0026-895X/90/050694-10$02.00/0

MOLECULAR PHARMACOLOGY, 37:694-703

Quantitative Analysis of Multiple κ-Opioid Receptors by Selective and Nonselective Ligand Binding in Guinea Pig Spinal Cord: Resolution of High and Low Affinity States of the κ_2 Receptors by a Computerized Model-Fitting Technique

MARIO TIBERI and JACQUES MAGNAN

Département de pharmacologie, Université de Móntréal, Montréal, Québec, Canada H3C 3J7 (M.T., J.M.) and Hôpital Louis-H. Lafontaine, Centre de Recherche, Montréal, Québec, Canada H1N 3M5 (J.M.)

Received September 7, 1989; Accepted January 9, 1990

SUMMARY

The binding characteristics of selective and nonselective opioids have been studied in whole guinea pig spinal cord, using a computer fitting method to analyze the data obtained from saturation and competition studies. The delineation of specific binding sites labeled by the μ-selective opioid [^{3}H]D-Ala^2,MePhe^4,Gly-ol^5-enkephalin (K_d = 2.58 nM, R = 4.52 pmol/g of tissue) and by the δ-selective opioid [^{3}H]D-Pen^2, D-Pen^5-enkephalin (K_d = 2.02 nM, R = 1.47 pmol/g of tissue) suggests the presence of μ and δ-receptors in the spinal cord tissue. The presence of κ receptors was probed by the κ-selective opioid [^{3}H]U69593 (K_d = 3.31 nM, R = 2.00 pmol/g of tissue). The pharmacological characterization of the sites labeled by [^{3}H] U69593 confirms the assumption that this ligand discriminates κ receptors in guinea pig spinal cord. The benzomorphan [^{3}H] ethylketazocine labels a population of receptors with one homogeneous affinity state (K_d = 0.65 nM, R = 7.39 pmol/g of tissue). The total binding capacity of this ligand was not different from the sum of the binding capacities of μ-, δ-, and κ-selective ligands. Under μ- and δ-suppressed conditions, [^{3}H]ethylketazocine still binds to receptors with one homogeneous affinity state (K_d = 0.45 nM, R = 1.69 pmol/g of tissue). Competition studies performed against the binding of [^{3}H]ethylketazocine under these experimental conditions reveal that the pharmacological profile of the radiolabeled receptors is similar to the profile of the κ receptors labeled with [^{3}H]U69593. Saturation studies using the nonselective opioid [^{3}H]bremazocine demonstrate that this ligand binds to spinal cord membranes with heterogeneous affinities (K_{d_1} = 0.28 nM, R_1 = 7.91 pmol/g of tissue; K_{d_2} = 3.24 nM, R_2 = 11.2 pmol/g of tissue). The total binding capacity obtained with [^{3}H]bremazocine (R_{total} = 19.1 pmol/g of tissue) was different from either the sum of the binding capacities of μ-, δ-, and κ-selective ligands or the binding capacity of [^{3}H]ethylketazocine obtained under unsuppressed conditions. These results suggest that [^{3}H]bremazocine labels additional opioid sites, namely the κ_2 receptors, in contrast to κ_1 sites labeled with [^{3}H] U69593. In experimental conditions where the binding of [^{3}H] bremazocine at μ, δ, and κ_1 receptors was quenched by selective blockers, [^{3}H]bremazocine recognizes the κ_2 receptors with one homogeneous affinity state (K_d = 3.45 nM, R = 8.23 pmol/g of tissue). However, competition studies suggest that some opioids bind to these κ_2 receptors with heterogeneous affinity states (high and low affinity states), whereas others bind with one apparently homogeneous affinity state. Thus, computer analyses have helped to establish the multiplicity of κ receptors in the guinea pig spinal cord. Results obtained in the presence of guanyl-5′-yl imidodiphosphate further lead to the conclusion that the interaction of opioids at the κ_2 receptors is more complex then was originally described. Finally, the relative proportions of the opioid receptors in whole spinal cord are as follows: 28% μ, 9% δ, 12% κ_1, and 51% κ_2 (κ_{2_H} plus κ_{2_L}).

The spinal cord is believed to be an important site of action for endogenous and synthetic opioids to produce analgesia and also to cause effects on motor, cardiovascular, gastrointestinal, and bladder function (for review, see Ref. 1). Immunohistochemical and autoradiographic studies have shown the presence of opioid peptides located mainly in the superficial laminae of the dorsal horn of the spinal cord (2–5). Moreover, other studies have demonstrated the presence of opioid binding sites in the spinal cord (6–8). Despite these lines of evidence, the exact mechanisms by which opioids interact with the spinal cord are not yet explained. This might be due, in part, to the uncertainty that remains about the nature of the opioid binding sites located on this tissue.

This work was supported by a C.A.F.I.R. grant from the Univérsité de Montréal. J.M. is a Chercheur-Boursier and M.T. is a recipient of a studentship of the Fonds de la Recherche en Santé du Québec.

ABBREVIATIONS: DADL, D-Ala^2,D-Leu^5-enkephalin; DAGO, D-Ala^2,MePhe^4,Gly-ol^5-enkephalin; DPDP, D-Pen^2,D-Pen^5-enkephalin, N-BNI, nor-binaltorphimine; U50488, (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; U69593, [(5α, 7α, 8β)-(−)-*N*-methyl-*N*-7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide; PD117302, (*trans*)-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[*b*] thiophene-4-acetamide; SKF10047, α-5,9-dimethyl-2-allyl-2′-hydroxy-6,7-benzomorphan; ICI197067, (2*S*)-*N*-[2-(*N*-methyl-3,4-dichlorophenylacetamido)-3-methylbutyl]pyrrolidine; BREM, (−)-bremazocine; EKC, (−)-ethylketazocine; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; G protein, GTP-binding protein.

Binding studies in mammalian brain have led to the characterization of the opioid receptors, which have been proposed to exist as three main types, termed μ, δ, and κ (9). However, several studies have suggested that other types of receptors could be included among this complex receptor family (10, 11). The concept of the multiplicity of opioid receptors has been enlarged because of numerous experimental data suggesting the existence of subtypes of opioid receptors. Indeed, it has been proposed that μ receptors might exist as two independent forms, named μ_1 and μ_2 (12). Using alkylating agents, Rothman *et al.* (13) have suggested that δ receptors were divided into δ type I and δ type II in rat brain. Finally, it has been proposed that κ receptors might be found as two separate entities, called κ_1 and κ_2 receptors (14–17). Castanas *et al.* (18) have even postulated the existence of κ_1-, κ_2-, and κ_3-opioid receptors in the adrenal medulla.

Studies performed in the rodent and human spinal cord have supported the concept of multiplicity of opioid receptors in this tissue (8, 19–28). Other studies done in the lumbosacral region of the spinal cord, however, have led to conflicting reports. On the one hand, it has been proposed that the rodent lumbosacral cord does not contain either μ or δ receptors (14, 29). This then led Attali *et al.* (14) to introduce the concept of heterogeneous κ receptors in the spinal cord, based essentially on the respective sensitivity of the two κ receptors to the mixed μ/δ-selective ligand DADL; thus, κ_1 receptors were defined as DADL insensitive, whereas κ_2 receptors were DADL sensitive. The same group of investigators have even proposed a specific interaction of μ- and δ-selective ligands with the κ_2 receptors (30). On the other hand, this heterogeneity of κ receptors based on μ- or δ-selective ligand sensitivity was questioned by studies that have demonstrated the presence of μ and δ binding sites in the spinal cord (25, 31). Moreover, it has been shown that κ_2 receptors in guinea pig brain were insensitive to μ- or δ-selective ligands (17). However, additional evidence obtained using radiolabeled benzomorphans in rodent spinal cord, in which the binding at the μ and δ sites was suppressed, has led to a renewed suggestion for the multiplicity of κ receptors (25, 32). Despite this evidence, no studies have really succeeded in obtaining a pharmacological characterization to demonstrate the existence of separate κ_1 and κ_2 receptors in the spinal cord, even when a computer-fitting model technique was used (25).

