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Discrimination of Three Types of Opioid Binding Sites in Rat Brain *in Vivo*

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SUMMARY

Opiate receptor sites in the rat brain were defined *in vivo* by measuring the binding of etorphine, sufentanil, diprenorphine, and naloxone in saturation and cross-competition experiments. The binding data were analyzed simultaneously, using a computerized curve-fitting technique with an extended least-squares nonlinear regression program. Three types of binding sites could be distinguished: site 1 (18 pmoles/g of brain), site 2 (15 pmoles/g of brain), and site 3 (20 pmoles/g of brain). Site 1 is bound selectively by sufentanil (the ratio of the apparent equilibrium dissociation constants $K_2/K_1 \approx 1200$), etorphine ($K_2/K_1 \approx 20$), and naloxone ($K_2/K_1 \approx 15$), and it resembles the *mu* binding site previously demonstrated *in vitro*. Diprenorphine binds to both site 1 and site 2 with high affinity and a slight (~3.7-fold) selectivity for site 1 over site 2. The latter site may represent a mixture of the *delta* and *kappa* binding sites. The third site displays relatively high affinity for naloxone, but it is clearly different from sites 1 and 2, as it exhibits a lack of affinity for sufentanil, etorphine, and diprenorphine. This binding site population does not resemble any of the known opiate binding sites. Recent *in vitro* binding studies revealed that site 3 (now termed *lambda* site) is highly labile *in vitro* and was, therefore, not previously detected. These results suggest significant differences between *in vitro* and *in vivo* opioid receptor binding characteristics.

INTRODUCTION

The concept of opioid receptor multiplicity was originally formulated on the basis of the differing pharmacodynamic profiles of selected opiates (1–4). *In vitro* binding studies support this concept and point to the existence of binding sites associated with the *mu* (morphine), *delta* (enkephalin), *kappa* (benzomorphan), and *sigma* (SKF 10,047) receptors in the rat central nervous system (5–7). A number of differences exist between the *in vivo* and *in vitro* properties of these binding site populations, such as differences in relative receptor affinity (8) and concentration of binding sites (9). Earlier studies (e.g., refs. 8–11) examined the *in vivo* binding of opiates but did not demonstrate which of the binding sites found corresponded to those shown in *in vitro* studies.

We have developed a method for measuring opiate receptor binding in the intact animal (*in vivo*). The approach involves s.c. administration of ^{3}H-labeled opiates to rats with and without various unlabeled opiates and the measurement of bound label in the cerebrum immediately after sacrifice, using a rapid membrane filtration technique (8, 9, 11). On the basis of this technique, we previously constructed saturation and cross-competition curves for both [^{3}H]etorphine and [^{3}H]diprenorphine and concluded that etorphine labels a subset of those binding sites in rat cerebrum labeled by diprenorphine in the intact rat (8). Additional cross-competition experiments with [^{3}H]naloxone and buprenorphine appeared to indicate that naloxone labeled an additional binding site *in vivo* not previously observed using the etorphine and diprenorphine tracers (12). In this paper we expand on these previous findings: a series of saturation and cross-competition experiments, using the *in vivo* labeling technique with [^{3}H]sufentanil, [^{3}H]etorphine, [^{3}H]diprenorphine, and [^{3}H]naloxone, was performed in order to address the question of *in vivo* opioid receptor multiplicity. The results were analyzed by using a multiple binding site model based on the law of mass action to describe the properties of the binding sites and ligands. The model is similar to those used previously to differentiate multiple binding sites *in vitro* (13, 14). The method analyzes untransformed binding data from a number of similar experiments simultaneously while allowing for the nonhomogeneity of variance among the data sets (14, 15). In contrast, the Scatchard technique (16) requires a linear transformation of experimental

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observations which distorts the error structure of the data and may result in biased parameter estimates (e.g., refs. 17 and 18). The method employed here allows the characterization of three distinct opiate binding sites in the intact rat. Furthermore, the binding site populations identified *in vivo* are compared with those established by *in vitro* techniques.

