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Opiate receptor binding in vivo and the correlation with pharmacological activity

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OPIATE RECEPTOR BINDING IN VIVO AND THE CORRELATION WITH PHARMACOLOGICAL ACTIVITY

by

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DISSERTATION

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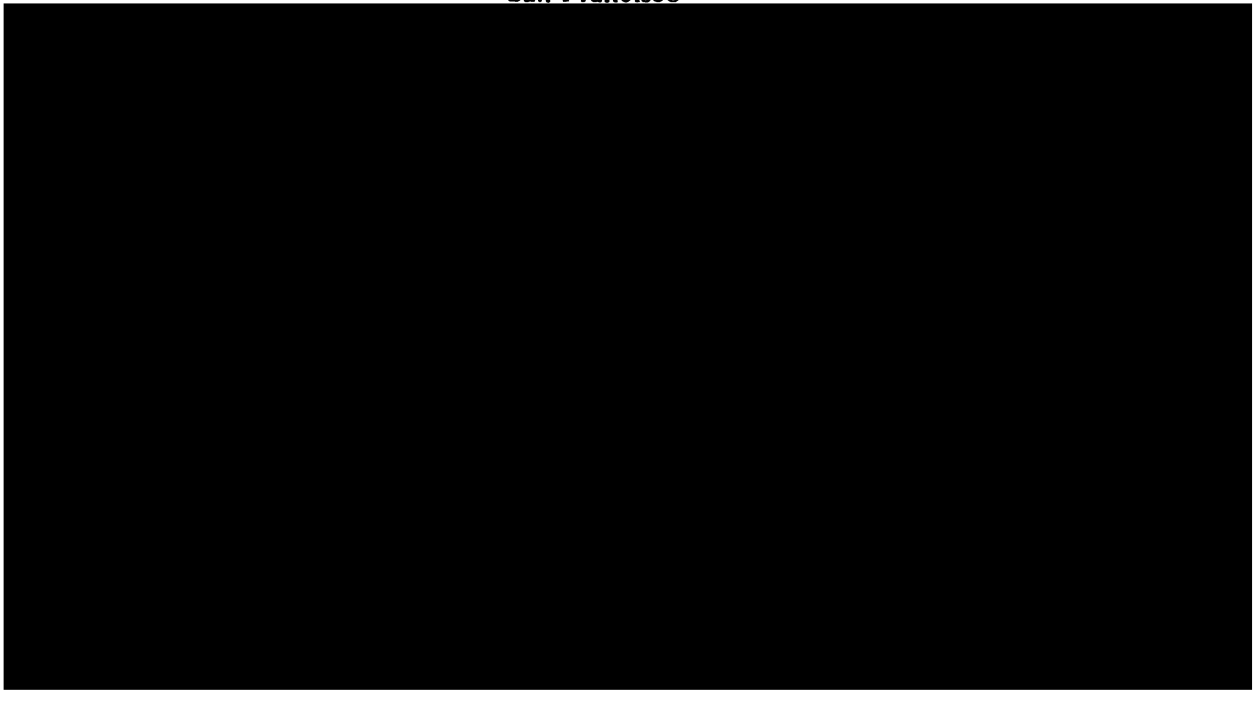

To my father, **HAROLD M. ROSENBAUM**, who taught me the difference between aleph-null and infinity, and to my mother, **DOROTHY S. ROSENBAUM, Ph.D.**, who taught me how to make chocolate chip cookies.

ABSTRACT

The relationship between opiate receptor binding and pharmacological effect was investigated in the intact rat. The *in vivo* model was chosen in order to understand the direct relationship between receptor binding and pharmacological effect and to circumvent the modifications which were demonstrated to occur to the receptor and its environment *in vitro*.

Bound drug in the rat cerebrum was measured after administration *in vivo* using a rapid membrane filtration technique. The agonists etorphine and sufentanil and the antagonists diprenorphine and naloxone were administered subcutaneously in both saturation and cross competition experiments, and the binding data were analyzed simultaneously using an extended least squares nonlinear regression program. Three classes of binding sites were discriminated with concentrations of 18, 15, and 10 pmol/g brain.

Site 1 binds sufentanil with ~ 1200-fold greater affinity than site 2. Etorphine and naloxone exhibit ~ 20-fold and 15-fold selectivity, respectively, for site 1 over site 2. This binding site resembles the μ binding site previously characterized *in vitro*. Diprenorphine binds to both site 1 and site 2 with a slight (~ 3.7-fold) selectivity for the former. Since site 1 resembles the μ sites, site 2 may represent a mixture of the δ and κ sites. Site 3 does not resemble any of the known opiate binding sites; it displays relatively high affinity for naloxone but lacks affinity for etorphine, sufentanil, and diprenorphine.

