revealed (Fig. 1A) that the presence of 150 mM NaCl decreased the number of high-affinity binding sites by 50% with no changes in the corresponding K_D (Table 2). Prior incubation of the membranes increased the number of high-affinity binding sites 1.6-fold without altering the K_D either in the presence or absence of Na^+. Thus, the pretreatment did not change the numerical value of the sodium ratio in [^{3}H]EKC binding (binding in the absence of Na^+/binding in the presence of Na^+). Neither initial incubation of membrane nor Na^+ altered the interaction between [^{3}H]EKC and the low-affinity specific binding sites. Computer analysis demonstrated that the model, consisting of two non-interacting saturable binding sites (Table 1), provided the best fit for the data obtained (Table 2). Although no statistically-significant difference between the two-saturable-site model and the one-cooperative-site model was obtained in the presence of NaCl, dissociation experiments, using the method of infinite

Table 2. Equilibrium binding parameters for [^{3}H]EKC assuming different models of ligand–receptor interactions

Binding parameters	Binding site models			
	1 saturable and 1 linear	2 saturable	2 saturable and 1 linear	1 co-operative
– Preincubation, – NaCl				
K_{D_1} (nM)	1.0 ± 0.2	0.58 ± 0.06	0.56 ± 0.08	6.1 ± 1.3
n_1 (fmol/mg protein)	228 ± 18	157 ± 10	154 ± 15	693 ± 120
K_{D_2} (nM)	—	38 ± 7	31 ± 20	—
n_2 (fmol/mg protein)	—	418 ± 19	357 ± 159	—
k_L (fmol/mg protein . nM)	2.8 ± 0.4	—	0.37 ± 1.0	—
n_H	—	—	—	0.53 ± 0.05
SWSD	8.28	0.521	0.506	5.65
Significance	$P < 0.001$	—	NS*	$P < 0.001$
– Preincubation, + NaCl				
K_{D_1} (nM)	2.5 ± 0.4	0.41 ± 0.20	0.30 ± 0.20	6.1 ± 0.05
n_1 (fmol/mg protein)	224 ± 20	60 ± 17	46 ± 20	451 ± 30
K_{D_2} (nM)	—	13 ± 3	9.1 ± 3.6	—
n_2 (fmol/mg protein)	—	319 ± 36	276 ± 36	—
k_L (fmol/mg protein . nM)	1.6 ± 0.3	—	0.53 ± 0.47	—
n_H	—	—	—	0.66 ± 0.03
SWSD	19.4	6.05	5.58	5.76
Significance	$P < 0.001$	—	NS	NS
+ Preincubation, – NaCl				
K_{D_1} (nM)	0.62 ± 0.07	0.40 ± 0.06	0.37 ± 0.08	2.1 ± 0.2
n_1 (fmol/mg protein)	330 ± 14	252 ± 22	237 ± 35	543 ± 32
K_{D_2} (nM)	—	22 ± 8	13 ± 11	—
n_2 (fmol/mg protein)	—	292 ± 24	237 ± 69	—
k_L (fmol/mg protein . nM)	2.0 ± 0.3	—	0.49 ± 0.71	—
n_H	—	—	—	0.58 ± 0.04
SWSD	26.3	10.1	9.98	18.5
Significance	$P < 0.001$	—	NS	$0.001 < P < 0.005$
+ Preincubation, + NaCl				
K_{D_1} (nM)	1.4 ± 0.2	0.40 ± 0.13	0.39 ± 0.13	3.5 ± 0.3
n_1 (fmol/mg protein)	273 ± 19	114 ± 25	112 ± 25	467 ± 24
K_{D_2} (nM)	—	11 ± 3	11 ± 3	—
n_2 (fmol/mg protein)	—	308 ± 21	309 ± 27	—
k_L (fmol/mg protein . nM)	1.5 ± 0.3	—	0.00001 ± 0.00061	—
n_H	—	—	—	0.64 ± 0.03
SWSD	36.6	9.58	9.58	9.75
Significance	$P < 0.001$	—	NS	NS

*Not significant ($P > 0.10$).

The values listed are the computer-estimated means and standard deviations for the best fit of the data to the binding models listed below and defined in Table 1. The experiments and the statistical treatment of the data are described in the legend to Figure 1 and in Methods. All of the experiments were carried out at 25°C in the absence and presence of 150 mM NaCl using untreated and initially incubated membranes, respectively. The concentration of [^{3}H]EKC in the assay ranged up to 100 nM. Significance, indicated by results of F-ratio tests, relates to the statistical difference of the data relative to that for the model with two saturable sites. The computer-estimated mean values and the corresponding standard deviations are shown. The estimation for each experimental condition was based on 18 data points obtained in 2 separate experiments. SWSD = sum of the weighted squared deviations (criterion of the variance ratio, or F-test).

dilution, revealed the lack of cooperative interactions (data not shown). Specific binding, determined by excess of unlabelled ligand, was completely stereospecific as shown by the use of UM1071-R, a potent κ agonist and its virtually inactive enantiomer UM1071-S as displacing agents (Fig. 1, inset).