MATERIALS AND METHODS

In vivo binding studies. The rapid membrane filtration technique described in detail by Perry *et al.* (11) was used to determine the amount of bound and unbound ligand in the cerebrum 20 min after s.c. administration to male Sprague-Dawley rats. This is the time at which brain concentrations of etorphine (11) and naloxone (19) reach their peak and the time at which one can expect minimal interference with the diprenorphine-receptor equilibrium from the postulated receptor microcompartment (19). This time point therefore is assumed to represent a time at which a steady state exists between the free drug and the drug-receptor complex, as at this time there is no net association or dissociation of the ligand-receptor complex. Sufentanil binding was also determined at this time point, but the pharmacokinetic disposition of this drug in the rat brain was not determined. The following doses of labeled ligands were used: [^{3}H]etorphine, 15–80 μCi/kg; [^{3}H]sufentanil, 150–300 μCi/kg; [^{3}H]diprenorphine, 100–150 μCi/kg; and [^{3}H]naloxone, 100–150 μCi/kg. Subcutaneous injection was selected over i.v. injection for the following reasons: (a) Most pharmacological data are derived from s.c. doses; therefore, labeling *in vivo* must use the same mode of administration in order to allow for direct comparison of pharmacological and *in vivo* binding data. (b) Absorption of the drug from s.c. sites is rather rapid and appears to be close to completion after ~10 min (11); moreover, s.c. administration produces a more shallow drug blood concentration-time profile than i.v. administration, and thereby, allows more time for equilibrium between the blood and brain compartments. (c) The measured variability between dose and concentration of unbound drug in the brain is sufficiently small for the purpose of the data analysis in this study (Fig. 1). However, for some drugs (most notably morphine and ethylketocyclazocine), very large s.c. doses are only partially absorbed. If observed, such experiments must be excluded from the study.

Confirmation of ligand stability during the in vivo and labeling period. The potential contribution of drug metabolites generated *in vivo* to the binding results was determined by using the following procedure: [^{3}H]Naloxone (143 μCi/kg) was administered to four rats. Two of these also received diprenorphine (2 mg/kg) since a binding experiment for naloxone was also determined under this condition (see Results). The animals were killed after 20 min. The brains were homogenized in 25 ml of 0.05 M Tris-HCl buffer (pH 7.4 at 37°) and next filtered in 250-μl aliquots through Whatman GF/B filters (six filters per rat). The filters were combined in groups of three and extracted overnight with 7.0 ml of methanol in the presence of 50 μg of unlabeled naloxone carrier. The next day, the methanol extract was decanted off the filters and evaporated with N_2 in a hot water bath (66°) to near dryness. The residue was taken up in 250 μl of the HPLC[2] eluent (65% methanol/35% sodium phosphate buffer, pH 6) and injected onto an Alltech C-18 reverse-phase column. At a flow rate of 2 ml/min, the retention time of naloxone in this system is 5.1–6.8 min. A similar procedure was followed for sufentanil, except that two rats received injections of [^{3}H]sufentanil (300 μCi/kg) and the resulting methanol extracts were obtained with 50 μg of unlabeled sufentanil carrier. The HPLC system for sufentanil was 70% acetonitrile/methanol (55:45)/30% 0.005 M K_2HPO_4. At a flow rate of 2.5 ml/min on the same column, sufentanil elutes after 10.5–12 min, during which period eluent is collected. For both naloxone and sufentanil, when the extraction recovery is taken into account, >95% of the radioactivity on the filters elutes from HPLC with a retention time characteristic of the native drug. This argues against the possibility of significant interference from metabolites in the quantitative determination of receptor-bound drug. Similar studies with etorphine (11) and diprenorphine (9) have shown a recovery of >90% of unchanged drug.

Estimation of binding parameters. For reasons discussed below (see Discussion), we analyzed our data using the three binding site model. Such a model may be derived using the law of mass action:

$$B_L = U_L\left[\frac{B_{max}^1}{U_L + K_{1L}} + \frac{B_{max}^2}{U_L + K_{2L}} + \frac{B_{max}^3}{U_L + K_{3L}} + NS_L\right] \quad (1)$$

where B_{max}^1, B_{max}^2, and B_{max}^3 are the brain concentrations of binding sites 1, 2, and 3. K_{1L}, K_{2L} and K_{3L} represent the apparent equilibrium dissociation constants of the ligand at these sites. These constants represent the concentration of ligand L occupying 50% of sites 1, 2, and 3, respectively, 20 min after the dose. U_L is the unbound concentration of ligand, determined experimentally as the difference between total and bound brain ligand concentration. The nonspecific binding parameter NS_L was estimated separately for each ligand. B_L is the predicted concentration of labeled and unlabeled ligand which is bound, i.e., the sum of specific and nonspecific binding. Equation 1 is used when the labeled and unlabeled drug are the same; the values of B_L are computed from the radiolabeled ligand binding, specific activity, and the concentration of unlabeled ligand.

In the presence of a different unlabeled competing ligand D, whose apparent equilibrium dissociation constants at the three sites are K_{1D}, K_{2D}, and K_{3D}, respectively, a similar model is used:

$$B_L = U_L\left[\frac{B_{max}^1}{U_L + K_{1L}(1 + D/K_{1D})} + \frac{B_{max}^2}{U_L + K_{2L}(1 + D/K_{2D})} + \frac{B_{max}^3}{U_L + K_{3L}(1 + D/K_{3D})} + NS_L\right] \quad (2)$$

Equation is therefore used to describe the cross-competition experiments. It should be noted that Eqs. 1 and 2 are valid only if ligand binding occurs at each site independently of binding at the other sites.