Both agonists produce analgesia in the rat tail flick test at the same (~ 2%) low fractional occupancy of site 1 whereas they display lower and largely different occupancies at site 2. Both antagonists reduce the potency of the agonists by 50% at a 50% occupancy of only site 1. Hence, these drugs probably exert their pharmacological effects at site 1 (μ sites) which may therefore be regarded as the receptor mediating analgesia in this test.

Examination of the in vivo μ site occupancy for a series of opiate mixed agonists-antagonists indicates that the activity of these drugs in three pharmacological test systems (i.e. thermal and pressure tail stimuli, paw pressure, and antiwrithing) cannot be explained on the basis of μ site interaction alone. Mixed agonist action can most easily be explained if the concepts of partial agonism and interaction with multiple receptor types are invoked.

In summary, this dissertation establishes certain criteria that facilitate an understanding of the relationships between drug dosage, opiate receptor binding, and pharmacological effects.

ACKNOWLEDGMENTS

While this dissertation has only one author on the front cover, I think that it is important for the reader to know that this work represents the culmination of my interaction with a number of individuals. The easiest way to acknowledge all these important people is to start at the beginning, so here goes:

I would like to thank all those who made the lab such a pleasant place in which to work; whose interactions were social and often emotional as well as scientific: in particular Drs. David Perry, Jonathan Maybaum, Marvin Cohen, and Joachim Grevel; an additional thanks to Mark Richards for his help in the final experimental push, and to both Joachim and Mark for listening and tolerating my ups and downs during the writing process.

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To my research advisor, Dr. Wolfgang Sadée, a very special thanks for all that you have taught me and for your friendship. I will always consider you as my mentor.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

A. OVERALL OBJECTIVE

The overall objectives of this dissertation are to determine: if there are any differences in opiate receptor binding properties *in vivo* and *in vitro*; which of the multiple opiate binding sites previously demonstrated *in vitro* exist in the intact rat; and which of these binding sites mediates analgesia.

B. BACKGROUND: OPIATES AND THE OPIATE RECEPTOR IN THE CNS

Pharmacology of the Opiates

The pharmacological properties of the opioids are diverse, and depend on the class (agonist, mixed agonist, or antagonist) of opioid investigated. The major effects of the opioid agonists result from their interactions with receptors in the central nervous system and the bowel. These include analgesia, respiratory depression, decrease in gastrointestinal motility, drowsiness, euphoria and mood alterations, and the induction of tolerance and physical dependence.[31] Opioid antagonists reverse or prevent the above effects, and will precipitate withdrawal in an opiate-dependent subject. Opioids which are classified as mixed agonists-antagonists exhibit both agonistic and antagonistic properties, depending on the dosage used, and the agonist or antagonist with which they are administered. Many of these drugs have a lower

addiction potential while exhibiting "morphine-like" analgesic effects; some mixed agonists-antagonists also produce psychotomimetic effects in humans and dysphoric effects in animals.[16]

Biochemical Demonstration of the Opiate Receptor

The first definitive demonstration of distinct opiate binding sites occurred in 1973, a result of investigations by three different laboratories.[1-3] The binding was saturable, stereospecific, and specific for narcotic analgesics and antagonists. The binding sites were determined to be the receptor responsible for opiate effects, as the rank order of affinities of a series of opiates correlated with their relative analgesic potencies (e.g.[4]). Opiate binding is sensitive to effects of protein modifying reagents, phospholipase, temperature, and the presence of mono- and divalent cations.[5]

A comparison of the *in vitro* receptor binding behavior of opiate agonists and antagonists led to the observation that Na^+ reduced agonist binding and enhanced antagonist binding.[6] Na^+ appears to affect the affinity of the opiates for the receptor rather than the number of binding sites, although the precise reason for the "sodium effect" remains controversial.[5] The "sodium ratio" (i.e. the ratio of the affinity of the drug in the presence to that in the absence of 100mM NaCl) has been used as a prediction of the relative agonist and antagonist properties of opiates *in vivo*,[7] although this is not an infallible indication of opiate activity.[5] In an effort to explain the difference in the binding properties of the opiate agonists and antagonists, the opiate receptor was envisioned as existing in

equilibrium between the "agonist" and "antagonist" conformation. These two conformations were theorized to be interconvertible with Na^+ favoring the antagonist conformer.[7]

Opiate Receptor Multiplicity

The concept of opiate receptor multiplicity was initially formulated by Matin[8,9] in order to explain the different pharmacodynamic profiles of a series of opiate agonists and mixed agonist-antagonists in dogs. Studies on the discriminative stimulus effects of opiate agonists[10] and the ability of various opiate agonists to produce tolerance and cross-tolerance[11] also support this concept.