In the present paper, we describe, using selective and nonselective opioid ligands, the pharmacological characterization of the κ_1- and κ_2-opioid receptors in whole guinea pig spinal cord with the assistance of computer modeling methods. The results show 1) that μ, δ, κ_1, and κ_2 receptors are present in the spinal cord; 2) that κ_1 receptors are few in number, as compared with the κ_2 receptors; 3) that κ_2 receptors might exist under two affinity states or forms; and 4) that computer-fitting techniques can be successfully applied to ligand binding data in order to demonstrate the multiplicity of opioids receptors in the spinal cord.

Materials and Methods

Drugs and Peptides

BREM hydrochloride was provided by Dr. D. Roëmer (Sandoz, Basel, Switzerland). EKC methanesulfonate and (−)-cyclazocine were generously donated by Dr. W. Michne (Sterling-Winthrop, Rensselear, NY). U50488 was provided by Dr. P. F. von Voigtlander (The UpJohn Company, Kalamazoo, MI). U69593 hydrochloride was purchased from Amersham. (+)- and (−)-SKF10047 hydrochloride were donated by Dr. R. Quirion (Douglas Hospital, Verdun, Québec). ICI197067 hydrochloride was from Cambridge Research Biochemicals (UK). N-BNI hydrochloride was obtained from Research Biochemical Industries (St. Louis, MO). PD117302 hydrochloride was kindly supplied by Dr. J. C. Hunter (Parke-Davis Research Unit, UK). Diprenorphine hydrochloride was from Reckitt and Colman. Naloxone hydrochloride was purchased from Sigma (St. Louis, MO). Morphine sulfate was obtained from BDH Pharmaceuticals. Unlabeled DADL, DAGO, and DPDP were from IAF Biochem (Laval, Québec).

Radiolabeled Ligands

[^{3}H]DAGO, (30 Ci/mmol), [^{3}H]DPDP, (54 Ci/mmol), [^{3}H]BREM, (17.3–21.3 Ci/mmol), and [^{3}H]EKC (28.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]U69593 (50–68.3 Ci/mmol) was obtained from Amersham.

Guinea Pig Spinal Cord Membrane Preparation

The whole spinal cord was rapidly isolated from male Hartley guinea pigs (350 to 500 g) and crude membranes were prepared as previously described (33). Briefly, the spinal cord was cut into pieces and homogenized with a Polytron (speed 4; 30 to 40 sec) in cold buffer A (50 mM Tris·HCl, pH 7.4 at 4°). The homogenate was then centrifuged at 49,000 × *g* for 10 min. At the end of the centrifugation, the supernatants were discarded and the pellets were resuspended in cold buffer A and centrifuged again under the same conditions. The final pellets were resuspended in cold buffer B (50 mM Tris·HCl, pH 7.4 at 25°) at a final concentration of 4–8 mg of original wet weight/ml.

[^{3}H]Opioid Binding Assays

The binding assays were carried out with 1.9 ml of membrane preparations incubated at 25° for 40 min, in a final volume of 2 ml. At the end of the incubation period, bound and free radioactivity were separated by vacuum filtration of the membrane preparations on glass fiber filters (GF/B; Whatman) that were presoaked in 0.3% polyethylenimine for 3 hr. The filters were then rinsed with 3 volumes of ice-cold buffer A, dried overnight, immersed in 2 ml of scintillation fluid (Optiscint Hi-Safe; Pharmacia), and counted at 50% efficiency in a Rackbeta counter (LKB).

Saturation experiments with the various tritiated opioid ligands were done by increasing the concentrations of the radiolabeled drug under study from 0 to 20 nM (10 to 17 points). Conversely, competition experiments were conducted using a constant concentration of the labeled opioid against various concentrations of the unlabeled drug under study (8 to 21 points). The level of nonspecific binding in each type of experiment was delineated by an addition of excess unlabeled diprenorphine (2.5 μM) or levallorphan (5 μM).

Data Analysis

All experimental data obtained from saturation and competition curves were analyzed by a weighted nonlinear least squares curve-fitting method that uses the Marquardt-Levenberg modification of the Gauss-Newton method (34). The curve-fitting programs used in the present study, called the EBDA/LIGAND programs, have been written and/or adapted for utilization on microcomputers. These programs have been previously described (34, 35). In order to overcome the unequal precision obtained from the measure of bound radioligand (Y), the deviations of the experimental points from their predicted values (i.e., residuals) were weighted according to the reciprocal of their expected variance (Var), based on the general weighting model:

$$\text{Weight} = 1/(\text{Var } Y)^2$$

The raw data are initially processed by the EBDA program, in order to give first estimates of the binding parameters and also to create data files that are used by the LIGAND program for the final evaluation of the exact mathematical "n by m" model developed by Feldman (36). This model is based on any number of ligands (n) interacting with any number of independent classes of binding sites or receptors (m) according to the law of mass action. The testing of the statistical differ-

ence between the weighted residual variance obtained with different $n \cdot m$ models (one-site, two-site, etc. . . .) is done by comparing those residuals according to the "extra sum of squares" principle, as explained by Munson and Rodbard (34) and also named the partial F test:

$$F = [(SS_1 - SS_2)/(df_1 - df_2)]/(SS_2/df_2)$$

The terms SS_1 and SS_2 are, respectively, the sums of squares of residuals for the fit with the simpler $n \cdot m$ model (fewer parameter estimates) and the more complex $n \cdot m$ model (more parameter estimates), and df_1 and df_2 correspond to the number of degrees of freedom assigned for the different fits. The calculated F ratio is then compared with a tabulated F statistic value with $(df_1 - df_2)$ degrees of freedom for the numerator and df_2 degrees of freedom for the denominator.

Saturation curves. The specific binding was obtained by a subtraction of the nonspecific binding from the total binding. The EBDA program processes the specific binding curve to give a Scatchard transformation and true Hill coefficient. Those transformations help us to seek a possible heterogeneity of the receptor sites probed by the radioligand under study. The LIGAND program is then used to obtain the best fitting model using the specific binding curve. To do so, the parameter N (coefficient of nonspecific binding) is held constant at zero, whereas the parameters K (affinity constant) and R (binding capacity) are allowed to float. When the two-site model was the best fit obtained, we performed an additional fit in which the N parameter was allowed to float with the parameters K and R. This was done to ensure that the two-site model was not likely due to another form of nonspecific binding displaceable by diprenorphine or levallorphan. The residual variance of the sum of squares of this fit was compared with the two-site fit using the partial F test. The reciprocal of the affinity constant of the labeled drug was calculated and was termed the equilibrium dissociation constant value (K_d).

Competition curves. The displacement curves were first analyzed by EBDA, using a sigmoidal curve-fitting program. The equation used to fit the displacement curve is a four-parameter logistic function:

$$Y = [(B_{max} - NS)/(1 + ((X/IC_{50})^p)] + NS$$

where X and Y are the concentration of competitor and bound radioligand, respectively, IC_{50} is the concentration of competitor inhibiting 50% of specific binding, B_{max} (upper limit of the curve) is the total amount of radioligand bound in the absence of any competitor, NS (lower limit of the curve) is the total amount of nonspecific binding, and p is the slope factor ("pseudo Hill coefficient"). The curves are then analyzed according to the LIGAND program, using data with the nonspecific binding not subtracted. Computer modeling of each displacement curve was done by setting the affinity constant of the radioligand (K_{11} and K_{12}) at the predetermined values measured with saturation studies. The coefficient for nonspecific binding for each competitor (N_2) was constrained to a value of zero, whereas N_1 (labeled drug) was allowed to float, as was the affinity constant of the competitor (K_{21}, K_{22}) and the concentration of receptor subtypes (R_1, R_2). The K_d value of the unlabeled drug was calculated by taking the reciprocal of the affinity constant.

Computations. For the purpose of this study, arithmetic means are reported for the receptor concentration and for the percentages calculated, whereas geometric means are calculated for values of the dissociation constant. The calculations of the geometric means and the standard error of the geometric mean were performed as previously described (37). It has been reported that affinity constants were log-normally distributed (37).

Statistical treatment. Before performing the statistical tests, we have assessed the homogeneity of the variances using both the Hartley and Bartlett tests. When two groups of data were compared, we have used the t test for unpaired values. In the case of multiple groups, *a posteriori* pair-wise comparisons were done, using Scheffé's analysis of variance. However, when variances were heteroscedastic, multiple pair-wise comparisons of means were done, according to the Games and Howell method. Those statistical tests, as well as correlation analysis, were described previously (38, 39). The level of significance was established at 5%.