Equation 2 requires that the expression for D be proportional to the *unbound* concentration of the competing ligand in the brain. Under *in vitro* conditions, the unbound concentration of each species may be predicted from the known total concentration of each ligand and the binding parameters. This involves the solution of a set of implicit equations, for example, using the method suggested by Feldman (20) and embodied in computer programs such as LIGAND (13) and SCAFIT (14). In contrast to the *in vitro* method, the total concentration of the competing ligand is not known *in vivo*.

Under the assumption of concentration-independent distribution and elimination, it can be predicted that the unbound concentration in any tissue will be proportional to the administered dose. We have experimentally verified this assumption for those ligands which were given simultaneously in labeled and unlabeled forms (Table 1; Fig. 1). Even for drugs whose excretion may be restricted by organ blood flow or capacity-limited elimination processes, the assumption of dose proportionality is likely to be valid shortly after drug administration, because concentrations will be determined largely by distribution rather than elimination mechanisms. The slope of the dose-unbound concentration line (Table 1) was used to convert dose to brain unbound concentration units when the unlabeled ligand is used as a competitor in a cross-competition experiment (Eq. 2).

The variance (VAR) of the ith observation in the jth data set was modeled by Eq. 3:

$$VAR_{ij} = V_j B_{ij}^Z \quad (3)$$

where V_j is a variance scale parameter for data in the jth data set and B_{ij} is the predicted bound concentration. The power Z was estimated as a common parameter for all experiments. The ligand combinations which were analyzed simultaneously using these models are indicated

[2] The abbreviation used is: HPLC, high-pressure liquid chromatography.

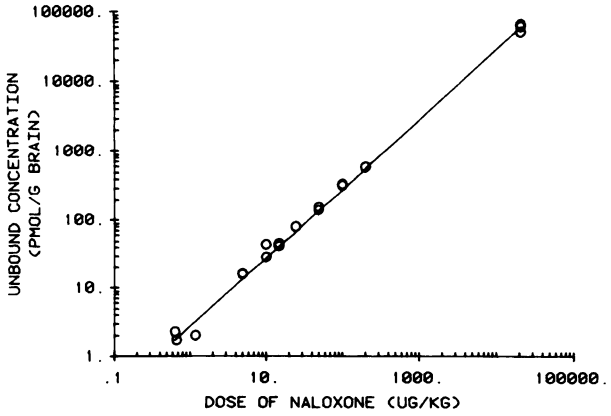

FIG. 1. *Dose-brain concentration relationship for naloxone.*
The unbound concentration was determined at each administered dose after a 20-min labeling period *in vivo*. Each point represents the mean of duplicate determinations in a single rat. Logarithmic scales are used only to aid in visualizing the data over a wide range of doses. The solid line is predicted by linear regression forced through the origin.

in Table 3. Simultaneous data analysis was performed, since each experiment indicated in Table 3 provides information about the ligand-binding site interaction which is useful in the analysis of data from another ligand-binding combination.

Materials. Tracers and unlabeled drugs were obtained from the following sources: [^{3}H]naloxone (21–50 Ci/mmole), [^{3}H]etorphine (33–51 Ci/mmole), etorphine·HCl, and diprenorphine·HCl from the National Institute on Drug Abuse (Rockville, Md.); [^{3}H]sufentanil (15 Ci/mmole) and sufentanil citrate from Janssen Pharmaceutica (New Brunswick, N. J.); naloxone·HCl from Endo Laboratories (Garden City, N. Y.). [^{3}H]Etorphine was also purchased from Amersham (Arlington Heights, Ill.), as was [^{3}H]diprenorphine (7.5–11 Ci/mmole). All tracers were purified by HPLC, using systems indicated here and in refs. 9 and 11) when purity was <90%. All doses of unlabeled ligands are expressed in terms of the free base. Male Sprague-Dawley rats (100–200 g) were purchased from either Simonsen Laboratories (Gilroy, Calif.) or Bantin and Kingman, Inc. (Freemont, Calif.).

All numerical, statistical, and graphical analyses were performed using the PROPHET system (21). Model specification and parameter estimation by nonlinear regression used MKMODEL (22) with an extended least-squares objective function (15).

RESULTS

Determination of the dose-brain concentration relationship. For each of the ligands (naloxone, diprenorphine, etorphine, and sufentanil), a plot such as the one illustrated in Fig. 1 for naloxone was constructed. The data are represented on logarithmic axes only to visualize the data throughout the entire concentration range. Figure 1 shows the linear relationship between the unbound ligand concentration in the cerebrum and the administered dose. Linear regression, forced through the origin, was used to estimate the slope of the dose-concentration relationship; the results are summarized in Table 1.