As described above for the receptor binding of [^{3}H]EKC, Scatchard analysis and computer fitting to binding models resolved specific binding of [^{3}H]etorphine into two components with no evidence of negative cooperativity (Fig. 1B). The respective K_D values (nM) in the absence and presence of Na^+ were 0.13 ± 0.02 and 22 ± 7 for [^{3}H]EKC, and 0.06 ± 0.03 and 5.3 ± 2.2 for [^{3}H]etorphine. The corresponding values for n_1 and n_2 (fmol/mg protein) were 220 ± 11 and 289 ± 24, while with Na^+ they were 114 ± 22 and 177 ± 24. As was the case for [^{3}H]EKC, prior incubation of the membrane did not alter the extent of the sodium ratio for [^{3}H]etorphine (data not shown). The slight, statistically-insignificant, discrepancies in the numerical values of the binding parameters for the high-affinity site listed in Tables 2 and 4 are due to different modes of fitting the data.

Competitive interactions assessed at equilibrium

Various concentrations of the radiolabelled ligand were incubated in the absence and presence of a constant concentration of a given unlabelled opiate (Table 4). The latter concentration was selected to inhibit the specific binding of 0.5 nM radiolabelled ligand by 30–50%. Initially, the results were evaluated by computer analysis for binding to a two-saturable-site model. Since no statistically-significant inhibition of the low-affinity binding site was observed, the data for all experiments within a given set of conditions (e.g. inhibition of radiolabelled ligand binding in either the absence and presence of NaCl) were fitted simultaneously, assuming no changes in the binding parameters of the low-affinity binding site. This approach has increased the statistical precision in evaluating the kinetic constants of the high-affinity binding sites. The unlabelled opiates increased the apparent K_Ds 2- to 7-fold for [^{3}H]EKC and [^{3}H]etorphine without decreasing the number of corresponding binding sites. This behaviour was indicative of a non-cooperative, competitive interaction

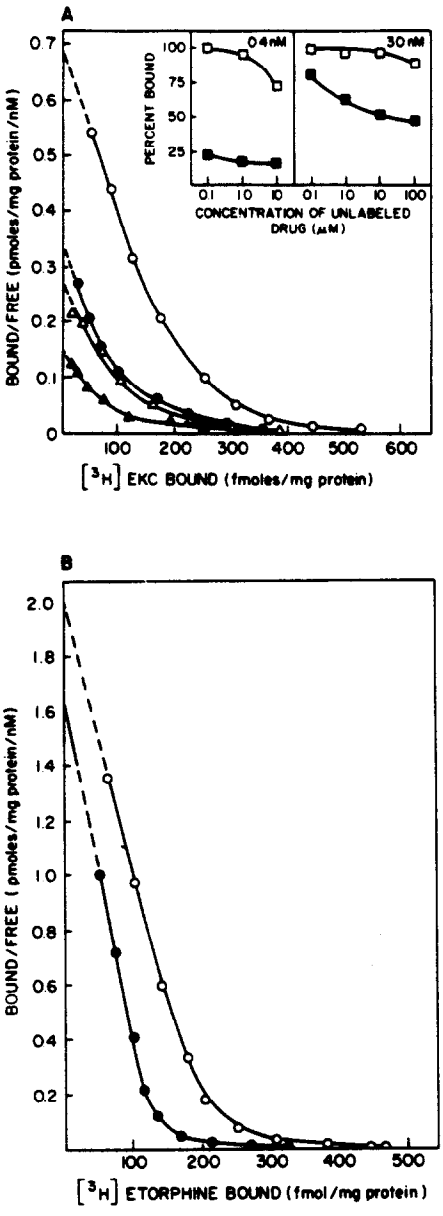

Fig. 1. Scatchard analysis of specific binding of [^{3}H]ethylketocyclazocine and [^{3}H]etorphine. The opioid receptor-related binding of 0.1–50 nM radiolabelled EKC (A) and etorphine (B) in the absence (○, △) and presence (●, ▲) of 150 mM NaCl was determined after incubation for 40 min at 25°C as described in Methods. Cerebral membranes were used either directly (△, ▲) or subjected to an initial incubation for 40 min at 37°C (○, ●). Specific interaction was defined either as the difference between ligand binding in the absence and presence of 10 μM labelled ligand, or as stereospecific binding determined by the use of UM1071-S (□) and UM1071-R (■) in the case of [^{3}H]EKC and dextrorphan and levorphanol in the case of [^{3}H]-etorphine. The optimal concentrations of these enantiomers to displace the radiolabelled ligands were determined as illustrated for [^{3}H]EKC binding (inset). The concentration of [^{3}H]EKC and [^{3}H]etorphine (not shown) in these experiments reflected the range used in the Scatchard analyses. The specific and sterospecific binding of [^{3}H]EKC and [^{3}H]etorphine were shown to be equivalent under all of the assay conditions used. The averages of data obtained in 2 experiments, each run with duplicate samples are shown.