Results

Saturation Studies

Binding characteristics of μ-, δ-, and κ-selective opioid ligands. The specific binding of [^{3}H][DAGO (μ-selective), [^{3}H]DPDP (δ-selective), and [^{3}H][U69593 (κ-selective), measured by saturation studies, were each best fitted to a one-site model, i.e., they display one homogeneous state of affinity. Fig. 1 shows a representation of the fitted specific binding of those selective opioids, using the Scatchard plot transformations. In crude membranes derived from guinea pig spinal cord, [^{3}H][DAGO has a K_d value of 2.58 ± 0.23 nM at the μ receptors, with a total binding capacity of 4.52 ± 0.52 pmol/g of wet tissue (four experiments). [^{3}H]DPDP has a K_d value of 2.02 ± 0.41 nM at the δ-receptors, with a binding capacity of 1.47 ± 0.27 pmol/g of wet tissue (three experiments). The κ-selective ligand [^{3}H]U69593 has a K_d value of 3.31 ± 0.20 nM and discriminates a total receptor population of 2.00 ± 0.17 pmol/g of wet tissue (four experiments). The sum of the binding capacities obtained with the different selective opioids yielded an R_{total} value of 7.99 pmol/g of tissue.

Binding characteristics of [^{3}H]EKC. The computerized fitting of the specific binding of [^{3}H] EKC in crude membranes of the spinal cord is described best by a one-site model (Fig. 2) with a K_d value of 0.65 ± 0.02 nM and a total receptor concentration of 7.39 ± 0.50 pmol/g of wet tissue (three experiments). Using a t test, we have been able to establish that this binding capacity value is not statistically different from the sum of the individual binding capacities measured with the selective ligands (R_{total} = 7.99). Saturation experiments were also performed under experimental conditions in which the μ receptors

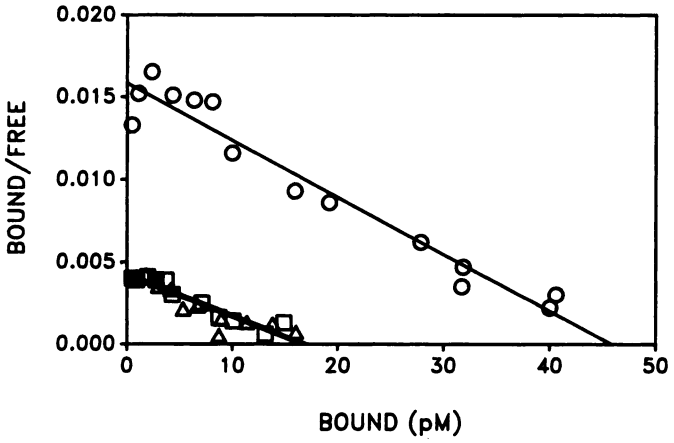

Fig. 1. Scatchard transformations of computerized fitting of the specific binding data obtained from saturation studies of [^{3}H]DAGO, [^{3}H]DPDP, and [^{3}H]U69593 in whole spinal cord. Guinea pig spinal cord membranes were incubated with increasing concentrations of the μ-selective opioid [^{3}H]DAGO (○), the δ-selective opioid [^{3}H]DPDP (□), or the κ-selective opioid [^{3}H]U69593 (△). Saturation curves were performed with 12 to 15 concentrations (0–20 nM) of the radioligand under study. The experimental data obtained for each of these representative examples are shown by *symbols*. Each point represents the mean of triplicate determinations (precision, >95%). The specific binding ranged approximately from 70 to 35% for [^{3}H]DAGO, from 50 to 15% for [^{3}H]DPDP, and from 50 to 20% for [^{3}H]U69593. *Lines*, computer-fitted lines of the observed data according to a one-site model. The binding parameters estimated by computer modeling are given in the text.

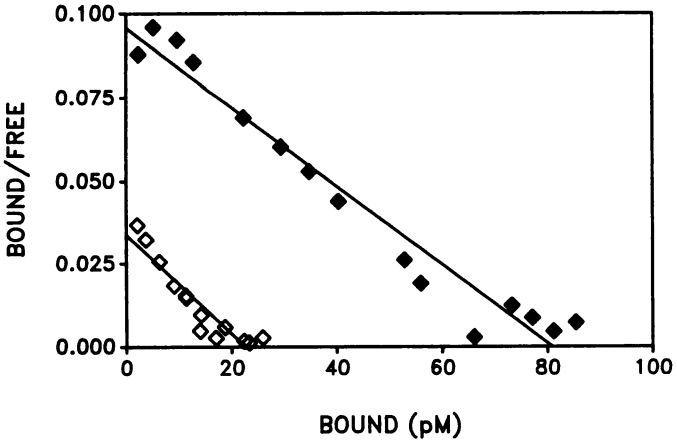

Fig. 2. Scatchard transformations of computerized fitting of the specific binding data from saturation studies of [^{3}H]EKC in whole spinal cord. Guinea pig spinal cord membranes were incubated with increasing concentrations of the nonselective benzomorphan [^{3}H]EKC in the absence (♦) or presence (◇) of 100 nM unlabeled DAGO and 100 nM unlabeled DADL, to prevent the binding of the radioligand at the μ and δ sites, respectively. Saturation curves were performed with 15 concentrations (0–20 nM) of [^{3}H]EKC. The experimental data obtained for each of these representative examples are shown by *symbols*. Each point represents the mean of triplicate determinations (precision, >95%). The specific binding ranged approximately from 85 to 35% under unsuppressed conditions and from 75 to 20% under μ- and δ-suppressed conditions. *Lines*, computer-fitted lines of the observed data according to a one-site model. The binding parameters estimated by computer modeling are given in the text.

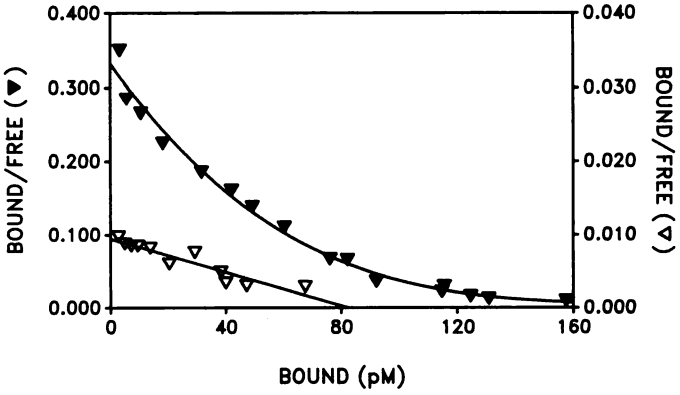

Fig. 3. Scatchard transformations of computerized fitting of the specific binding data obtained from saturation studies of [^{3}H]BREM in whole spinal cord. Guinea pig spinal cord membranes were incubated with increasing concentrations of the nonselective benzomorphan [^{3}H]BREM in the absence (▼) or presence (▽) of 100 nM unlabeled DAGO, 100 nM unlabeled DADL, and 120 unlabeled U69593, to prevent the binding of the radioligand to the μ, δ, and κ sites, respectively. Saturation curves were performed with 17 concentrations (0–20 nM) of [^{3}H]BREM. The experimental data obtained for each of these representative examples are shown by *symbols*. Each point represents the mean of triplicate determinations (precision, >95%). The specific binding ranged approximately from 90 to 35% under unsuppressed conditions and from 45 to 20% under μ-, δ-, and κ-suppressed conditions. *Lines*, computer-fitted lines of the observed data according to a two-site model (unsuppressed conditions) or to a one-site model (μ-, δ-, and κ-suppressed conditions). The binding parameters estimated by computer modeling are given in the text.

and δ receptors were selectively blocked using 100 nM unlabeled DAGO (μ selective) and 100 nM unlabeled DADL (mixed μ and δ selective). Under these blocking conditions, the specific binding of [^{3}H]EKC was still best fitted to a one-site model (as represented by a linear Scatchard plot in Fig. 2), with a K_d value of 0.45 ± 0.03 nM and an R value of 1.69 ± 0.10 pmol/g of tissue (four experiments). Experimental conditions used to block μ and δ receptors did not alter statistically the K_d value of [^{3}H]EKC, as compared with that obtained under unsuppressed conditions, but they did reduce significantly the binding capacity of [^{3}H]EKC. Interestingly, the R value obtained for [^{3}H]EKC under μ- and δ-suppressed conditions is not statistically different from the R value observed using the κ-selective [^{3}H]U69593. We performed an additional set of saturation experiments (three experiments) in which the binding of [^{3}H]EKC at the μ, δ, and κ binding sites was suppressed, respectively, by 100 nM unlabeled DAGO, 100 nM unlabeled DADL, and 120 nM unlabeled U69593. Simultaneous analysis of the three specific binding curves by LIGAND revealed that the binding capacity of [^{3}H]EKC in whole spinal cord was totally abolished (>90% inhibition; data not shown). This suggests that, in our binding assay conditions, [^{3}H]EKC labels only the μ, δ, and κ binding sites with high affinity in whole spinal cord membranes.