Binding site populations differentiated. The estimates of B_{max} (given in Table 2) were obtained from simultaneously analyzing the data sets indicated in Table 3, and the results of fitting the sufentanil, diprenorphine, and naloxone binding data to a three binding site model are illustrated in Fig. 2. The etorphine saturation curve has been published previously (11). Note that the sufentanil binding curve appears to saturate at a B_{max} of 18 pmoles/g of brain. Diprenorphine binds to an additional type of binding sites not labeled by sufentanil, whose concentration is 15.3 pmoles/g of brain. The binding of naloxone was not explicable by these two types of sites alone but could be explained by postulating a third type of site with a B_{max} of 19.8 pmoles/g of brain. The three binding site model, therefore, appeared to be (see Discussion) the model of minimal complexity for describing the binding data.

The binding of naloxone to an additional binding site with extremely low affinity for diprenorphine is illustrated in Fig. 3 by two sets of naloxone saturation binding curves that were obtained using naloxone in the absence or the presence of 2 mg/kg diprenorphine. No changes in tracer naloxone binding were observed with 1 mg/kg or 10 mg/kg diprenorphine (data not shown). The 2 mg/kg dose of diprenorphine essentially saturates all of the diprenorphine binding sites *in vivo* (refs. 8 and 9 and this study). Therefore, the binding of naloxone in the presence of this dose of diprenorphine indicates the existence of binding sites for which diprenorphine has negligible affinity. Because the existence of substantial [^{3}H]naloxone binding *in vivo* in the presence of saturating doses of diprenorphine has not been observed in previous *in vitro* studies, we tested a series of opioid drugs for their ability to compete for [^{3}H]naloxone *in vivo* (Table 4). The doses selected for competitors were those that completely inhibited [^{3}H]etorphine binding *in vivo*. (The complete [^{3}H]etorphine displacement data will

TABLE 1

Dose-concentration relationships

Values are the slope of the dose (micrograms per kilogram) versus unbound brain concentration (picomoles per gram of brain) curves as determined by least-squares regression obtained after a 20-min labeling period *in vivo*. Data for naloxone are illustrated in Fig. 1.

Drug	Slope (conversion factor)	Standard error of slope	Dosage range	r^2	No. of data points
			μg/kg		
Etorphine	0.7912	0.0187	0–200	0.9714	54
Sufentanil	0.7061	0.0555	0–900	0.9152	16
Diprenorphine	0.7377	0.0263	0–10,000	0.9764	20
Naloxone	2.724	0.0603	0–20,000	0.9903	21
Naloxone, in the presence of a 2 mg/kg diprenorphine dose	1.361	0.0143	0–20,000	0.9980	19

TABLE 2

Binding site populations differentiated from simultaneous fitting of the naloxone, etorphine, sufentanil, and diprenorphine in vivo competition curves indicated in Table 3

Binding site	B_{max}	SE	Tentative receptor assignments[a]
	pmoles/g brain	*pmoles/g brain*	
1	18.0	0.7	*Mu*
2	15.3	1.7	*Delta + kappa*
3	19.8	0.3	Additional naloxone site [= *lambda* site (30)]

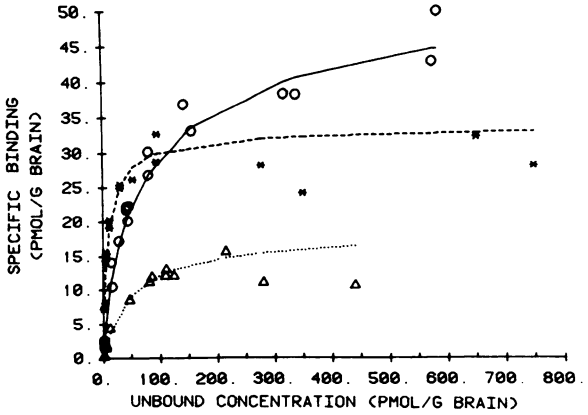

FIG. 2. *Saturation isotherms obtained in vivo after a 20-min labeling period.*

Each curve represents the computer-generated fits of the data using the three binding site model (see Materials and Methods). Each point represents the mean of duplicate determinations in a single rat: sufentanil (Δ), diprenorphine (*), and naloxone binding (O), respectively. The lines represent the sufentanil (····), diprenorphine (– – –), and naloxone (——) predicted values. Specific binding was derived by subtracting the model predicted values for the nonspecific binding from each experimentally determined data point.