In vitro binding studies point to the existence of multiple binding sites in the CNS, and have resulted in the differentiation of at least four distinct opiate binding sites. The μ binding site is selective for the opiate alkaloids, while the δ site selectively binds the opiate peptides (i.e. enkephalins).[12] The κ site is thought to be selective for opiates in the benzomorphan structural class;[13] the endogenous ligand for this binding site appears to be dynorphin.[14,15] At present, it is debatable as to whether the σ binding site, selective for n-allylnormetazocine (SKF 10,047) is actually an opiate binding site, and/or receptor,[16] as this binding site appears analogous with the phencyclidine receptor in rat brain.[16,17] An additional type of binding site, the ε site, has also been postulated; this is the site at which β-endorphin is thought to exert its action. However, this site has only been demonstrated in the rat vas deferens preparation.[18]

The concept of opiate receptor multiplicity is also reinforced by data obtained from studies using isolated peripheral organ preparations such as the guinea pig ileum (GPI), mouse vas deferens (MVD), and rat vas deferens (RVD). Since striking differences in the rank order potencies of various opioids to inhibit electrically stimulated contractions are observed in the GPI and MVD, these preparations are thought to contain different receptor populations.[68] The rank order potencies seen in these preparations are similar to those observed for the various binding sites seen in the CNS: The GPI is highly responsive to morphine whereas it is much less sensitive to the enkephalins. Hence, this preparation is thought to predominately contain μ receptors.[68] However, this preparation has also been shown to contain κ receptors.[14,15] In contrast, the mouse vas deferens preparation is much more responsive to enkephalins than to the opiate alkaloids; this preparation is therefore thought to contain δ receptors.[68] Moreover, the receptors is the RVD appear to be different than those in the GPI and MVD; that is, this preparation is more responsive to β-endorphin than it is to either morphine or the enkephalin pentapeptides; in fact, the RVD is relatively non-responsive to these latter agents.[68]

The classification scheme that results from these <u>in vitro</u> investigations differs somewhat from that proposed by Martin, whose classification (discussed below) is based solely upon pharmacological criteria in the intact animal. He has postulated three receptor types, μ, κ, and σ, whose prototypic ligands are morphine, ketocyclazocine, and SKF 10,047, respectively.[8]

Pert's group has classified the opiate receptor system somewhat differently, based on the sensitivity of opiate receptor binding to

inhibition by GTP or the non-hydrolyzable analog GPP(NH)P. Hence, Type 1 sites are GTP-sensitive (i.e., agonist binding is decreased by GTP) and adenylate-cyclase coupled whereas Type 2 sites are GTP resistant and uncoupled to adenylate cyclase.[19] Type 1 sites exhibit brain distribution and binding characteristics similar to the μ sites, and Type 2 sites resemble the δ sites.[19] Depending upon incubation conditions in vitro, the μ and δ sites appear to be interconvertible as regards their ligand-binding characteristics.[20] Moreover, the Type 1 (GTP-sensitive) receptor is thought to consist of the μ-agonist, μ-antagonist, and δ-agonist conformations; modification of key sulfhydryl groups can "freeze" the receptor into either of these states.[21]

The Opiate Receptor and Adenylate Cyclase

The opiate receptor system is thought to be coupled to a variety of effectors: opiate administration results in changes in the cellular movement and content of Ca^{2+},[31,122] inhibition and/or alteration in neurotransmitter release,[31] alterations in ATPase activity,[122] and an inhibition of adenylate cyclase in certain systems.

The biochemical characterization of the opiate receptor-adenylate cyclase interaction has been most extensively studied in the NG108-15 cell system, and it has been demonstrated that both Na^{+} and guanine nucleotide are required for opiate agonist mediated inhibition of cyclase activity.[22,23,24]