Binding characteristics of [^{3}H]BREM. Saturation curves of [^{3}H]BREM performed under unsuppressed binding conditions are represented best by curvilinear Scatchard plots (Fig. 3). The specific binding of [^{3}H]BREM under unsuppressed conditions was modeled best to a two-site fit. Computer analysis has resolved the binding of [^{3}H]BREM into two components, a high affinity form (K_{d_1}, R_1) with a K_{d_1} value of 0.28 ± 0.02 nM and an R_1 value of 7.91 ± 0.76 pmol/g of wet tissue and a low affinity form (K_{d_2}, R_2) with a K_{d_2} value of 3.24 ± 0.35 nM and an R_2 value of 11.2 ± 1.88 pmol/g of wet tissue (four experiments). The total binding capacity (R_1 plus R_2) of [^{3}H]BREM is 19.1 ± 1.93 pmol/g of wet tissue, a value that is significantly different from both the total binding capacity of [^{3}H]EKC (under unsuppressed conditions) and the R_{total} value established with the selective opioids. This might indicate that [^{3}H]BREM delineates, in whole spinal cord, other opioid sites in addition to the well known μ, δ, and κ receptors. To verify this assumption, we have done a series of saturation experiments in which 100 nM unlabeled DAGO, 100 nM unlabeled DADL, and 120 nM unlabeled U69593 were added to the membrane mixture to suppress the binding of [^{3}H]BREM at the μ, δ, and κ receptor sites. Under these conditions, the specific binding of [^{3}H]BREM was modeled best to a one-site model (Fig. 3). The high affinity binding of [^{3}H]BREM was totally eliminated, whereas the low affinity binding form remained, with a K_d value of 3.45 ± 0.22 nM and a binding capacity of 8.23 ± 0.88 pmol/g of wet tissue (four experiments). The K_d and R values observed under these suppressed conditions were not statistically different from the respective values of the low affinity binding form of [^{3}H]BREM seen under unsuppressed conditions. Moreover, the binding capacity of [^{3}H]BREM under suppressed conditions is statistically different from the total binding of [^{3}H]BREM (under unsuppressed conditions).

Competition Studies

Comparison of the pharmacological profile of the binding of [^{3}H]U69593 and [^{3}H]EKC at κ receptors in guinea pig spinal cord. A series of competition studies using 16 compounds have been done in order to fully ascertain the κ-opioid nature of the sites labeled by [^{3}H]U69593 or by [^{3}H]EKC (under μ- plus δ-suppressed conditions). The binding parameters derived from computer analyses of the displacement

curves obtained against the two radioligands are summarized in Tables 1 and 2. All opioids tested against both of these radiotracers at the κ receptors display slope factors that are not statistically different from unity. As expected, they are best fitted to one homogeneous affinity state. It is quite clear that the κ sites labeled by [^{3}H]U69593 or [^{3}H]EKC are stereoselective (Tables 1 and 2). Indeed, using the benzomorphans (−)-SKF10047 and (+)-SKF10047, we have demonstrated that the (−)-isomer is over 600-fold more potent than the (+)-isomer when the sites are labeled by [^{3}H]U69593. Similarly (−)-SKF10047 is over 1500-fold more potent than its (+)-congener when κ binding sites are labeled by [^{3}H]EKC.

With both radiolabeling methods, μ-selective substances (such as DAGO or morphine) and δ-selective substances (such as DPDP or DADL) were all weak inhibitors of the radioactivity bound at the κ receptors. The K_d values of DAGO and morphine were about 1000 to 2000 nM and 300 to 400 nM, respectively, against [^{3}H]U69593 and [^{3}H]EKC at the κ receptors (Tables 1 and 2). The δ-selective drugs DADL and DPDP display K_d values that were greater than 10,000 nM. On the other hand, all benzomorphans [except (+)-SKF10047] assayed were good inhibitors of the radioactivity bound at the κ receptors. However, against the binding of [^{3}H]U69593, bremazocine was the most potent benzomorphan tested, with a K_d of 0.17 nM being 2 times more potent than cyclazocine and 10 times more potent than EKC. Conversely, against the binding of [^{3}H]EKC (under μ- and δ-suppressed conditions), cyclazocine was the most potent benzomorphan tested, with a K_d of 0.09 nM. In these experimental conditions, cyclazocine was 8 times more potent than bremazocine and 35 times more potent than EKC. The κ-selective opioids were all potent displacers of κ binding. The benzeneacetamide drugs (U69593, U50488, PD117302) were all equipotent at the κ receptors when those sites were labeled by either [^{3}H]U69593 or [^{3}H]EKC, whereas the pyrrolidine ICI197067 was the most potent κ-opioid tested. At the [^{3}H]U69593-labeled κ sites ICI197067 displayed a K_d value of 0.48 nM (Table 1), whereas at the [^{3}H]EKC-labeled κ sites it has an affinity of 4.73 nM (Table 2). Overall, it has a potency that is 3 to 8 times higher than the potency of the benzeneacetamide compounds. Among the nonselective antagonists tested, diprenorphine was the most potent, with a K_d value of 0.47 nM (at the [^{3}H]U69593-labeled sites) or 3.13 nM (at the [^{3}H]EKC-labeled sites), whereas naloxone has K_d values of 8.94 and 30.4 nM, respectively. The putative κ-selective antagonist N-BNI was also tested, and the results show that it is a strong inhibitor of binding at the κ receptors, with a K_d value of about 1 to 2 nM (Tables 1 and 2). The binding capacity values obtained from computer analyses of displacement curves were compared pair-wise with the ones measured by saturation studies, using the Scheffé test. Neither the R values derived from displacement curves against [^{3}H]U69593 (Table 1) nor those derived from displacement curves against [^{3}H]EKC were statistically different from the respective R values established by saturation studies (see Tables 1 and 2).

Altogether, these data suggest that κ receptors can be labeled using either the nonselective opioid [^{3}H]EKC (under μ- plus δ-suppressed conditions) or the selective κ agonist [^{3}H]U69593. To further assess the similarity between these two pharmacological profiles, we performed a correlation analysis of the K_d values obtained with the different κ-labeling methods in the

TABLE 1

Computer-derived binding parameters from competition experiments of various opioids against the binding of [^{3}H]U69593 in whole guinea pig spinal cord membranes

For each competition curve against [^{3}H]U69593, the computer programs provide estimates of the slope factor (pseudo-Hill coefficients), the binding capacity (R), and the affinity of the unlabeled ligand, expressed in the present report as the equilibrium dissociation constant (K_d). The displacement curves were performed with 10 to 12 concentrations in duplicate (precision, >95%). Curves were done against a constant concentration of [^{3}H]U69593 (≈1.1 nM). Under these conditions, the specific binding was between 40 and 45%. All binding parameters obtained with the opioids were fitted best to a one-site model. Results are expressed as the mean ± standard error of the number of independent experiments indicated by n. For K_d values, the data were transformed to logarithmic form and geometric means were calculated.