Note that the computer-generated curves represent simultaneous fits obtained with the use of all binding data (see Table 3). Since each set of binding data was obtained on different occasions with different batches of rats, such fits may show more deviation for individual binding curves than fits that come from analyzing each binding curve separately. Moreover, the scatter of the data in the high dosage range is fairly large because of a relatively high ratio of nonspecific binding over specific binding. This is a particularly large problem for diprenorphine and sufentanil, for which small changes in nonspecific binding cause large changes in the estimate of total specific binding. Nevertheless, we feel that the computer-estimated values for total binding capacity largely overcome these problems by simultaneous data analysis and, thus, provide a best estimate of the *in vivo* binding parameters.

be presented elsewhere.) Since both [^{3}H]etorphine and [^{3}H]naloxone selectively label site 1 (*mu* site) and to a small extent site 2 *in vivo*, failure of these opioids to compete fully for [^{3}H]naloxone binding indicates lack of affinity for site 3 *in vivo*. Of the drugs tested, only the closely related analogues of naloxone, e.g., naltrexone and nalorphine, were capable of competing fully for [^{3}H]naloxone binding, whereas diprenorphine, buprenorphine (12), ethylketocyclazocine, cyclazocine, and sufentanil only partially prevented [^{3}H]naloxone binding *in vivo*.

Binding selectivity of etorphine, sufentanil, diprenorphine, and naloxone. Figures 4 and 5 illustrate part of the data used to estimate the binding parameters of several ligands to the three sites. Figure 4 shows the decrease in the binding of [^{3}H]sufentanil, [^{3}H]naloxone, and [^{3}H]diprenorphine when co-administered with increasing doses of unlabeled naloxone. The ability of sufentanil to compete for the binding of [^{3}H]sufentanil, [^{3}H]etorphine, and [^{3}H]naloxone is depicted in Fig. 5. Because the fraction and amount of the tracer entering the brain varied with each experiment, the actual binding is a function of both competitor dose and the amount of tracer in the brain. Both of these independent variables were used to predict binding. Therefore, the predicted binding cannot be readily shown in graphical form because of the separate variation in both of these dosing variables. For the purposes of illustration, the data in Figs. 4 and 5 have been normalized to the amount of tracer in the cerebellum. Since the cerebellum does not contain the classical opiate receptors (10), it can be used as a reference to account for changes of the tracer pharmacokinetics (see legend to Fig. 4 for further discussion). The actual data points thus represent the ratio of [^{3}H]naloxone binding in the cerebrum over the total ^{3}H-labeled tracer concentration in the cerebellum. A similar cerebrum/cerebellum ratio approach has previously been used by Herz and co-workers (e.g., ref. 10) to normalize *in vivo* opioid receptor binding.

Compared with its ability to compete with itself, naloxone is more potent in competing for sufentanil but less potent in competing for diprenorphine (Fig. 4). The lower potency of naloxone in competing for [^{3}H]naloxone as compared with [^{3}H]sufentanil stems from the substantial binding of naloxone to site 3 with lower affinity than to site 1, while sufentanil only binds to site 1. Similarly,

TABLE 3

In vivo binding ligand combinations

The estimates of the variance scale parameters (see Eq. 3) are shown for each experiment. Numbers in parentheses indicate the number of observations in each data set.

Displacer	[^{3}H]Etorphine	[^{3}H]Sufentanil	[^{3}H]Diprenorphine	[^{3}H]Naloxone
Etorphine	0.0514 (54)	—	0.2140 (15)	—
Sufentanil	0.0449 (18)	0.0072 (16)	—	0.0490 (23)
Diprenorphine	0.0216 (16)	—	0.0239 (20)	—
Naloxone	0.5918 (20)	0.0143 (15)	0.1336 (18)	0.0199 (21)
Naloxone + 2 mg/kg diprenorphine	—	—	—	0.019 (19)

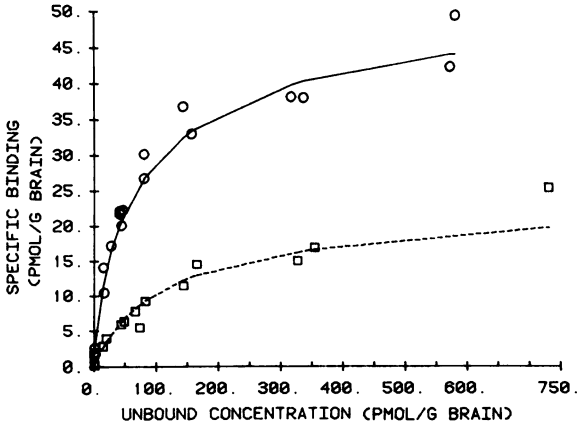

FIG. 3. *Naloxone saturation binding curves obtained in vivo after a 20-min labeling period.*