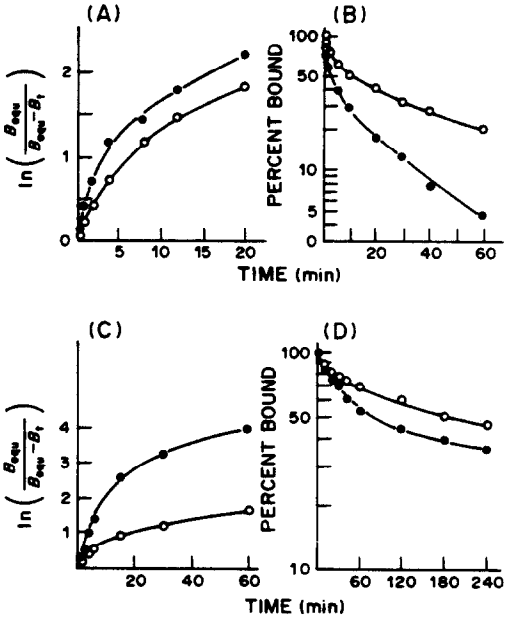

Fig. 2. Kinetics of specific binding and dissociation of [^{3}H]ethylketocyclazocine and [^{3}H]etorphine. These experiments were carried out at 25°C in the absence (○) or presence (●) of 150 mM NaCl with cerebral membranes which had been subjected to prior incubation. To investigate the association kinetics, specific binding of 0.5 nM of [^{3}H]-EKC (A) or [^{3}H]etorphine (C) were determined as described in Methods. The ordinate represents the natural logarithm of the specific binding at equilibrium divided by the difference between that quantity and the specific binding at the times indicated (Bennett, 1978). In the experiments on ligand dissociation, the membrane suspension was incubated with 0.5 nM of [^{3}H]EKC (B) or [^{3}H]etorphine (D). Reassociation was prevented by the addition of unlabelled ligand to yield a final concentration of 10 μM as described in Methods. The percentage of specifically bound [^{3}H]EKC or [^{3}H]etorphine at various times after the dilution is plotted. The averages of two experiments with duplicate samples are shown. The corresponding results of the individual experiments differed by less than 5%.

between both μ and κ opiates at the high-affinity binding sites for the radiolabelled ligands (Table 4).

Kinetics of ligand binding and dissociation

The rates of association and dissociation of [^{3}H]EKC and [^{3}H]etorphine revealed two phases (Fig. 2 and Table 3) of the high-affinity ligand-receptor interaction, which appeared homogeneous under conditions of equilibrium binding (Fig. 1 and Table 2). Both in the absence and presence of Na^+ the two phases were characterized by fast and slow equilibration, respectively. The K_D values calculated from the respective rate constants were similar to those obtained for the high-affinity binding sites from the data of the equilibrium binding experiments (Tables 2 and 3, and text).

Competitive ligand dissociation

In these experiments, the interaction between different opiates and the two dissociating phases of [^{3}H]EKC (not shown) and [^{3}H]etorphine binding

Table 3. Rate constants for specific binding of [^{3}H]EKC and [^{3}H]etorphine

NaCl (nM)	Component 1 k_1 ($min^{-1}M^{-1}$)	Component 1 k_{-1} (min^{-1})	Component 1 K_{Dcalc} (nM)	Component 2 k_2 ($min^{-1}M^{-1}$)	Component 2 k_{-2} (min^{-1})	Component 2 K_{Dcalc} (nM)
[^{3}H]EKC						
0	8.1×10^7	9.7×10^{-3}	0.12	5.4×10^8	1.0×10^{-1}	0.19
150	1×10^8	2.5×10^{-2}	0.25	4×10^8	5.0×10^{-1}	1.0
[^{3}H]Etorphine						
0	2.8×10^7	2.2×10^{-3}	0.079	2.9×10^8	5.8×10^{-2}	0.20
150	5.4×10^7	1.4×10^{-3}	0.026	5.5×10^8	4.1×10^{-2}	0.075

The listed data are the computer-estimated values for the experiments described in Fig. 2. K_{Dcalc} is defined as k_{-1}/k_1 or k_{-2}/k_2.