Compound	n	K_d	R	Slope factor
		nM	pmol/g of tissue	
DPDP	3	>10,000[a]	ND[b]	ND
DADL	3	>10,000[c]	ND	ND
DAGO	3	1647 ± 150	2.20 ± 0.08	1.21 ± 0.17
Morphine	3	321 ± 15.8	1.78 ± 0.16	0.87 ± 0.12
Bremazocine	3	0.17 ± 0.01	2.37 ± 0.09	0.92 ± 0.18
Cyclazocine	3	0.35 ± 0.06	2.47 ± 0.17	1.09 ± 0.14
EKC	3	1.71 ± 0.19	2.50 ± 0.16	1.07 ± 0.15
(−)-SKF10047	3	2.33 ± 0.30	2.17 ± 0.16	0.95 ± 0.13
(+)-SKF10047	3	1534 ± 263	2.36 ± 0.19	1.02 ± 0.16
Diprenorphine	3	0.47 ± 0.02	2.23 ± 0.18	1.31 ± 0.24
Naloxone	3	8.94 ± 1.29	2.02 ± 0.03	0.86 ± 0.08
N-BNI	3	1.30 ± 0.12	1.80 ± 0.21	0.85 ± 0.13
PD117302	3	6.03 ± 0.69	2.52 ± 0.25	1.02 ± 0.15
U50488	3	2.34 ± 0.26	1.94 ± 0.06	1.06 ± 0.09
U69593	6	3.88 ± 0.54	2.09 ± 0.18	0.92 ± 0.05
ICI197067	3	0.48 ± 0.04	1.91 ± 0.20	1.10 ± 0.12

[a] At a concentration of 13,000 nM, the inhibition was 18 ± 5% (n = 3).
[b] ND, not determined.
[c] At a concentration of 18,000 nM, the inhibition was 51 ± 18% (n = 3).

TABLE 2

Computer-derived binding parameters from competition experiments of various opioids against the binding of [^{3}H]EKC (under μ- and δ-suppressed conditions) in whole guinea pig spinal cord membranes

For each competition curve against [^{3}H]EKC under suppressed conditions, the computer programs provide estimates of the slope factor (pseudo-Hill coefficients), the binding capacity (R), and the affinity of the unlabeled ligand, expressed in the present report as the equilibrium dissociation constant (K_d). The displacement curves were performed with 10 to 12 concentrations in duplicate (precision, >95%). Curves were obtained against a constant concentration of [^{3}H]EKC (≈0.6 nM) in the presence of 100 nM DAGO and 100 nM DADL to suppress μ and δ sites. Under these conditions, the specific binding was between 60 and 70%. All binding parameters obtained with the opioids were fitted best to a one-site model. Results are expressed as the mean ± standard error of the number of independent experiments indicated by n. For K_d values, the data were transformed to logarithmic form and geometric means were calculated.

Compound	n	K_d	R	Slope factor
		nM	pmol/g of tissue	
DPDP	3	>10,000[a]	ND[b]	ND
DADL	3	>10,000[c]	ND	ND
DAGO	3	2350 ± 94.1	2.05 ± 0.19	0.87 ± 0.13
Morphine	3	389 ± 11.2	2.70 ± 0.03	0.86 ± 0.08
Bremazocine	3	0.70 ± 0.01	2.89 ± 0.07	1.18 ± 0.07
Cyclazocine	3	0.09 ± 0.004	2.65 ± 0.05	0.93 ± 0.06
EKC	3	3.14 ± 0.25	2.48 ± 0.23	0.92 ± 0.08
(−)-SKF10047	3	2.40 ± 0.21	2.44 ± 0.08	0.91 ± 0.06
(+)-SKF10047	3	3742 ± 519	3.37 ± 0.24	1.29 ± 0.12
Diprenorphine	3	3.13 ± 0.03	3.03 ± 0.14	1.19 ± 0.10
Naloxone	3	30.4 ± 0.01	2.81 ± 0.25	0.98 ± 0.09
N-BNI	3	2.22 ± 0.10	2.55 ± 0.20	0.87 ± 0.05
PD117302	3	12.6 ± 0.67	2.90 ± 0.10	0.89 ± 0.12
U50488	3	17.7 ± 0.14	2.75 ± 0.07	0.92 ± 0.04
U69593	3	18.5 ± 1.62	2.59 ± 0.02	0.86 ± 0.05
ICI197067	3	4.73 ± 0.68	2.79 ± 0.10	0.90 ± 0.04

[a] At a concentration of 13,000 nM the inhibition was 2 ± 1% (n = 3).
[b] ND, not determined.
[c] At a concentration of 18,000 nM, the inhibition was 21 ± 6% (n = 3).

guinea pig spinal cord. The K_d values derived from [^{3}H]U69593 and [^{3}H]EKC displacement curves were found to correlate strongly ($r = 0.96$, $p < 0.05$, 14 experiments; data not shown). However, some discrepancies can be noted in the absolute affinity values of the unlabeled opioids measured under these two labeling conditions. The presence of relatively large concentrations of unlabeled opioids in the incubation medium (100 nM DAGO and 100 nM DADL) during the labeling of κ sites with [^{3}H]EKC (but not in the case of [^{3}H]U69593) might possibly explain these. On the one hand, the blockers might compete, to a certain extent, with the κ sites and, thus, cause an increase in the absolute quantity needed for the opioid under study to displace [^{3}H]EKC bound at these sites. On the other hand, there is a possibility that the opioid under study might be quenched by the presence of an excess concentration of unlabeled blocker, and this quenching might vary with the different compounds that have been tested, leading to the observed differences in the variations (2- to 10-fold) in absolute K_d values.

Pharmacological profile of the binding of [^{3}H]BREM under μ-, δ-, and κ-suppressed conditions. The same 16 compounds used for the pharmacological characterization of the κ receptors were tested against the binding of [^{3}H]BREM (under μ-, δ-, and κ-suppressed conditions). Fig. 4 shows that some opioids interact at these sites with one homogeneous state of affinity, whereas some others are represented best by a two-site model. Table 3 summarizes the binding parameters obtained from individual analysis of displacement curves against the binding of [^{3}H]BREM (under suppressed conditions) that are best fitted to the one-site model. It is important to note that unlabeled EKC displays a K_d of about 45 nM at those sites. This might explain why we have not been able to label these sites with [^{3}H]EKC. Indeed, at these high concentrations of radioactivity, the level of specific binding is very low (<10%), which makes it difficult, if not impossible, to delineate those additional sites with [^{3}H]EKC.

Furthermore, the binding of [^{3}H]BREM at the additional sites was stereoselective, inasmuch as (−)-SKF10047 was 150-fold more potent than (+)-SKF10047. The nonselective antagonists naloxone and diprenorphine were also best fitted to a one-site model, with respective K_d values of 280 and 56.6 nM. This would tend to establish the opioid character of these additional sites. Under our experimental conditions, μ and δ peptides were inactive, which would indicate the non-μ and non-δ nature of these sites (Table 3). To further assess the identity of these sites, we performed a correlation analysis between the affinities of the opioids tested at the κ sites labeled with [^{3}H]U69593 (Table 1) and the ones established at the [^{3}H]BREM sites (Table 3). This yields a r value of 0.99 ($p < 0.05$, six experiments), showing a high degree of association between the two profiles.