The adenylate cyclase system is composed of at least three parts: the receptor protein, a nucleotide binding protein (N), and the

depending upon the receptor system and whether it couples to an inhibitory (N_i) or stimulatory (N_s) N protein, when these three units are coupled to one another.[24,25] The observation that GTP is required for opiate agonist inhibition of cyclase activity, and that the non-hydrolyzable analog of GTP (GPP(NH)P) as well as GTP-S and NaF eliminate this inhibition has led to the hypothesis that opiates mediate cyclase activity indirectly *via* an N unit.[26] Alternately, it has been proposed that opiates inhibit adenylate cyclase *via* stimulation of a GTPase which is involved in the "turn-off mechanism" of the enzyme[27,28] rather than *via* interaction with the N_i protein. However, it has also been proposed that the negative effect of GTP on opiate receptor binding is due to an allosteric effect of GTP directly on the opiate receptor.[29]

The affinity of opiates for the various binding sites is differentially affected by guanine nucleotide; binding to the μ sites appears to be more profoundly regulated by GTP than is binding to the δ sites.[30] This is consistent with the initial classification of the opiate receptor into Type 1 and Type 2 sites,[19] although Type 1 sites are now thought to include the δ agonist conformation as well.[21] The relationship between the coupling of the receptor to adenylate cyclase (by whatever mechanism) and opiate analgesia has not been established.

Functions of the Opiate Receptor Types

The functions of the opiate *receptor* types, as distinct from the *binding site* types, have been delineated by Martin, who based his classification of the opiate receptor system on pharmacological data. Hence, the "μ receptor" mediates supraspinal analgesia, respiratory

depression, euphoria, and physical dependence. The "κ receptor" mediates spinal analgesia, miosis, and sedation. The dysphoric effects of some opiates are thought to be mediated *via* the "σ receptor".[31]

The pharmacological significance of the opiate binding sites is not as clearly defined. Hence, it is not known if the μ site represents the "μ receptor" responsible for opiate analgesia, although there is suggestive evidence. Such evidence is based on the comparison of the rank order affinities of a series of drugs for the μ binding site *in vitro* and their potencies as analgesics *in vivo*.[e.g. 32,33] Similarly, the κ[34,35] and δ[36] binding sites have also been implicated in the mediation of analgesia produced by ligands which selectively bind to these sites. Pasternak has proposed that the μ binding site contains two sub-types, μ_1 and μ_2. The μ_1 has been suggested to be the common high affinity site which mediates both morphine and enkephalin analgesia,[37] while the μ_2 site has been implicated in the respiratory depressant actions of those agonists which bind selectively to the μ sites.[37,76,123] At present, the evidence for a single analgesic receptor or receptor sub-type is controversial. Furthermore, all conclusions regarding the functional significance of the various opiate binding sites are based on the indirect comparison of *in vitro* binding activity and *in vivo* pharmacological activity.

Limitations to the In Vitro Methodology

There are a number of questions regarding the drug-receptor interaction which are not readily addressed using the *in vitro* methodology alone. Sadée and co-workers[38,39] have demonstrated that the

receptor binding properties of the opiate agonist etorphine and the opiate antagonist diprenorphine are altered to some extent by *in vitro* homogenization and washing procedures.[38,39] A comparison of the receptor binding properties of opiates after administration to the intact animal (*in vivo*) and to the brain homogenate (*in vitro*) such as that discussed in Chapter I provides information as to the degree of alteration in the receptor environment by common *in vitro* work-up procedures. Due to these modifications *in vitro* it is not known which of the opiate binding sites demonstrated *in vitro* actually exist in the intact animal. Hence, comparisons of *in vitro* rank order binding site affinities and *in vivo* pharmacological potencies are not adequate to determine the binding site(s) which mediate(s) a given pharmacological effect. Moreover, the possibility that the multiple binding sites observed *in vitro* may be different conformations of a single receptor molecule[20,21] bears consideration. To avoid *in vitro* artifacts which may favor a specific conformation, binding can be analyzed *in vivo* in the presence of all the regulatory factors which promote the conformational changes. Therefore, any discrete conformations or sites are analyzed as they exist in the intact animal. However, it must be noted that the actual determination of the binding does take place *in vitro* (homogenized brain), and that one can never be entirely certain that modifications have not taken place during the homogenization process.

A number of studies have investigated opiate receptor binding *in vivo*.[e.g. 5,38-41] However, these previous studies have failed to address the question of opiate receptor multiplicity. Only by investigating opiate binding in the intact animal can the relationships

among drug dosage, receptor occupancy at each type of opiate binding site, and drug effect be established.

C. SPECIFIC AIMS

1. To compare the sensitivity of the opiate receptor to the effects of Na^+ and guanine nucleotides after *in vitro* and *in vivo* labeling, since these regulators are known to be necessary for the coupling of the receptor to adenylate cyclase.

2. To demonstrate and describe multiple binding sites in the intact rat in order to determine which of the binding sites demonstrated *in vitro* exist *in vivo*.

