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Opiate receptor: Cooperativity of binding observed in brain slices

(receptor/morphine/naloxone/Hill slope/homotropic interactions)

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ABSTRACT **Kinetic constants for binding of ^{3}H-labeled morphine and naloxone were determined from Hill plot analyses and from experiments in which the concentration of tritiated drug was constant and that of nonlabeled drug varied. With brain slices, the binding of either drug exhibited strong positive cooperativity (Hill slope >3); this was not observed in brain homogenates. Thus, in slices the relationship between opiate binding site and ligand may be more relevant physiologically and pharmacologically.**

Specific *in vitro* binding is a currently popular method used to quantify the interaction of receptor molecules with both endogenous and exogenous ligands such as drugs. Drug–receptor binding is frequently measured in crude tissue preparations by using varying concentrations of radiolabeled drug. The unbound material is removed by filtration or centrifugation, and binding to the receptor is assumed to be that portion of bound radioactive drug that can be displaced by excess amounts of nonlabeled drug. With this method, receptors for neurotransmitters (e.g., norepinephrine, dopamine, and serotonin) and the opiate analgesics and antagonists have been characterized as to their binding properties (1, 2). *In vitro* binding experiments are valuable as tools for elucidating mechanisms of drug action and for screening new compounds (e.g., opiate-like drugs). However, with this technique, the nonsaturable, nonspecific binding increases linearly with the radiolabeled drug concentration so, at high drug concentrations which are necessary to show saturation and to determine maximum receptor binding accurately, receptor binding constitutes only a small portion of the total binding of radiolabeled ligand. We have developed a method for determining receptor binding parameters *in vitro* in which a fixed, low concentration of radiolabeled drug is used and the binding of labeled drug is assayed in the presence of increasing concentrations of nonlabeled drug. By using this analysis, the concentration of saturable opiate binding sites and their affinity for morphine and its antagonist, naloxone, were estimated. These studies have yielded data indicating that, in brain slices, the receptor sites for opiate agonists and antagonists exhibit positive cooperativity. This cooperativity is not observed in brain homogenates.

MATERIALS AND METHODS

Materials. [^{3}H]Morphine (30 Ci/mmol) was purchased from Amersham/Searle Corp. (Arlington Heights, IL) and [^{3}H]naloxone (20 Ci/mmol), from New England Nuclear Corp. (Boston, MA). Morphine sulfate was purchased from Mallinkrodt Chemical Works (St. Louis, MO) and naloxone hydrochloride, from Endo Laboratories, Inc. (Garden City, NY).

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Male rats (Sprague–Dawley) weighing 175–225 g were used, and brain slices or homogenates were prepared in a cold room (5°). A McIlwain tissue chopper was used to prepare slices (0.5 mm thick) of basal ganglia and diencephalon from freshly obtained rat brain, or the same brain areas were homogenized with a motor-driven Teflon-pestle homogenizer (Potter–Elvehjem). The tissue preparations were incubated with one concentration of either of the tritiated drugs in the presence of varying concentrations of the nonlabeled form of the same drug for 20 min at 35°. Tris·HCl buffer, 50 mM, was used. The nonsaturable binding of tritiated drug was determined by incubation in the presence of excess (10 μM) nonlabeled drug. After incubation, slices were immediately homogenized, and, for both tissue preparations, duplicate aliquots were filtered on nitrocellulose membranes (0.8 μm pore size, Millipore Corp., Bedford, MA). The filters were dissolved, and their radioactivity was measured by liquid scintillation spectrometry. Protein concentration was determined by the biuret method (3). Binding constants were determined directly from the data as described below.

Theoretical Considerations and Determination of Binding Constants. Drug-receptor binding (b) is postulated to be governed by the law of mass action and, as such, can be expressed as:

$$b = [DR] = \frac{B_{max}[D]^n}{K_D{}^n + [D]^n} \qquad [1]$$

in which $[DR]$ is the concentration of drug–receptor complex and $[D]$ is the concentration of drug. This is analogous to the Hill equation of enzyme kinetics. B_{max} is the concentration of sites available to drug, K_D is the dissociation constant or concentration of drug required to bind half the sites, and n is an index of cooperativity among binding sites. Eq. **1** predicts that a plot of receptor binding versus ligand concentration will be a hyperbola if the binding sites are independent of each other (n = 1, solid line in Fig. 1) but will be sigmoidal if there is positive cooperativity among sites (n = 2 or 4, dashed and broken lines in Fig. 1).