On the other hand, the competition curves of other opioids against the binding of [^{3}H]BREM (under suppressed conditions) were best fitted to a two-site model (Table 4). For instance, N-BNI discriminated 59% of the total receptor population with high affinity (R_H) with a dissociation constant (K_{d_H}) of 6.22 nM, whereas 41% of the receptors were of low affinity (R_L) with a dissociation constant of (K_{d_L}) 923 nM. As for the benzeneacetamides, PD117302 delineated 44% R_H (K_{d_H} = 40.1 nM) and 56% R_L (K_{d_L} = 3744 nM), U50488 delineated 45% R_H (K_{d_H} = 96.6 nM) and 55% R_L (K_{d_L} = 3776 nM), and

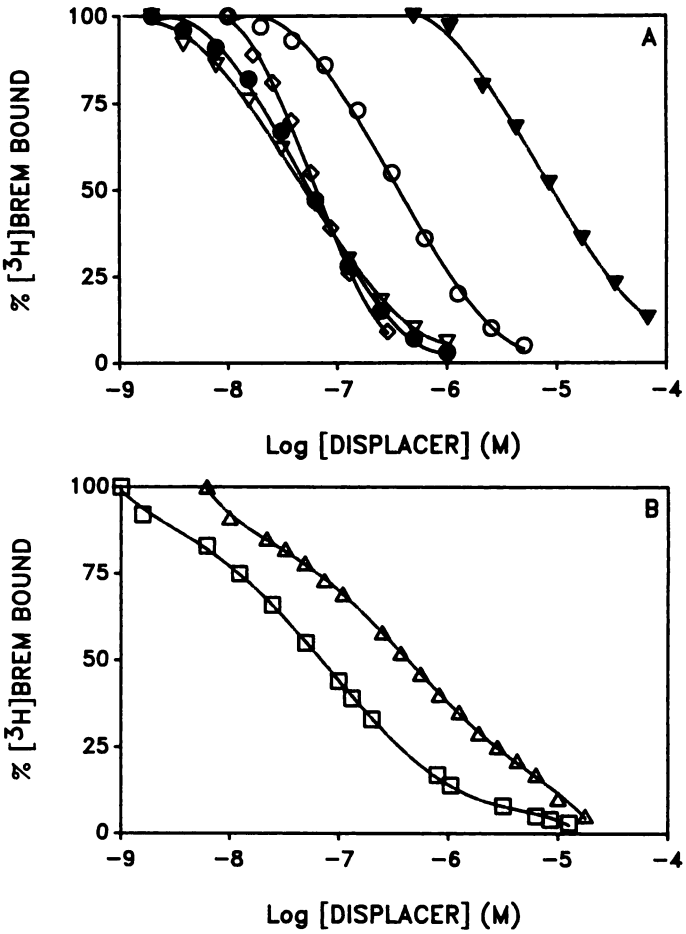

Fig. 4. Computerized curve-fitting of binding data from competition curves against [^{3}H]BREM under μ-, δ-, and κ-suppressed binding conditions. Guinea pig spinal cord membranes were incubated with about 1.7 nM [^{3}H]BREM in the presence of 100 nM unlabeled DAGO, 100 nM unlabeled DADL, and 120 nM unlabeled U69593. The experimental data obtained for each of these representative examples are shown by *symbols*. Each point represents the mean of duplicate determinations (precision, >95%). Displacement curves were performed with 10 to 21 concentrations. For details, see legends to Tables 3 and 4. A, These are representative displacement curves done with increasing concentrations of EKC (●), (−)-SKF10047 (▽), (+)-SKF10047 (▼), naloxone (○), or diprenorphine (◇). *Lines*, computer-fitted lines of the observed data to a one-site model. The binding parameters estimated by computer modeling are given in Table 3. B, These are representative displacement curves done with increasing concentrations of ICI97067 (□) or PD117302 (△). *Lines*, computer-fitted lines of the observed data to a two-site model. The binding parameters estimated by computer modeling are given in Table 4.

U69593 delineated 52% R_H (K_{d_H} = 346 nM) and 48% R_L (K_{d_L} = 51,644 nM). Similar results were obtained with the pyrrolidine ICI197067, which discriminated 49% R_H (K_{d_H} = 12.3 nM) and 51% R_L (K_{d_L} = 2667 nM). Another striking feature was that the interaction of cyclazocine (benzomorphan) and morphine were also best fitted to a two-site binding model. This apparent heterogeneity was supported by slope factors that were significantly different from 1 (Table 4). With the assistance of computer methods, we have established that cyclazocine interacts with 58% R_H (K_{d_H} = 1.5 nM) and 42% R_L (K_{d_L} = 80.9 nM). These results are in contrast to those obtained with morphine, which recognizes 27% R_H (K_{d_H} = 37.7 nM) and 73% R_L (K_{d_L} = 12,159 nM) (Table 4). We observe that cyclazocine is 6-fold more potent than bremazocine and 30-fold more potent than

TABLE 3

Computer-derived binding parameters from competition experiments of various opioids against the binding of [^{3}H]BREM (under μ- δ- and κ-suppressed conditions) in whole guinea pig spinal cord membranes

For each competition curve against [^{3}H]BREM under suppressed conditions, the computer programs provide estimates of the slope factor (pseudo-Hill coefficients), the binding capacity (R), and the affinity of the unlabeled ligand, expressed in the present report as the equilibrium dissociation constant (K_d). The displacement curves were obtained against a constant concentration of [^{3}H]BREM (≈1.7 nM) in the presence of 100 nM DAGO, 100 nM DADL, and 120 nM U9593 to suppress the μ, δ, and κ sites. Under these conditions, the specific binding was between 40 and 50%. All binding parameters obtained with the opioids were fitted best to a one-site model. Results are expressed as the mean ± standard error of the number of independent experiments indicated by n. For K_d values, the data were transformed to logarithmic form and geometric means were calculated.

Compound	n	K_d	R	Slope factor
		nM	pmol/g of tissue	
DPDP	3	>10,000[a]	ND[b]	ND
DADL	3	>10,000[c]	ND	ND
DAGO	3	>10,000[d]	ND	ND
Bremazocine	5	8.82 ± 0.96	7.92 ± 0.54	1.37 ± 0.08
EKC	3	44.6 ± 2.00	12.1 ± 0.59	1.04 ± 0.07
(−)-SKF10047	3	38.9 ± 2.93	10.6 ± 0.60	0.92 ± 0.02
(+)-SKF10047	3	5942 ± 198	11.7 ± 0.45	1.05 ± 0.17
Diprenorphine	4	56.6 ± 5.51	7.84 ± 1.51	1.30 ± 0.17
Naloxone	4	280 ± 12.8	7.35 ± 1.37	1.04 ± 0.09

[a] At a concentration of 13,000 nM, the inhibition was 8 ± 5% (n = 3).
[b] ND, not determined.
[c] At a concentration of 18,000 nM, the inhibition was 10 ± 5% (n = 3).
[d] At a concentration of 20,000 nM, the inhibition was 55 ± 4% (n = 3).

EKC or (−)-SKF10047 at the R_H sites. At these R_H sites, U69593 was the least potent benzeneacetamide, whereas PD117302 was the most potent drug in this chemical group. Overall, N-BNI was the most potent κ-selective opioid tested. Interestingly, the use of simultaneous analyses improves the precision and the appropriateness of the binding parameters (34).

Preliminary experiments using the nonhydrolyzable analogue Gpp(NH)p have indicated that the two-site model obtained with the compounds listed in Table 4 might be explained by the fact that these [^{3}H]BREM sites are coupled to a G protein. According to the saturation studies, the binding parameters of [^{3}H]BREM obtained under suppressed conditions and in the presence of 100 μM Gpp(NH)pp were not significantly different, as compared with the ones measured in the absence of guanyl nucleotides (data not shown). Furthermore, competition studies of U50488 against the binding of [^{3}H]BREM (under suppressed conditions) in the presence of 100 μM Gpp(NH)pp have revealed that this κ agonist is now interacting with the labeled sites with one homogeneous state of affinity (Fig. 5B). The affinity of unlabeled BREM at those sites was not changed in the presence of Gpp(NH)p (Fig. 5A).

Each total receptor quantity determined by computer analysis of competition curves at the [^{3}H]BREM sites was compared in a pair-wise manner with the ones obtained by saturation studies. Because the data between samples were inherently heteroscedastic, we used the test of equality of means based on the Games and Howell method (see Materials and Methods). The statistical test allows us to conclude that no significant differences exist between the different total binding capacities established by either competition or saturation studies ($p > 0.05$).