(Fig. 3) was investigated. The binding of 0.5 nM [^{3}H]EKC or [^{3}H]etorphine was allowed to reach equilibrium in the absence and presence of an unlabelled opiate. After initiating dissociation of radiolabelled ligand with excess unlabelled opiate, the rates of the fast and slow phases and their relative contribution to total radiolabelled ligand binding were determined. None of the unlabelled opiates significantly altered the rate of dissociation of [^{3}H]EKC or [^{3}H]etorphine under any of the experimental conditions. These findings indicated that the competing ligands bound to the fast and slow dissociating sites in a non-cooperative manner, in agreement with the corresponding results of equilibrium binding (Table 4). In the absence of NaCl, competing opiates altered the relative contribution of the slowly- and rapidly-dissociating components to total specific binding of [^{3}H]EKC and [^{3}H]etorphine: significantly greater inhibition of the slow component occurred. However, the extent of inhibition by putative μ and κ ligands was indistinguishable (Fig. 3). On the other hand, in the presence of Na^+, a differential effect of μ and κ opiates on the dissociation of [^{3}H]etorphine (but not of [^{3}H]EKC) became apparent. Whereas μ agonists reduced the contribution of the slow component to specific binding at equilibrium from 53 to 35%, κ agonists had little or no effect. Putative antagonists of either type of opioid receptor produced no significant changes in the distribution of the two dissociative phases (Fig. 3).

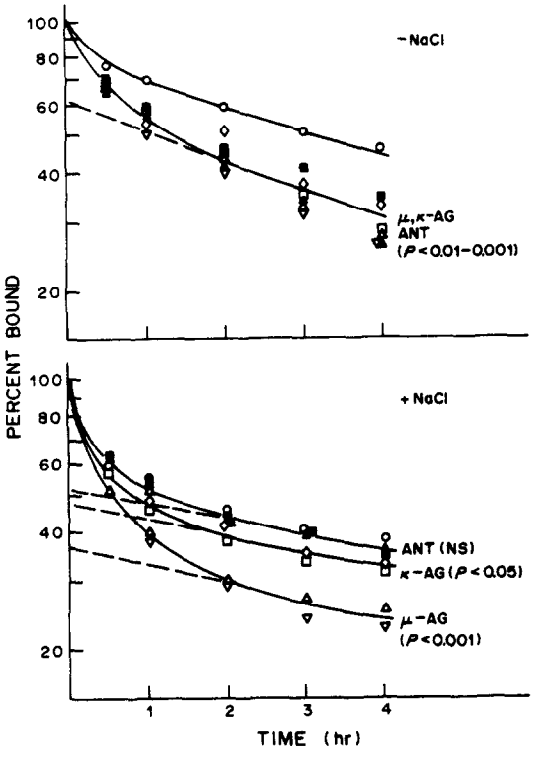

Fig. 3. Dissociation of [^{3}H]etorphine from opioid receptors in the presence of competing ligands. Untreated cerebral membranes were incubated with 0.5 nM [^{3}H]etorphine to equilibrium at 25°C alone (○) or with enough unlabelled opiates to inhibit the specific binding by approx. 20% in the absence or presence of 150 mM NaCl. Unlabelled opiates included the antagonists (ANT) naltrexone (▲) and MR2266 (■), the μ agonists (AG) morphine (△) and levorphanol (▽) and the κ agonists EKC (□) and UM1071-R (◇). Two to four drugs were tested simultaneously in single experiments. Ligand dissociation was initiated by the addition of 10 μM unlabelled etorphine. The results of one to three determinations for each drug were evaluated by nonlinear least squares regression analysis as described in Methods. The statistical significance of differences between the percentage contributions of the slow dissociative component in the presence and absence of unlabelled drugs was determined by the two-tailed Student's *t*-test with 9 to 28 *df*. The *P*-values listed in the Figure indicate the significance of the data compared to the control. NS = not significant.

DISCUSSION

Analysis of nonlinear receptor binding.

Biphasic specific binding of opiates, as shown in this study for [^{3}H]EKC and [^{3}H]etorphine, has frequently been ascribed to the presence of two different populations of receptor sites. However, other biological (DeMeyts, Roth, Neville, Gavin and Lesniak, 1973) or methodological (Fischel and Medzihradsky, 1981) factors can be responsible for such findings. Negatively cooperative ligand-receptor interactions were shown to be responsible for the biphasic binding of insulin to a variety of tissues (DeMeyts *et al.*, 1976). This conclusion was based primarily on the ability of unlabelled insulin to accelerate the dissociation rate of previously bound [^{125}I]insulin (DeMeyts *et al.*, 1973). By this criterion, as well as by the "goodness of fit" of the data to allosteric or multiple site models by a computer-modelling procedure, the opioid receptor binding of [^{3}H]EKC and [^{3}H]etorphine in the present work was shown to be best described by two populations of non-interacting