The lines represent the predictions by the three binding site model. Each point represents the mean of duplicate determinations in a single rat: naloxone binding alone (O——O) and naloxone binding in the presence of a 2 mg/kg diprenorphine dose (□- - -□). Specific binding was determined as in Fig. 2.

sufentanil is less potent in competing for naloxone and etorphine as compared with its ability to compete with itself (Fig. 5). A 1.5 mg/kg dose of sufentanil reduces etorphine and naloxone only to 16% and 26% of control binding, respectively, while it fully prevents [^{3}H]sufentanil binding. Higher doses of sufentanil could not be tested since the rats generally did not survive sufentanil doses exceeding 2.5 mg/kg for the duration of the receptor labeling period.

The selectivity of the competing ligands for the binding sites labeled by the tracers is obtained primarily from analysis of the cross-competition curves. Visual examination of Fig. 4 reveals that naloxone is, therefore, most selective for the binding site labeled by sufentanil, as it competes with this ligand with the highest potency. Similarly, sufentanil is apparently most selective for the binding sites labeled by itself (Fig. 5). The actual selectivity of a ligand for a binding site, however, is obtained by comparing the apparent equilibrium dissociation constants for a given ligand to the various binding sites, and estimates of the dissociation constants of the four ligands for the three types of sites are summarized in Table 5. Sufentanil is highly selective for the first type of binding

TABLE 4

Reduction of [^{3}H]naloxone tracer (143 μCi/kg = 1 μg/kg) binding in vivo by unlabeled opioid drugs in doses that fully inhibit [^{3}H]etorphine binding

	Dose	Reduction of [^{3}H]naloxone binding
	mg/kg	%
Diprenorphine	2	89
Ethylketocyclazocine	25	83
Cyclazocine	10	85
Sufentanil[a]	1	75
Naltrexone	10	99
Nalorphine	20	98
Naloxone	10	100

[a] This dose of sufentanil also failed to displace [^{3}H]etorphine binding completely (~85% of control; see Fig. 5).

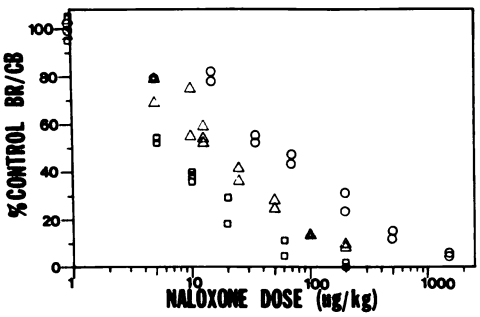

FIG. 4. *Ability of naloxone to compete for various ligands in vivo.*

Each point represents the mean of duplicate determinations in a single rat. The data show naloxone's ability to compete for sufentanil (□), naloxone (△), and diprenorphine (○) tracer, respectively. The competitor was co-administered with the tracer, and sacrifice occurred after a 20-min labeling period. The ordinate represents the percentage of control (tracer only) specific binding. The range of control values is indicated on the *Y*-axis. All data points have been normalized to the amount of radioactivity in the cerebellum to account for the tracer pharmacokinetics in the brain (see text). This is based on the assumption that the cerebellum does not contain high-affinity, saturable opioid receptor sites, which is correct for sufentanil and diprenorphine but not for naloxone, which binds to *lambda* sites in the cerebellum (30). However, this binding of [^{3}H]naloxone represents less than 10% of total [^{3}H]naloxone in the cerebellum, so that the maximal error introduced by normalizing the data relative to the cerebellum content is 10%. This value represents a negligible error for the purpose of the figure, and it does not enter into the computer-estimated binding parameters. Also, nonspecific binding, defined as the difference in the presence and absence of a 1.5 mg/kg naloxone dose (□), or a 10 mg/kg naloxone dose (△, ○), was subtracted from the observed binding. The control (100%) represents the ratio of [pmoles [^{3}H]tracer bound per g cerebrum − nonspecific binding]/[pmoles [^{3}H]tracer per g cerebellum], when no unlabeled competitor is co-administered.

site, having virtually no affinity to sites 2 and 3. Similarly, etorphine is selective for the first type of binding site, although not nearly as selective as is sufentanil. Diprenorphine exhibits ~4-fold selectivity for site 1 over