3. To determine which of the three opiate binding sites demonstrated *in vivo* mediate the analgesic actions of etorphine and sufentanil, two extremely potent opiate analgesics which exhibit very high affinity *in vivo*. (This very high affinity makes these drugs optimal for use in *in vivo* binding studies which by design require large amounts of tracer.)

4. To determine whether a series of opiate mixed agonists-antagonists exert their analgesic and antagonistic actions *via* interaction with one, or several, receptor sites *in vivo*.

CHAPTER I

OPIATE RECEPTOR BINDING AFFECTED BY GUANINE NUCLEOTIDE: PARTIAL LOSS OF SENSITIVITY TO GPP(NH)P EVIDENT IN VITRO

1. SUMMARY

The sensitivity of the receptor dissociation rate of the opiate agonist [^{3}H]etorphine to the effects of GPP(NH)P and Na^+ was measured in washed rat brain membrane homogenates after *in vitro* labeling and in fresh rat brain homogenate after *in vivo* labeling. Comparison to the previously measured rapid *in vivo* dissociation curve ($t_{1/2}$ ~ 50 sec)[42] revealed that brain homogenization and membrane washing procedures significantly prolonged the dissociation rate, even when measured in the presence of Na^+ and guanyl nucleotide. The *in vivo* dissociation rate could only be reproduced *in vitro* when labeling occurred *in vivo* and brain homogenization occurred in the presence of these regulatory factors. The prolonged *in vitro* [^{3}H]etorphine dissociation curve was predominately a result of a decreased sensitivity to guanine nucleotide rather than to Na^+. These data suggest that partial functional uncoupling of the opiate receptor-effector system may occur *in vitro*.

2. INTRODUCTION

It is currently believed that the opiate receptor system is coupled to the adenylate cyclase system, and that both Na^+ and guanine nucleotide are required for opiate mediated inhibition of cyclase activity.[22,23,24] Previous work with the adenylate cyclase coupled

β-adrenergic receptor shows that the in vitro preparative procedures result in a disrupted coupling of the receptor to the adenylate cyclase (effector) system.[43,44] This has been demonstrated as a decrease in the high coupling efficiency of agonists in isolated membranes relative to that in whole cells,[43] and as a decrease in agonist affinity for the receptor evident in whole cells relative to cell homogenates.[44] Moreover, we have recently observed that the in vivo receptor dissociation rate of the opiate agonist [^{3}H]etorphine is considerably faster than in vitro rates, even in the presence of added Na^+ and guanyl nucleotides. These data suggested a change in the sensitivity of etorphine binding towards the combination of guanyl nucleotides and Na^+ in vitro, an event that might reflect a loss of the high coupling efficiency of etorphine observed in vivo.[42]

This chapter demonstrates that common in vitro preparative procedures such as brain homogenization and membrane washing result in a partial loss in sensitivity to the effect of guanine nucleotides on opiate agonist binding.

3. MATERIALS AND METHODS

3.1 Materials

[15-16-^{3}H]Etorphine (60 Ci/mmol) was purchased from Amersham, Arlington Heights, IL. GPP(NH)P* was purchased as the tetralithium salt from Boehringer Mannheim, Indianapolis, IN. D-Ala^2-D-Leu^5-enkephalin 3

*Abbreviations used: GPP(NH)P: guanosine-5'-(β,γ-imido)triphosphate, DADL: D-Ala^2-D-Leu^5-enkephalin, DHM: Dihydromorphine.

as purchased from Peninsula Laboratories, Inc., San Carlos, CA. Dihydromorphine and Etorphine HCl were generously supplied to us by the NIDA, Rockville, MD.

All tracers were purified when purity was below 90% using an HPLC method described elsewhere.[42] Male Sprague Dawley rats (100-150g) were obtained from Simonsen, Gilroy, CA.

3.2 Methods

3.2a Tracer Dissociation in Washed Membranes

Washed rat brain membrane homogenates were prepared as described previously.[45] After incubating with [^{3}H]etorphine (7.7×10^{-11} to 1.65×10^{-10}M) ± 10^{-6}M cold etorphine for 45 min at a Tris buffer homogenate dilution of 1:17 for a 1.5 g brain, aliquots of washed membrane homogenate were diluted in Tris buffer to a final dilution of 1:350 and dissociation initiated at 37°C in the presence of 10^{-6}M unlabeled etorphine, as described previously.[42] Additions of NaCl and GPP(NH)P were made to the dilute homogenate as indicated in each experiment. Residual binding was measured by rapid filtration of the homogenate through Whatman GF/B filters.[42]

The initial binding (binding at time 0, prior to the initiation of tracer dissociation) was assessed either by filtering the concentrated homogenate (1:17 dilution) directly prior to the initiation of dissociation or by computer fitting the subsequent data points to a biexponential equation and thereby obtaining a fitted value for the initial binding (zero point). In all cases, the initial binding results

in an occupancy of 2-10% of the total receptor population for [^{3}H] etorphine (.54 - 1.9 pmol bound/g brain).[42] Nonspecific binding was subtracted from all values and was defined as the percent of total radioactivity bound in the presence of 10^{-6}M unlabeled etorphine.