In the present studies the concentration of tritiated drug was fixed and the concentration of nonlabeled drug varied. Thus, the measured ^{3}H-labeled drug binding by receptor is a fraction of the actual drug–receptor binding. In experiments in which ^{3}H-labeled drug is being displaced by increasing concentrations of nonlabeled drug, binding of ^{3}H-labeled drug (b_1) can be expressed as:

$$b_1 = \frac{B_{max}(d + a)^n}{K_D{}^n + (d + a)^n} \cdot \frac{d}{d + a} + C \qquad [2]$$

in which d is the concentration of ^{3}H-labeled drug, a is the concentration of nonlabeled drug, and C is the nonspecific binding of tritiated drug (measured in the presence of excess concentrations of nonlabeled drug). On a plot of ^{3}H-labeled

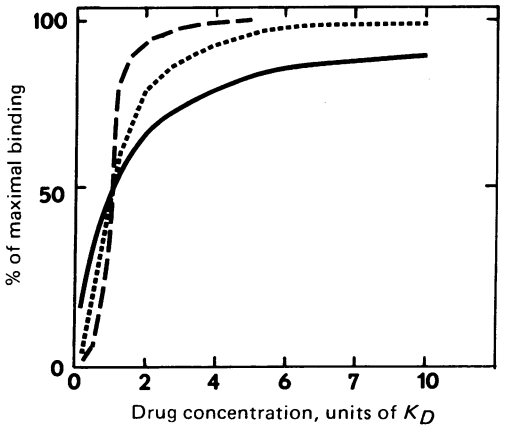

FIG. 1. Theoretical curves for drug-receptor binding determined by the saturation method. Calculation was based on Eq. 1, with B_{max} and K_D held constant and n (number of interacting sites) equal to 1 (—), 2 (- - - -), and 4 (– – –).

drug bound versus total concentration of drug (log scale), the binding is similar to an isotope dilution curve if the sites are independent (n = 1, solid line in Fig. 2). If there is positive cooperativity among binding sites and the concentration of ^{3}H-labeled drug is less than the K_D, the amount of labeled drug bound initially increases when relatively low concentrations of nonlabeled drug are added (n = 2 or 4, dashed and broken lines in Fig. 2).

This experimental design and analysis offers several advantages over the more conventional saturation experiment in which the binding of various concentrations of ^{3}H-labeled drug is assayed. Foremost, the analysis of the data is not invalidated by cooperativity among sites, nor does it require prior accurate determination of B_{max}. Additionally, the problem of linearly increasing nonsaturable binding, which can obscure receptor binding, is avoided and the use of radiolabeled drug is decreased, because a fixed, low concentration of radiolabeled drug is used. The values of B_{max}, K_D, and n were estimated directly from all data points by an iterative, nonlinear least squares regression analysis using Brown's method (4) to solve the normal equations or the Marquardt algorithm (5) for minimizing a sum of squares by nonlinear functions. A CDC 6500 computer was used in the analysis.

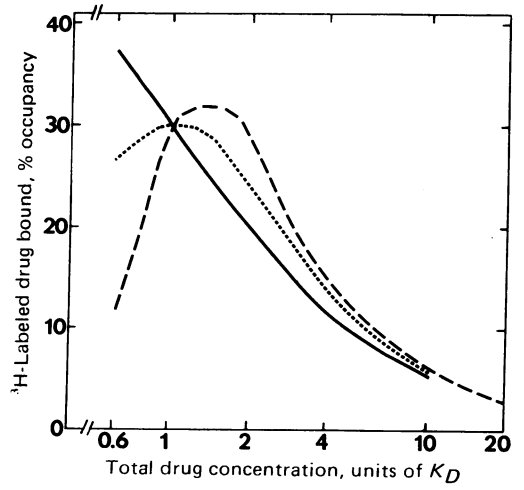

FIG. 2. Theoretical curves for drug–receptor binding determined by the displacement method. Calculation was based on Eq. 2, with B_{max}, K_D, and n as in Fig. 1.

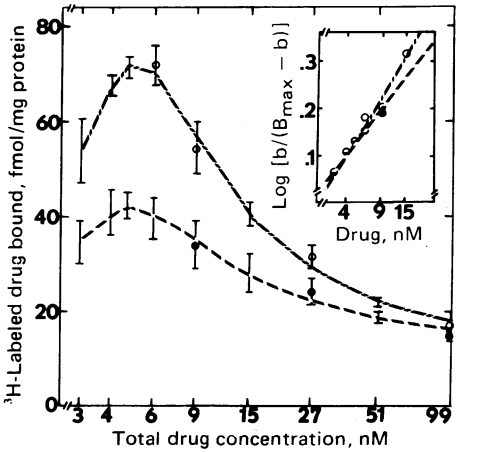

FIG. 3. Receptor binding to slices determined by the displacement method. The concentration indicated on the abscissa is for nonlabeled drug plus 3 nM tritiated drug. Lines are shown as nonlinear least squares regression curves fitted to all data points. Data are expressed as mean ± SEM (of at least four experiments) except where regression line overlaps the mean. (*Inset*) Hill plot. Data were converted into saturation data and calculated for graphic analysis. Lines were fitted by linear least squares regression. The slope, n, is 2.6 for morphine (– – –) (r^2 = 0.987) and 3.4 for naloxone (- - - -) (r^2 = 0.972).