Table 4. Interaction between μ and κ opiates and specific binding of [^{3}H]EKC and [^{3}H]etorphine at equilibrium

Competing ligand	−NaCl		+NaCl	
	Apparent K_D (nM)	n (fmol/mg protein)	Apparent K_D (nM)	n (fmol/mg protein)
[^{3}H]EKC: *High-affinity binding site*				
None	0.61 ± 0.08	163 ± 12	0.58 ± 0.23	75 ± 18
Morphine	0.87 ± 0.13	139 ± 13	1.12 ± 0.33	104 ± 21
EKC	1.05 ± 0.14	159 ± 14	1.37 ± 0.37	111 ± 22
Low-affinity binding site				
None	39 ± 8	405 ± 20	16 ± 3	312 ± 17
[^{3}H]Etorphine: *High-affinity binding site*				
None	0.11 ± 0.01	204 ± 7	0.065 ± 0.021	122 ± 12
Morphine	0.35 ± 0.03	218 ± 9	0.39 ± 0.09	172 ± 17
Levorphanol	0.65 ± 0.07	200 ± 12	ND*	ND
Naltrexone	0.51 ± 0.05	204 ± 10	0.25 ± 0.06	160 ± 16
EKC	0.37 ± 0.03	184 ± 8	0.33 ± 0.08	154 ± 16
UM 1071	0.65 ± 0.07	200 ± 12	ND	ND
MR 2266	0.86 ± 0.08	251 ± 12	0.38 ± 0.09	127 ± 17
Low-affinity binding site				
None	20 ± 3	319 ± 12	14 ± 5	235 ± 21

*Not determined.

Untreated cerebral membranes were incubated with a constant amount of unlabelled ligand and varying concentrations (0.1–50 nM) of [^{3}H]EKC or [^{3}H]etorphine to equilibrium at 25°C. The concentration of unlabelled opiate was sufficient to inhibit the specific binding of 0.5 nM radiolabelled ligand by 30%. Specific binding was determined in the absence and presence of 10 μM of the respective unlabelled ligand. Samples were placed on ice for 10 min immediately prior to filtration. The data obtained in the absence and presence of 150 mM NaCl were pooled separately, and subjected to simultaneous nonlinear least squares regression analysis as described in the text. The significance of the data was determined using the Student's t-test. Shown are the computer-estimated means and standard deviations for 27 to 58 points obtained in 3–6 experiments ([^{3}H]EKC) and for 41 to 80 data points obtained in 5–7 experiments ([^{3}H]etorphine).

saturable binding sites with different affinities, rather than by an occupancy-dependent isomerization of a single site (Tolkovsky, 1982). In contrast to the receptor model with distinctive non-interacting binding sites for the opioid alkaloids investigated in this work, the binding of morphine and leucine enkephalin in membranes from the rat brain was best described by two allosterically-coupled populations of sites (Rothman and Westfall, 1982a, 1982b). According to that model, the binding of morphine (and etorphine) masks, and thus modulates, the binding sites for the opioid peptide. A similar model, depicting allosteric interaction between κ and δ opioid ligands binding to different sites on a common receptor molecule (endorphin receptor), has also been proposed (Smith and Lee, 1983). It is of interest to point out that these allosteric models of the opioid receptor are based on the binding patterns of pairs of chemically-dissimilar ligands, i.e. an alkaloid and peptide. In contrast, in the previous study (Fischel and Medzihradsky, 1981) and present work, the receptor binding of opioid alkaloids has been described, whereby the data favored a two-site rather than an allosteric model. A recent kinetic study of the binding of opioid agonists and antagonists in membranes from the rat brain has shown that opioid alkaloids, in contrast to opioid peptides, interact with multiple receptor sites (Scheibe, Bennett, Spain, Roth and Coscia, 1984).

The kinetics of receptor binding carried out in this study confirmed the relevance of the proposed model and provided further resolution. The kinetic data revealed that high-affinity ligand binding consisted of two components, equilibrating at different rates, not distinguishable by equilibrium binding methods. Using [^{3}H](D-Ala^2-D-Leu^5)enkephalin, a slowly dissociating ligand-opioid receptor complex has previously been described in synaptic membranes from bovine brain (Pryhuber, Roth and Coscia, 1982). This observation has led to the postulation of a multistep mechanism for opioid agonists, whereby the rapidly reversible ligand-receptor complex initially formed is converted into a slowly-dissociating high-affinity conformation (Scheibe *et al.*, 1984). The comparison of these findings with the present data is hampered by the use of opioid agonist peptides in one and alkaloids in the other. Nonetheless, in apparent contrast to the multistep binding model for agonists, multiple dissociation components for the antagonist [^{3}H]naltrexone have also been described (Fischel and Medzihradsky, 1981). It should also be noted that similar K_D values for the receptor binding of [^{3}H]naltrexone (Fischel and Medzihradsky, 1981), [^{3}H]EKC and [^{3}H]etorphine (see Results) were obtained at equilibrium and kinetically. However, in neither study an occupancy-dependent shift in ligand binding affinity and pattern of dissociation has been specifically tested for.