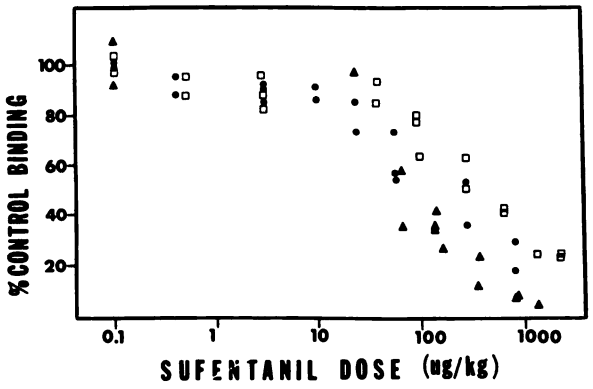

FIG. 5. *Ability of sufentanil to compete for various ligands in vivo.*

Each point represents the mean of duplicate determinations in a single rat. The data show sufentanil's ability to compete for sufentanil (▲), etorphine (●), and naloxone (□) tracers, respectively. The competitor was co-administered with the tracer, and sacrifice occurred after a 20-min labeling period. The ordinate represents the percentage of control (tracer only) specific binding. All data points have been normalized to the amount of radioactivity in the cerebellum to account for the tracer pharmacokinetics in the brain (see text and Fig. 4). For the purposes of the figure, nonspecific binding, defined for each tracer as the difference in the presence and absence of a 10 mg/kg naloxone dose, was subtracted from the observed binding.

TABLE 5

Binding selectivity for the three receptor sites differentiated in vivo

The dissociation constants for each drug at the three binding sites were estimated using the data sets in Table 3 and Eqs. 1, 2, and 3.

Drug	K_1[a]	k_2	K_3
	μg/kg	μg/kg	μg/kg
Sufentanil	69	>70,000	>70,000
Etorphine	28	650	>70,000
Diprenorphine	6.8	25	>70,000
Naloxone	7.1	108	36

[a] K represents the s.c. drug dose that occupies 50% of the respective binding site population at 20 min after the dose.

site 2, but also binds to site 2 with a high affinity (K_2 = 25 μg/kg). Naloxone is most selective for site 1, exhibiting ~15-fold selectivity for the first binding site population as compared with site 2. Of the four ligands employed, naloxone is the only one that binds appreciably to site 3, and it binds to site 3 (K_3 = μg/kg) with a 3-fold selectivity for this site over site 2 (K_2 = 108 μg/kg).

Nonspecific binding and variance parameter estimates. The nonspecific binding was constant for all diprenorphine and sufentanil experiments and was equal to 3.8% and 7.2% of the unbound concentration, respectively. The nonspecific binding of naloxone was 1.8% of the unbound concentration in all experiments except those in which the labeled ligand was administered in the presence of 2 mg/kg diprenorphine, where the value was 2.4%. The nonspecific binding was constant for etorphine when etorphine and diprenorphine were the unlabeled ligands and was equal to 3.3% of the unbound concentration; the value increased to 8.2% and 18.3% when naloxone and sufentanil, respectively, were the unlabeled ligands. Separate NS_L parameters were estimated for a given ligand when the indicated ligand combinations were obtained with different tracer batches.

The variance scale parameter estimates are listed in Table 3 for each ligand combination. The size of the variance scale parameter is an indication of the relative goodness of fit of each data set. That is, the best "fits" of the data to this model occur for those data sets having the lowest variance scale parameters. The estimate of the power Z was 1.84.

DISCUSSION

We have simultaneously analyzed saturation and cross-competition binding data obtained *in vivo* using sufentanil, etorphine, diprenorphine, and naloxone. There are several lines of evidence which point to the existence of multiple binding sites *in vivo*: we have previously shown that etorphine labels a subset of sites labeled by diprenorphine (8). Furthermore, we have shown that naloxone labels a binding site population distinct from that labeled by diprenorphine (12). On the basis of these observations, at least three types of opiate binding sites appear to exist in the rat brain *in vivo* with different binding affinities for the ligands employed in this study; therefore, no attempts were made to fit the data to a two binding site model. However, attempts were made to fit the data from the experiments listed in Table 3 to a four binding site model, and the fit was not significantly improved over that for a three binding site model, indicating that the three binding site model is adequate. This does not rule out the possibility of the existence of a fourth binding site *in vivo*; rather, the ligands employed here do not allow us to discriminate an additional type of binding site.

We have shown that a linear relationship between administered dose and unbound brain concentration (Fig. 1; Table 3) exists for the four ligands studied here. The linearity observed for these ligands is not surprising, however, when one considers that the amount of drug bound to high-affinity sites is very small in comparison to that administered (<1% of the dose); in this case it is to be expected that the unbound concentration is directly proportional to dose at a time when concentrations are largely determined by distribution phenomena. Moreover, the linear relationship between dose and unbound drug concentration in the brain suggests that the assumption is valid that low-affinity drug binding to general tissue molecules is linear and not saturable over the concentrations used.