RESULTS AND DISCUSSION

The initial increase in binding, characteristic of positive cooperativity, was seen in slice experiments for both [^{3}H]morphine and [^{3}H]naloxone binding (Fig. 3). This is in contrast to results obtained with homogenates (Fig. 4), in which the data followed the pattern predicted for the absence of cooperativity. The parameters B_{max}, K_D, n, and C for slice data, determined by nonlinear regression analysis, are given in Table 1. The index of cooperativity, n, is greater than 1 in the slice experiments for both the agonist morphine and antagonist naloxone. When the data were analyzed graphically by using a Hill plot (Fig. 3 *inset*), n was slightly lower than these values but still >1 (2.6 for morphine, 3.4 for naloxone). Therefore, binding of one ligand molecule facilitates the binding of other molecules.

This cooperativity among binding sites was not observed in crude brain homogenates in the present experiments (n = 0.5 for morphine and 0.8 for naloxone) nor in earlier studies of naloxone and dihydromorphine binding to a particulate fraction of brain (6). Pert and Snyder (7) reported data for [^{3}H]naloxone

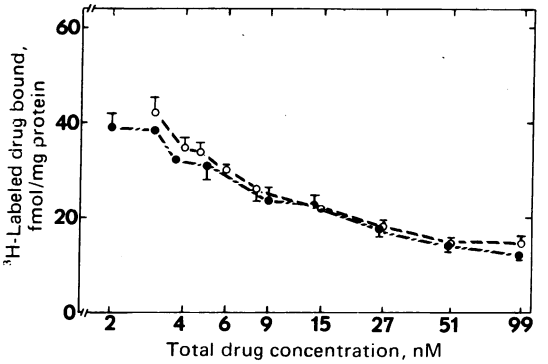

FIG. 4. Receptor binding to homogenates determined by the displacement method. Binding of ^{3}H-labeled drug to brain homogenate was determined as described in the *text*. Data are expressed as mean ± SEM (of at least three experiments). Concentration of [^{3}H]morphine (– – –) was 3 nM; [^{3}H]naloxone (- - - -) was 2 nM. Positive cooperativity was not observed.

Table 1. Binding parameters for opiate binding sites in brain slices

	B_{max}, fmol/mg protein	K_D, nM	n	C, fmol/mg protein
Morphine	64	3.8	3.1	14
Naloxone	132	3.8	3.6	14

Binding parameters were determined by nonlinear least squares regression analysis of all data points. B_{max} is the binding site concentration, K_D (dissociation constant) is the concentration of drug at which half of the sites are bound, n is the number of interacting sites, and C is the nonsaturable binding. Binding was assayed in the absence of sodium. The values for C estimated by regression analysis and determined directly (in the presence of excess nonlabeled drug) are in good agreement.

binding to brain homogenates in which no evidence of cooperativity was seen (saturation and displacement curves both closely resembled the respective theoretical curve for $n = 1$). This was later confirmed by Simantov and Snyder (8), who calculated Hill slopes of 0.8–1.2 for inhibition of [^{3}H]naloxone binding by enkephalin, morphine, or naloxone. In contrast, Simon and coworkers (9) found that, for [^{3}H]naltrexone and [^{3}H]etorphine binding, Scatchard plots were nonlinear and resembled the case of $n > 1$; Simon (2) later reported n to be 1.5. These findings are interesting because they indicate that, if detectable at all, cooperativity is much less pronounced in preparations in which the cell membrane has been damaged. It is not surprising that interaction among sites is greater in a system in which the cell membrane is relatively unperturbed. Moreover, this cooperativity of binding could explain the relative steepness of morphine analgesia logarithmic dose-response curves.

The binding site concentration, B_{max}, is greater for the antagonist naloxone (131 fmol/mg of protein) than for the agonist morphine (64 fmol/mg of protein); these values are in good agreement with those reported by other investigators using different drugs and tissue preparations (10, 11). From these results it appears that naloxone has access to some sites that are not available to morphine. We have previously demonstrated (12) that [^{3}H]morphine bound to brain slices is displaced by nonlabeled naloxone in a concentration-dependent fashion. Thus, there may be a set of naloxone-specific sites as well as a group of sites that interact with both naloxone and morphine. Lee *et al.* (6) reported similar results with a particulate fraction of brain tissue.

The finding of positive cooperativity among narcotic receptors is interesting because it supports a multiple subunit model for the receptor. Thus, alterations of subunit interaction, such as a conformational change, may be involved in changes of receptor binding due to sodium or chronic morphine treatment.

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