Significance of high and low affinity binding sites

In early studies on opioid receptor binding, the marked depression of the high-affinity component for [^{3}H]dihydromorphine in the presence of sodium ions led to the suggestion that antagonists and agonists bind to the high and low-affinity sites, respectively (Pasternak and Snyder, 1975). However, subsequently pure opioid agonists were shown to displace high-affinity binding sites (Lord *et al.*, 1977).

Indeed, as shown in this study, both agonists and antagonists competed preferentially for the high-affinity binding sites (Table 4). In this respect the present data, obtained with membranes from rat brain, provide no support for the recently proposed receptor model of separate recognition sites for opioid agonists and antagonists (Portoghese and Takemori, 1983).

In membranes from transformed neural cells, unlabelled κ and δ opiates competed preferentially with the low-affinity component of the [^{3}H]EKC binding isotherm (West, McLawhon, Dawson and Miller, 1981). Correspondingly, it was proposed that the high-affinity binding site, termed μ_1, is common to all opiates and is responsible for analgesia, whereas ligand interaction at the lower affinity receptor sites mediates the specific effects of the different types of opiates (Pasternak, 1980; Wolozin *et al.*, 1982). On the other hand, the results in this paper show that in membrane preparations from rat cerebra, a partial resolution in the dissociation of μ and κ opiates was achieved at ligand concentrations which favored the binding to the high-affinity sites of [^{3}H]EKC and [^{3}H]etorphine.

Discrimination between the receptor binding of [^{3}H]EKC and [^{3}H]etorphine

The classification of μ and κ opiates in membranes from the rat brain has recently been described on the basis of their differential sensitivity toward sodium ions in binding to receptor (Medzihradsky *et al.*, 1984). A possible molecular correlate for this resolution was revealed by the kinetics of ligand binding determined in the present study. The high affinity binding of [^{3}H]EKC and [^{3}H]etorphine consisted of two components. In the presence of sodium, the rapidly-dissociating phase of [^{3}H]etorphine binding became refractory to inhibition by μ but not κ agonists, thus revealing a degree of discrimination between these types of opiates at the high-affinity binding sites. Since the unlabelled opiate alkaloids altered the relative contribution of the rapidly and slowly equilibrating components to total ligand binding without affecting the respective dissociation rates, their interaction with receptor was competitive rather than cooperative in nature. In view of the different patterns of inhibition displayed by μ and κ agonists in the absence and presence of NaCl (Fig. 3), it will be of interest to evaluate the significance of an interconversion of multiple populations of opioid receptor sites (Bowen, Gentleman, Herkenham and Pert, 1981), suggested for other receptors to be regulated by intracellular Na^+ (Insel and Motulsky, 1984).

At the present level of resolution in membranes from rat brain, it is difficult to identify the two newly resolved components of the binding of [^{3}H]EKC and [^{3}H]etorphine in relation to postulated opioid receptor binding sites. For instance, since the slowly equilibrating component of the dissociation of [^{3}H]EKC and [^{3}H]etorphine was inhibited by both μ and κ opiates, it would have been tempting to assume that it represents the μ_1 sites, and the rapidly equilibrating component the κ binding sites. However, in such a case one of the dissociative components of [^{3}H]EKC should have been insensitive to inhibition by μ agonists. To what extent conformational changes between receptor forms of different affinity (Scheibe *et al.*, 1984) represent a general phenomenon for the binding of opioid ligands remains to be assessed. A higher degree of resolution of the κ receptor in membranes from the rat brain can be expected from the use of either radiolabelled opiates with increased specificity (e.g. von Voigtlander *et al.*, 1983; Lahti *et al.*, 1985) or unlabelled irreversible ligands specifically alkylating discrete populations of opioid receptor sites (James and Goldstein, 1984; Clark and Medzihradsky, 1986). An alternative and comprehensive approach is the study of opioid receptor heterogeneity in systems where receptor-effector coupling represents an additional molecular diversity which contributes to the biological differentiation of the action of opiates (Barchfeld *et al.*, 1982; Barchfeld and Medzihradsky, 1984; Clark and Medzihradsky, 1986). In this respect, the heterogeneity of opioid receptors can be expressed by the nature of the effector system, and the mechanism and efficacy of coupling. However, the results of this study show that even under conditions of unfavorable ligand and tissue specificity, the analysis of ligand association and dissocation kinetics, in addition to the assessment of equilibrium binding, represents a valuable approach in characterizing the interaction between ligands and opioid receptors.