3.2b Tracer Dissociation in Untreated Membranes:

Tracer dissociation following *in vivo* labeling was determined immediately after sacrifice of the animals in order to minimize any changes that might occur to the receptor system *in vitro* according to the method of Perry et al.[42] Briefly: Male Sprague Dawley rats, 120-150g were injected with 100-200 µCi/kg [^{3}H]etorphine and sacrificed after 20 min. A 160 µCi/kg (1.1 µg/kg) dose of etorphine results in a total brain tracer concentration of approximately 4.5×10^{-10}M, ~ 50% of which (.21 pmol/g brain) is specifically bound.[42]

3.2c Computer Analysis of the Dissociation Curves:

The dissociation curves of [^{3}H]etorphine in the presence of 100 mM NaCl and 50 µM GPP(NH)P were analyzed using the FITFUN Program of the Prophet Computer System.[46]. All curves were fitted to the following biexponential equation: $Y = B_1 \cdot EXP(-k_1 \cdot x) + B_2 \cdot EXP(-k_2 \cdot x)$, where x = time after dilution and the ordinate values are % initial binding. In this case, the computer generated constants B_1 and B_2 correspond to the proportion of binding sites dissociating with a half-life described by the rate constants k_1 and k_2, respectively. Initial binding is defined as the sum of B_1 and B_2.

4. RESULTS

4.1 Analysis of the Receptor Dissociation of [^{3}H]Etorphine in the Presence of NaCl + GPP(NH)P Under Various Labeling and Homogenization Conditions

Figure 1 demonstrates clear differences in the [^{3}H]etorphine off-rate under different labeling and homogenization conditions. The slowest rate is obtained when labeling takes place in washed membranes. This rate is accelerated when labeling occurs *in vivo* and the dissociation is observed in crude homogenates immediately after sacrifice and brain homogenization. A further increase in the dissociation rate occurs when labeling takes place *in vivo* and the brain is homogenized in 50μM GPP(NH)P and 100mM NaCl immediately after sacrifice. There were no differences in the dissociation curves obtained after *in vivo* labeling, however, when GPP(NH)P and NaCl were added either alone or in combination to the ice cold Tris buffer in the initial homogenization step (data not shown).

The results of computer fitting of the curves in Fig. I-1 to a biexponential decay expression are indicated in Table I-1. The computer-generated values indicate a substantially longer dissociation half-life of both the slow and fast phases of the curve after *in vitro* relative to *in vivo* receptor labeling conditions. Furthermore, there is an additional decrease in half-life of the slow phase of the curve from 13 to 4 min when the brain is homogenized in Na^+ + GPP(NH)P. The half-life of the fast phase is approximately 1 min in both cases, corresponding to the dissociation half-life of ~ 50 sec. for [^{3}H]etorphine *in vivo*.[42]