The properties of the first type of binding sites (B_{max}^1 = 18 pmoles/g of brain) resemble those of the *mu* receptor, i.e., the high selectivity (K_2/K_1 ~ 1500) of the *mu*-specific ligand sufentanil (23), and the high affinity (K_1 = 7 μg/kg) and selectivity (K_2/K_1 ~ 15) of the *mu*-selective antagonist naloxone (24) for this binding site population. *In vivo*, etorphine behaves as a site 1-selective ligand. This result contrasts with the finding by Chang and Cuatrecasas (6) that etorphine is equipotent in competing for ligands binding to *mu* and *delta* binding sites in rat membrane preparations and behaves as a universal ligand for the *mu*, *delta*, and *kappa* sites in homogenates of guinea pig brain (25). However, more recently Wood *et al.* (5) and Rothman and Westfall (26) found that etorphine is, in fact, *mu*-selective in rat brain membranes. On the other hand, etorphine binds to an additional type of site, site 2, as shown by its ability to completely block [^{3}H]diprenorphine binding *in vivo*, at suitably high doses (8). Thus, etorphine's behavior *in vivo* (K_2/K_1 ~ 20) is compatible with its moderately *mu*-selective binding properties *in vitro* (5). Diprenorphine is thought to be a universal ligand for the opiate receptor system, binding with equal affinity to the *mu*, *delta*, and *kappa* binding sites *in vitro* (27). Since diprenorphine also binds to site 2 with relatively high affinity *in vivo*, it may therefore be surmised that site 2 represents a mixture of the *delta* and *kappa* sites. With the present data it is not possible to differentiate the *delta* and *kappa* sites which may comprise site 2, and additional studies with *delta*- and *kappa*-selective ligands are needed.

An additional binding site for naloxone has previously been demonstrated by others *in vitro* (28, 29). However, this naloxone *in vitro* binding site displayed rather low affinity relative to the *mu* sites and was, therefore, largely ignored subsequently. We have previously demonstrated that a substantial fraction of naloxone bound *in vivo* cannot be fully competed for by doses of buprenorphine that saturate the diprenorphine binding sites *in vivo* (12). Therefore, this residual naloxone site (site 3) does not resemble the known *mu*, *delta*, and *kappa* sites. The present study further characterized the naloxone-selective site 3 by providing an estimate of the concentration of binding sites (20 pmoles/g of brain) as well as its

affinity for naloxone *in vivo*. Surprisingly, naloxone displays a greater affinity at site 3 than at the putative *delta* and *kappa* sites (site 2). Moreover, its selectivity for site 1 over site 3 is only 5-fold, whereas *in vitro* a much greater selectivity was observed (28–30). These results clearly demonstrate that *in vivo* and *in vitro* binding characteristics can differ substantially from each other. Further experiments on the nature of the *in vitro* binding behavior of naloxone at site 3 have shown that this binding site is extremely labile *in vitro*, as it is only observable in a high-affinity form at low temperatures immediately after sacrifice of the animals and brain homogenization (30). Furthermore, binding site 3 (now termed the *lambda* site) (30) appears to be selective for those opiates in the 4,5-epoxymorphinan structural class, e.g., naloxone, morphine, naltrexone, and nalorphine. The identity of site 3 observed here *in vivo* and the *lambda* site observed *in vitro* (30) in fresh brain homogenates is strongly suggested by the results presented in Table 4. Only naloxone, naltrexone, and nalorphine were capable of fully reducing *in vivo* binding of [^{3}H]naloxone, a result that is identical with the binding pattern of opioids to the new *lambda* site *in vitro*. Accordingly, diprenorphine is unable to compete completely for [^{3}H] naloxone binding at doses well beyond those which saturate diprenorphine binding sites *in vivo* (2 mg/kg). We therefore recommend that diprenorphine no longer be considered a "universal ligand" for the system of opioid binding sites in rat brain. The pharmacological significance of the third type of binding site (i.e., site 3 = *lambda* site) remains unknown at present. Nonclassical naloxone effects that may be linked to the *lambda* site include reversal of strokelike symptoms, shock syndrome, and other effects of naloxone *in vivo* and *in vitro* (30).

Analyses such as the one described here enable us to determine the binding constants of a series of opioid ligands to the three types of binding sites in the intact rat brain, circumventing any modifications that might take place *in vitro* (8, 9). Nevertheless, one must consider that the data analysis rests on two major assumptions, namely, that equilibrium has indeed been established *in vivo* and that the law of mass action applies. Although the results are self-consistent, these two assumptions cannot be readily tested by the present method, unless further tracers with yet different relative affinities to individual receptor types are introduced to the data analysis. Thus the current study must be viewed as a first step in quantitatively determining drug interactions with multiple *in vivo* binding sites, which must be validated in the future by independent approaches.

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