Acknowledgements—We wish to thank Patricia J. Dahlstrom for her expert technical assistance. This work was supported in part by USPHS Grant DA-00254 and by a grant from the Rackham School of Graduate Studies at The University of Michigan (S.V.F.).

REFERENCES

Barchfeld C. C., Maassen Z. and Medzihradsky F. (1982) Receptor-related interactions of opiates with PGE-induced adenylate cyclase in brain. *Life Sci.* **31:** 1661–1665.

Barchfeld C. C. and Medzihradsky F. (1984) Receptor-mediated stimulation of brain GTPase by opiates in normal and dependent rats. *Biochem. biophys. Res. Commun.* **121:** 641–648.

Bennett J. P. (1978) *Neurotransmitter Receptor Binding* (Yamamura H. I., Enna S. J. and Kuhar M. J., Eds), pp. 57–90. Raven Press, New York.

Bowen W. D., Gentleman S., Herkenham M. and Pert C. (1981) Interconverting μ and δ forms of the opiate receptor in rat striatal patches. *Proc. natn. Acad. Sci. U.S.A.* **78:** 4818–4822.

Boxenbaum H. G., Riegelman S. and Elasoff R. M. (1981) Statistical estimations in pharmacokinetics. *J. Pharmacokinet. Biopharmac.* **2:** 123–148.

Chang K.-J., Hazum E. and Cuatrecasas P. (1980) Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative kappa and sigma agonists). *Proc. natn. Acad. Sci. U.S.A.* **77:** 4469–4473.

Chang K.-J. Hazum E. and Cuatrecasas P. (1981) Novel

opiate binding sites selective for benzomorphan drugs. *Proc. natn. Acad. Sci. U.S.A.* **78:** 4141–4145.

Clark M. J. and Medzihradsky F. (1986) Heterogeneity and specificity in the coupling of opioid receptor to brain GTPase. *Subst. Alcohol Actions/Misuse.* In press.

DeMeyts P., Roth J., Neville D. M. Jr, Gavin J. R. III and Lesniak M. A. (1973) Insulin interactions with its receptors: Experimental evidence for negative cooperativity. *Biochem. biophys. Res. Commun.* **55:** 154–161.

DeMeyts P., Bianco A. R. and Roth J. (1976) Site–site interactions among insulin receptors. Characterization of the negative cooperativity. *J. biol. Chem.* **251:** 1877–1888.

Fischel S. V. and Medzihradsky F. (1981) Scatchard analysis of opiate receptor binding. *Molec. Pharmac.* **20:** 269–279.

Garzon J., Sanchez-Blazquez P. and Lee N. M. (1984) [^{3}H]Ethylketocyclazocine binding to mouse brain membranes: evidence for a kappa opioid receptor type. *J. Pharmac. exp. Ther.* **231:** 33–37.

Goldstein A. and James I. F. (1984) Multiple opioid receptors, criteria for identification and classification. *Trends Pharmac. Sci.* **5:** 503–505.

Hiller J. M. and Simon E. J. (1980) Specific, high affinity [^{3}H]ethylketocyclazocine binding in rat central nervous system: lack of evidence for kappa receptors. *J. Pharmac. exp. Ther.* **214:** 516–519.

Insel P. A. and Motulsky H. J. (1984) A hypothesis linking intracellular sodium, membrane receptors, and hypertension. *Life Sci.* **34:** 1009–1013.

James I. F. and Goldstein A. (1984) Site-directed alkylation of multiple opioid receptors. I. Binding selectivity. *Molec. Pharmac.* **25:** 337–342.

Kosterlitz H. W. and Paterson S. J. (1980) Characterization of opioid receptors in nervous tissue. *Proc. R. Soc. Lond. B* **210:** 113–122.

Lahti R. A., Mickelson M. M., McCall J. M. and Voigtlander P. F. von (1985) [^{3}H]-U-69593 a highly selective ligand for the opioid κ receptor. *Eur. J. Pharmac.* **109:** 281–284.

Lord J. A. Waterfield A. A., Hughes J. and Kosterlitz H. W. (1977) Endogenous opioid peptides: multiple agonists and receptors. *Nature, Lond.* **267:** 495–499.

Lowry O. H., Rosebrough N. A., Farr A. W. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193:** 265–275.

Magnan J., Paterson S. J., Tavani A. and Kosterlitz H. (1982) The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn-Schmiedebergs Arch. Pharmac.* **319:** 197–205.

Medzihradsky F. (1976) Stereospecific binding of etorphine in isolated neural cells and in retina, determined by a sensitive microassay. *Brain Res.* **108:** 212–219.