Discussion

In the present study, we have documented the concept of multiplicity of opioid receptors in the guinea pig spinal cord, with the assistance of computerized methods. Our results demonstrate the presence of specific binding sites for [^{3}H]DAGO (μ selective) and [^{3}H]DPDP (δ selective opioid), suggesting that μ and δ receptors are indeed located on this nervous tissue and confirming the results of Traynor and Rance (31). Moreover, saturation studies have revealed the existence of specific binding sites for the κ-selective agonist [^{3}H]U69593. The binding of the nonselective benzomorphan [^{3}H]EKC is explained best by a one-site model. This ligand labels a total population of opioid receptors (R) that is not different from the summation of R_{DAGO} plus R_{DPDP} plus R_{U69593}. We are, therefore, proposing that [^{3}H]EKC binds with one homogenous high affinity state to μ, δ,

TABLE 4

Computer-derived binding parameters of various opioids obtained from the simultaneous analysis of competition curves against [^{3}H]BREM (under μ-, δ-, and κ-suppressed conditions) in whole guinea pig spinal cord membranes

The data obtained for each competition curve performed against [^{3}H]BREM (under μ-, δ-, and κ-suppressed conditions) were first processed using individual computerized analysis (data not shown), and the binding parameters estimated by this method were then refined by the use of simultaneous analysis with unlabeled EKC competition curves. Because EKC had one homogeneous affinity at the sites labeled by [^{3}H]BREM (see Table 3), affinities of EKC at both sites were constrained to share the same value during the computerized fitting process. The use of simultaneous analyses improves the validation of the two-site model over the one-site model and, thus, provides more reliable binding parameters for the opioids listed below. For details of the methodology and computation used, see Materials and Methods and the legend to Table 3. H, high; L, low. n, number of experiments.

Compound	n	K_d		R		R_H (%)	Slope factor
		K_{d_H}	K_{d_L}	R_H	R_L		
		nM		pmol/g of tissue			
Morphine	5	37.7 ± 3.29	12,159 ± 1,609	3.24 ± 0.43	8.79 ± 0.44	27 ± 4[a]	0.71 ± 0.09[b]
Cyclazocine	4	1.50 ± 0.56	80.9 ± 24.6	6.95 ± 1.34	5.02 ± 1.30	58 ± 11[c]	0.79 ± 0.05[b]
N-BNI	3	6.22 ± 0.80	923 ± 251	7.15 ± 0.43	4.90 ± 0.41	59 ± 4[d]	0.52 ± 0.54[b]
PD117302	4	40.1 ± 8.06	3,744 ± 324	5.23 ± 0.64	6.77 ± 0.62	44 ± 5[e]	0.65 ± 0.03[b]
U50488	4	96.6 ± 5.45	3,776 ± 114	5.43 ± 1.37	6.56 ± 1.35	45 ± 11[f]	0.87 ± 0.11
U69593	4	346 ± 56.7	51,644 ± 11,090	6.34 ± 0.48	5.63 ± 0.49	52 ± 4[g]	0.71 ± 0.04[b]
ICI197067	4	12.3 ± 2.01	2,667 ± 787	6.09 ± 0.72	5.91 ± 0.69	49 ± 6[h]	0.61 ± 0.03[b]

[a] $R_H + R_L$ = 12.0 ± 0.09 pmol/g of tissue.
[b] Statistically different from 1, using t test ($p < 0.05$).
[c] $R_H + R_L$ = 12.0 ± 0.06 pmol/g of tissue.
[d] $R_H + R_L$ = 12.0 ± 0.04 pmol/g of tissue.
[e] $R_H + R_L$ = 12.0 ± 0.03 pmol/g of tissue.
[f] $R_H + R_L$ = 12.0 ± 0.02 pmol/g of tissue.
[g] $R_H + R_L$ = 12.0 ± 0.01 pmol/g of tissue.
[h] $R_H + R_L$ = 12.0 ± 0.03 pmol/g of tissue.

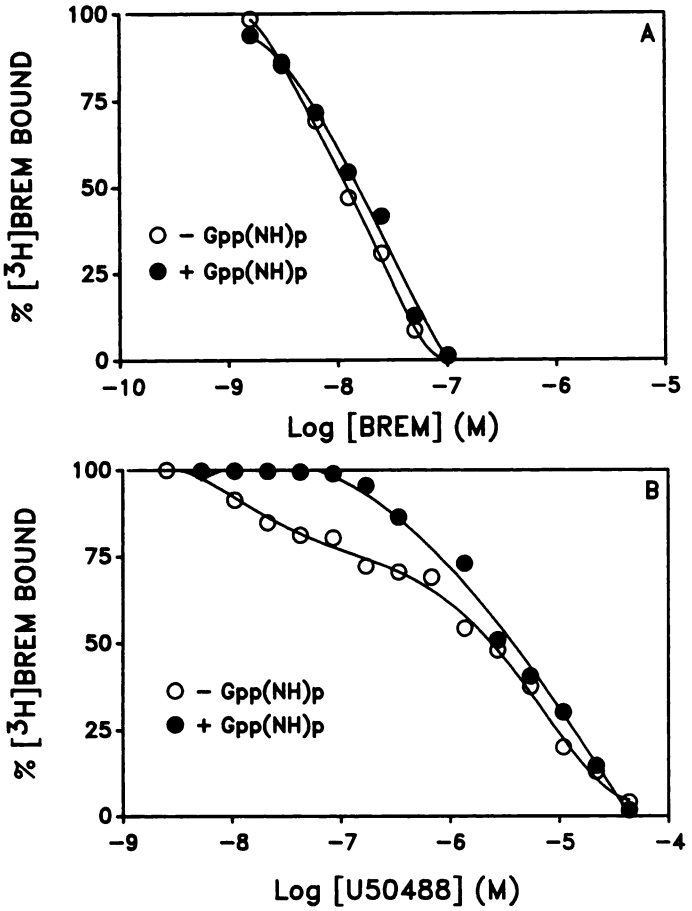

Fig. 5. Computerized curve-fitting of binding data from competition curves against [^{3}H]BREM (μ-, δ-, and κ-suppressed) in the absence and presence of guanyl nucleotides. Competition studies were performed in guinea pig spinal cord membranes against the binding of 1.7 nM [^{3}H] BREM (μ-, δ-, and κ-suppressed) in the absence or presence of 100 μM Gpp(NH)p. Each point represents the mean of duplicate determinations (precision, >95%). Displacement curves were done with 10 to 20 concentrations. All curves were adjusted by the use of simultaneous analysis. A, These are representative displacement curves done with increasing concentrations of BREM. The displacement curves done in the absence or presence of 100 μM Gpp(NH)p were both best fitted to a one-site model. The binding parameters were not significantly different from those shown in Table 3. B, These are representative displacement curves done with increasing concentrations of U50488. The displacement curve performed in the absence of guanyl nucleotides was best fitted to a two-site model. The binding parameters were not significantly different from those shown in Table 4. The competition curve done in the presence of 100 μM Gpp(NH)p was best fitted to a one-site model with a K_d value of 3236 nM and a R value of 9.07 pmol/g of tissue. During the computerized fitting process, affinities of unlabeled BREM at the high and low affinity sites (in the absence or presence of guanyl nucleotides) were constrained to share the same value.

and κ receptors in whole guinea pig spinal cord membranes. Under μ- and δ-suppressed conditions, the binding capacity of [^{3}H]EKC was not significantly different from that measured for [^{3}H]U69593. Displacement curves performed against the binding of either [^{3}H]U69593 or [^{3}H]EKC (μ plus δ suppressed) clearly show that these two radiotracers label a κ receptor subtype with similar pharmacological profiles. This is supported by a significant correlation between the K_d values derived from opioid competition curves against [^{3}H]EKC and those obtained against [^{3}H]U69593. These results are in disagreement with those of Nock *et al.* (40) in rat brain, where [^{3}H]U69593 was suggested to label a κ receptor subtype that differs from the one labeled by [^{3}H]EKC. Nonetheless, the data obtained with [^{3}H]U69593 and [^{3}H]EKC in the guinea pig spinal cord agree with a previous study from our laboratory, which reported that [^{3}H]U69593 and [^{3}H]EKC discriminate the same κ subtype in guinea pig brain membranes (33).