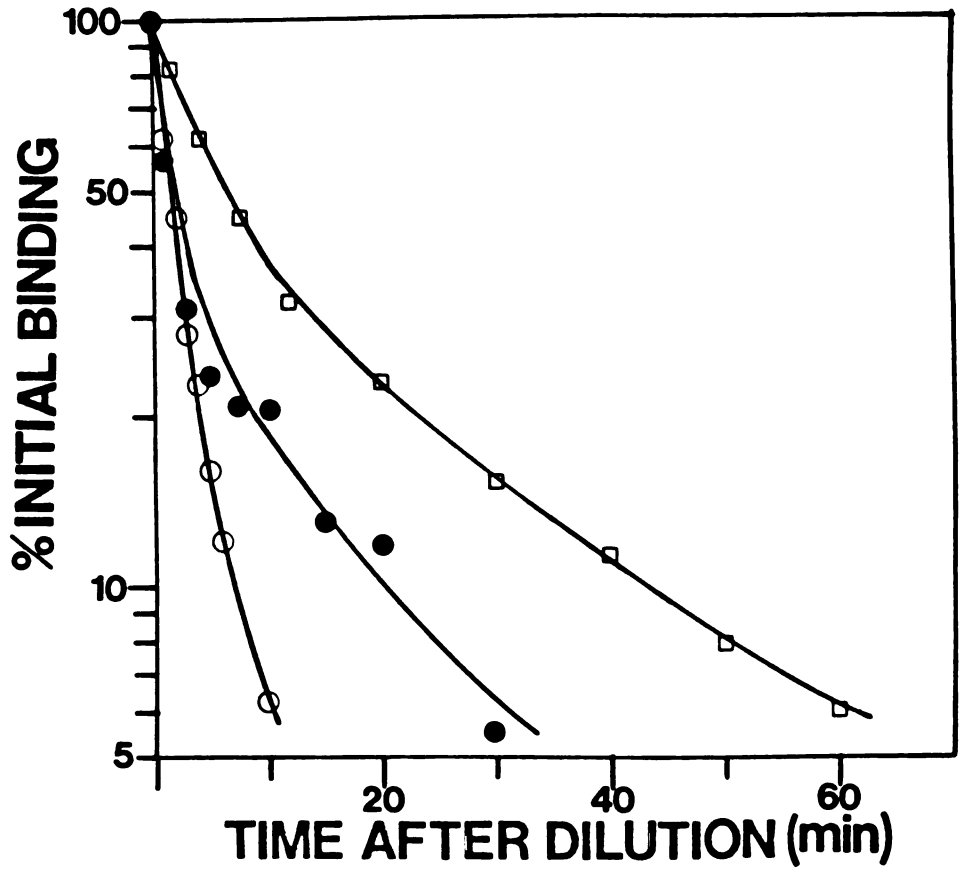

Figure I-1. Effect of different labeling and homogenization conditions on the dissociation of [^{3}H]etorphine in the presence of 100 mM NaCl + 50 M GPP(NH)P. Dissociation was measured in vitro at 37°C in the presence of 10^{-6}M unlabeled etorphine, 100 mM NaCl and 50 M GPP(NH)P at a 1:350 homogenate dilution.

□): Labeling occurred in vitro in washed membranes for 45 min with a tracer concentration of 1.65×10^{-10}M. Initial binding is determined from the computer fitted parameters (see text).

●): Labeling occurred in vivo using a 160 Ci/kg [^{3}H]etorphine dose over 20 min. Initial binding is determined directly in the concentrated homogenate.

○): Labeling occurred in vivo using a 200 Ci/kg [^{3}H]etorphine dose over 20 min. In this case the brains were homogenized in ice cold Tris buffer + 50 M GPP(NH)P + 100 mM NaCl. Initial binding is determined directly in the concentrated homogenate. Each curve was replicated once with similar results.

Table I-1: Computer Fitted Determinations of the Constants in the Following Biexponential Equation:

$$Y = B_1 \cdot EXP(-k_1 \cdot X) + B_2 \cdot EXP(-k_2 \cdot X)$$

Binding Condition	FITTED VALUES $B_1 \pm SD$	$k_1 \pm SD$	$B_2 \pm SD$	$k_2 \pm SD$	% Phases ± SD	Corresponding $t_{1/2}$ (min)	Mult r^2
IN VITRO*	41.50 ±1.59	.2039 ±.0148	27.82 ±1.87	.0139 ±.0020	59.87 ± 2.29 40.13 ± 2.70	3.40 49.86	.9999
IN VIVO	68.87 ±2.21	.9321 ±.0662	31.17 ±1.89	.0524 ±.0056	68.87 ± 2.21 31.17 ± 1.89	.744 13.23	.9995
IN VIVO Homogenize in NaCl + GPP(NH)P	70.38 ±27.86	.5932 ±.1721	29.25 ±28.15	.1653 ±.1167	70.38 ± 27.86 29.25 ± 28.15	1.17 4.19	.9991

*Extrapolated zero value = 69.32

The data were fitted to a biexponential equation using the 'FITFUN' program of the Prophet system (see text). The % of phases dissociating with the corresponding half-life is determined from the computer generated parameters B_1 and B_2, where initial binding is defined as $B_1 + B_2$. The dissociation half-life = .693/k. All dissociation curves are measured in the presence of 100mM NaCl + 50μM GPP(NH)P. Data used in the analysis are taken from Figure I-1.