Medzihradsky F., Dahlstrom P. J., Woods J. H., Fischel S. V and Mitsos S. E. (1984) Resolution in the receptor binding of putative mu and kappa opiates. *Life Sci.* **34:** 2013–2022.

Metzler C. M. (1969) Technical Report 72/69/7292/0005, Upjohn Co., Kalamazoo, Michigan.

Neal J. L. (1972) Analysis of Michaelis kinetics for two independent, saturable membrane transport functions. *J. theor. Biol.* **35:** 113–118.

Pasternak G. W. and Snyder S. H. (1975) Identification of novel high affinity opiate receptor binding in rat brain. *Nature, Lond.* **253:** 563–565.

Pasternak G. W. (1980) Multiple opiate receptors: [^{3}H]ethylketocyclazocine receptor binding and ketocyclazocine analgesia. *Proc. natn. Acad. Sci. U.S.A.* **77:** 3691–3694.

Peterson G. L. (1979) Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Analyt. Biochem.* **100:** 201–220.

Pfeiffer A. and Hertz A. (1981) Demonstration and distribution of an opiate binding site in rat brain with high affinity for ethylketocyclazocine and SKF 10,047. *Biochem. biophys. Res. Commun.* **101:** 38–44.

Pfeiffer A. and Herz A. (1982) Discrimination of three opiate receptor binding sites with the use of a computerized curve-fitting technique. *Molec. Pharmac.* **21:** 266–271.

Portoghese P. S. and Takemori A. E. (1983) Different receptor sites mediate opioid agonism and antagonism. *J. med. Chem.* **26:** 1341–1343.

Pryhuber K. G., Roth B. L. and Coscia C. J. (1982) Demonstration of a slowly dissociating form of bovine hippocampal synaptic membrane opiate receptors. *Eur. J. Pharmac.* **83:** 47–53.

Romer D., Hill R. C. and Maurer R. (1982) Receptor interactions and reinforcing capacities of different opioids. In: *Learning and Memory, Drugs and Reinforcer* (Saito S. and Yamagita T., Eds), pp. 286–293. Excerpta Medica, Amsterdam.

Rothman R. B. and Westfall T. C. (1982a) Morphine allosterically modulates the binding of [^{3}H]leucine enkephalin to a particulate fraction of rat brain. *Molec. Pharmac.* **21:** 538–547.

Rothman R. B. and Westfall T. C. (1982b) Allosteric coupling between morphine and enkephalin receptors *in vitro*. *Molec. Pharmac.* **21:** 548–557.

Scheibe S. D., Bennett D. B., Spain J. W., Roth B. L. and Coscia C. J. (1984) Kinetic evidence for differential agonist and antagonist binding to bovine hippocampal synaptic membrane opioid receptors. *J. biol. Chem.* **259:** 13,298–13,303.

Sedman A. J. and Wagner J. G. (1976) CSTRIP, a Fortran IV computer program for obtaining initial polyexponential parameter estimates. *J. Pharmac. Sci.* **65:** 1006–1010.

Simon E. G., Hiller J. M. and Edelman I. (1973) Stereospecific binding of the potent narcotic analgesic [^{3}H]etorphine to rat brain homogenates. *Proc. natn. Acad. Sci. U.S.A.* **70:** 1947–1949.

Smith A. P. and Lee N. M. (1983) The multiple site β-endorphin receptor. *Trends Pharmac. Sci.* **4:** 163–164.

Snyder S. H. and Goodman R. R. (1980) Multiple neurotransmitter receptors. *J. Neurochem.* **35:** 5–15.

Tolkovsky A. M. (1982) Etorphine binds to multiple opiate receptors of the caudate nucleus with equal affinity but with different kinetics. *Molec. Pharmac.* **22:** 648–656.

Voigtlander P. F. von, Lahti R. A. and Ludens J. H. (1983) U-50,488: a selective and structurally novel non-mu (kappa) opioid agonist. *J. Pharmac. exp. Ther.* **224:** 7–12.

West R. E. Jr, McLawhon R. W., Dawson G. and Miller R. J. (1981) Opiate binding sites in the lumbo-sacral spinal cord from various species. In: *Advances in Endogeneous and Exogeneous Opioids* (Tagaki H. and Simon E., Eds), pp. 18–20. Kodensha Press, Tokyo.

Wolozin B. L., Nishimura S. and Pasternak G. W. (1982) The binding of kappa- and sigma-opiates in rat brain. *J. Neurosci.* **2:** 708–713.

Woods J. H., Smith C. B., Medzihradsky F. and Swain H. H. (1979) Preclinical testing of new analgesic drugs. In: *Mechanisms of Pain and Analgesia* (Beers R. F. Jr and Bassett E. G., Eds), pp. 429–445. Raven Press, New York.