It is evident from our results that the nonselective benzomorphan [^{3}H]BREM labels the opioid receptor sites found on the spinal cord with heterogeneous binding affinity states. The total binding capacity obtained from saturation studies was higher than R_{EKC} or the sum of R_{DAGO} plus R_{DPDP} plus R_{U69593}, indicating that [^{3}H]BREM delineates other opioid sites in addition to μ, δ, and κ receptors. Interestingly, the R value for the [^{3}H]BREM high affinity binding sites (R_1) is not significantly different from R_{EKC} or the sum of R_{DAGO} plus R_{DPDP} plus R_{U69593}. It seems likely that μ, δ, and κ receptors are recognized by [^{3}H]BREM with one homogeneous high affinity state in the guinea pig spinal cord. This confirms a previous study, which has reported that BREM had similar high affinities at these three receptors (41). What then is the identity of the low affinity binding sites (R_2) discriminated with the use of [^{3}H] BREM? After suppression of μ, δ, and κ receptors with selective unlabeled blockers, saturation studies with [^{3}H]BREM have revealed the presence of residual binding sites that display one homogeneous affinity state. Competition studies performed against the binding of [^{3}H]BREM (under μ-, δ-, and κ-suppressed conditions) show that μ- and δ-selective opioids have little or no affinity for these sites. Furthermore, there is a high degree of correlation between the affinities of opioids at these sites (Table 3) and their affinities at the κ sites (Table 1). These observations suggest the κ nature of these additional sites labeled with [^{3}H]BREM. However, the pharmacological profile of [^{3}H]BREM was obtained under conditions in which its binding at the κ receptors was effectively suppressed. Obviously, the additional sites labeled with [^{3}H]BREM cannot represent the same κ sites that are labeled with high affinity by [^{3}H]U69593. Consequently, we propose that these sites be named κ_2 sites. Accordingly, the κ sites labeled with high affinity by [^{3}H]U69593 or by [^{3}H]EKC (μ plus δ suppressed) should be called κ_1 sites. This is in agreement with the nomenclature already proposed in rat (16) and guinea pig brain (17).

Unexpectedly, competition studies performed against the binding of [^{3}H]BREM under μ-, δ-, and κ_1-suppressed conditions yield two different pharmacological profiles, suggesting the possible heterogeneity of these κ_2 binding sites. Indeed, computer analyses reveal that binding of unlabeled BREM, EKC, (+)- and (–)-SKF10047, diprenorphine, and naloxone at these sites was best explained by one homogeneous affinity state. In contrast, simultaneous analyses show that the displacement curves of cyclazocine, morphine, U69593, U50488, PD117302, ICI197067, and N-BNI can be best represented by a two-site model. These latter results are different from those observed in guinea pig brain, where all displacement curves were best fitted to a one-site model. These observations are not due to improper blocking conditions. Indeed, saturation studies of [^{3}H]EKC performed in the presence of μ-, δ-, and κ_1-selective blockers in both tissues have indicated that the binding capacity was reduced by more than 90%, as compared with unsuppressed conditions (data not shown).

One striking feature is that the κ_2 pharmacological profile originally described in the lumbosacral region of the guinea pig

spinal cord by Attali *et al.* (14) does not correlate with the profile observed at our putative κ_2 receptors ($r = 0.11$, $p > 0.05$, seven experiments; data not shown). For instance, at their κ_2 receptor DADL displayed an affinity of 57 nM, whereas in the present study the affinity of DADL was over 10,000 nM. However, morphine had similar affinities in both studies (37.7 versus 35 nM). These discrepancies are not easily resolved but they might be explained by several factors. Firstly, they can be explained by the use of different methods for the κ_2 receptor labeling. Attali *et al.* (14) have used the nonselective ligand [^{3}H]etorphine (under unsuppressed conditions) to probe the κ_2 receptors in the lumbosacral region, as opposed to our use of [^{3}H]BREM in whole guinea pig spinal cord. According to these authors, [^{3}H]etorphine labels a homogeneous population of opioid receptors, namely the κ_2 subtype, which are DADL sensitive, in contrast to their κ_1 receptors (labeled with [^{3}H] EKC under μ- and δ-suppressed conditions), which are DADL insensitive in the same preparation. Secondly, these discrepancies might be related to the use by these authors of graphical methods to derive their binding parameters. Because the level of nonspecific binding of the radioligands in the spinal cord is higher, as compared with other nervous tissue, graphical methods can be a major source of error. The use of curve-fitting programs will give, in comparison with the classical methods, more precise binding parameters (34). Thirdly, there is also a possibility that the κ receptor family in the guinea pig spinal cord is composed of more than two subtypes and that our two different approaches have separately identified one of the two subtypes.

In fact, we would like to propose that κ_2 receptors exist under two affinity forms in the guinea pig spinal cord, namely a high affinity form (κ_{2_H}) and a low affinity form (κ_{2_L}). Indeed, we have shown that the nonhydrolyzable guanyl nucleotide Gpp(NH)p can modulate the interaction of opioids at the κ_2 sites. Certain drugs recognize both the high and low affinity components of the κ_2 sites in the absence of GPP(NH)p. However, in the presence of Gpp(NH)p the high affinity form is converted to the low affinity form. This is illustrated by the interaction of U50488 at the κ_2 sites with one homogeneous affinity state (Fig. 5). The data obtained in the present study may be consistent with earlier reports explaining the molecular pharmacology of the β-adrenergic receptors. It has been demonstrated that agonists bind to adenylate cyclase-coupled β-adrenergic receptors with a high and low affinity state, whereas antagonists interact with the receptor with one homogeneous affinity (42). This typical interaction (which also involves G proteins) was further ascribed to the binding model called the ternary complex (43). Interestingly, the opioids listed in Table 3 have all been described as having antagonistic activity (41). This would be in agreement with the description of one homogeneous binding affinity for these compounds at the κ_2 receptors. In contrast, most opioids listed in Table 4 have been described as having agonistic properties in several biological preparations (41, 44–47). Thus, the presence of heterogeneous affinity states for the κ_2 receptors discriminated by these putative κ_2 agonists agrees with previous findings observed with the β-adrenergic receptor system. As for the κ-selective antagonist N-BNI (48), the manifestation of heterogeneous binding at κ_2 receptors is not inconsistent with the binding model proposed herein. There are several findings of opioids having agonistic properties at one receptor subtype while they have antagonistic properties at other opioid receptor subtypes. For instance, BREM is an agonist at the κ-opioid receptors and an antagonist at the μ receptors located in the guinea pig myenteric plexus (49). Moreover, diprenorphine has agonist activity at the κ-opioid receptors of the guinea pig myenteric plexus while it has antagonist activity at the κ-opioid receptors in the rabbit vas deferens (50).

Evidence obtained from other laboratories is also consistent with the G protein-coupled receptor model to explain opioid ligand properties of the κ_2-opioid receptor in guinea pig spinal cord. For example, in both spinal cord and spinal cord cocultures, the κ agonist U50488 produces inhibition of forskolin-stimulated adenylate cyclase by receptors that are negatively coupled to adeylate cyclase via a pertussis toxin-sensitive G_i protein (51). Concomitantly, a study in rat spinal cord-dorsal root ganglion cocultures has shown that κ receptors are negatively coupled to voltage-dependent Ca^{2+} channels via a pertussis toxin-sensitive G protein and that this interaction involves cyclic AMP-dependent and -independent mechanisms (52). This latter study has also revealed that inhibition of the stimulated Ca^{2+} influx by U50488 clearly suggests two distinct interactions namely a high affinity interaction with an ED_{50} value of 52 nM and a percentage of R_H of 37% and a low affinity interaction with an ED_{50} value of about 5300 nM and a percentage of R_L of 63%. In our study, U50488 has a K_{d_H} of 97 nM with a percentage of R_H of 45% and a K_{d_L} of 3600 nM with a percentage of R_L of 55%. Strikingly, after pertussis toxin treatment, U50488 depresses the stimulated Ca^{2+} influx in treated coculture cells with only a low affinity interaction (ED_{50} = 20 μM) combined to ADP-ribosylation of a 40–41-kDa protein (52).

In conclusion, the present study demonstrates the presence of μ, δ, κ_1, and κ_2 receptors in the guinea pig spinal cord. Computer-fitting methods have permitted the resolution of high and low affinity forms for the κ_2 receptor. Unfortunately, the data presented here cannot fully ascertain whether κ_1 and κ_2 subtypes constitute the same κ protein existing under multiple affinity forms or are represented by two independent proteins that are encoded by two separate genes. Further investigations are needed to elucidate the exact mechanisms by which opioids interact at the κ_1 and κ_2 receptors. Undoubtedly, the development of new, highly selective, κ_2 agonists and antagonists will be a good step in that direction.

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Send reprint requests to: Mario Tiberi, Département de Pharmacologie, Université de Montréal, C.P. 6128, Succursale A, Montréal, Québec, Canada H3C 3J7.