4.2 Effect of Homogenization Conditions on [^{3}H]Etorphine Association In Vitro

The effect of Na^+ and GPP(NH)P on the in vitro [^{3}H]etorphine receptor equilibrium binding was determined by adding 2.7×10^{-11} to 1.6×10^{-10}M concentrations of the tracer to crude brain homogenate immediately after homogenate preparation. At a 1:350 Tris buffer homogenate dilution, the presence of 50μM GPP(NH)P plus 100 mM NaCl reduces [^{3}H]etorphine equilibrium binding to 60% of control. Moreover, when these regulatory factors are present in the initial homogenization step, the equilibrium binding of [^{3}H]etorphine in the presence of GPP(NH)P + NaCl is further reduced to 40% of the control value. (Control = 6.2 pmol/g brain specifically bound). This finding parallels the changes in [^{3}H]etorphine receptor dissociation rates shown in Fig. I-1.

In order to test whether the effects of Na^+ and GPP(NH)P on [^{3}H]etorphine receptor binding kinetics change during the incubation of the crude membrane homogenate, the binding was determined 5 min after tracer addition to a 1:17 homogenate dilution. This homogenate dilution was chosen in order to increase the association rate and to allow rapid changes to be assessed. At this dilution tracer binding reaches a plateau after 10 min, at which time > 70% of the tracer is bound. The 5 min binding values are compared in Tris buffer alone and in the presence of 50μM GPP(NH)P + 100 mM NaCl (both present in the initial ice cold homogenate as well as in the incubation medium). The ratio of the binding values in the presence to that in the absence of these two factors (i.e. the "Na^+ + GPP(NH)P ratio")[47] is .417 $\pm$ .073 (n = 6) when

the tracer is added immediately after homogenization. However, the ratio increases to 1.16 ± .266 (n = 6) when the homogenate is preincubated at 37°C for 30 min prior to tracer addition. This result demonstrates that further changes in the sensitivity of the opiate receptor system to Na^+ and GPP(NH)P occur after homogenization during the incubation of the crude homogenate at 37°C.

4.3 [^{3}H]Etorphine Dissociation After the Labeling of Various Receptor Subpopulations In Vitro

Differences in the *in vitro* receptor dissociation of [^{3}H]etorphine after *in vivo* and *in vitro* labeling (Fig. I-1) could have arisen from differential labeling of opiate receptor subtypes under these conditions. Therefore, the receptor system was labeled with [^{3}H]etorphine (considered a universal ligand for the opiate receptor subsets *in vitro*)[13] in the presence of selective blocking agents in order to alter the labeling pattern. Under these conditions it is possible to observe different receptor subpopulations in washed membranes.

Prior to the initiation of dissociation, the tracer (5×10^{-9}M) was incubated at a 1:40 homogenate dilution either alone, or in the presence of 10^{-7}M D-Ala^2-D-Leu^5-enkephalin (DADL) (which blocks μ and δ sites[13,48] or in the presence of 5×10^{-9}M dihydromorphine (DHM) which selectively blocks μ sites.[13] At this homogenate dilution 14.5 pmol/g brain are membrane bound in the presence of [^{3}H]etorphine alone (control). This value decreases to 84% of control in the presence of 5×10^{-9}M DHM and to 16% of control in the presence of 1×10^{-8}M DHM in

two separate experiments. A 10^{-7}M DADL concentration reduced binding to 36% of control (average of two separate experiments). In all cases the shape of the [^{3}H]etorphine receptor dissociation curves observed at a 1:80 homogenate dilution in the presence of 100mM NaCl and 25μM GPP(NH)P is identical (data not shown). This result provides evidence against the argument that the differences observed in the dissociation of [^{3}H]etorphine after labeling in vivo and in vitro (Fig.I-1) are a result of selectively labeling a subset of the opiate receptor system.

4.4 Differential Effects of GPP(NH)P and Na^+ on Tracer Dissociation in Untreated and Washed Brain Homogenates

The dissociation of [^{3}H]etorphine in untreated and treated brain homogenates was examined in the presence of GPP(NH)P and Na^+ added either alone or in combination (Fig. I-2). There is a marked effect of GPP(NH)P alone on [^{3}H]etorphine dissociation (Fig. I-2a) in untreated homogenates (immediately following in vivo binding). At 40 minutes after homogenate dilution binding is approximately 30% less in the presence of the nucleotide when compared to that in Tris alone. In contrast, the effect of the nucleotide on [^{3}H]etorphine dissociation is largely attenuated in washed membranes (Fig. I-2b); at 40 min there is only ~ 10% less binding in the presence of GPP(NH)P. Furthermore, the enhancement in the dissociation rate by GPP(NH)P in the presence of NaCl is again more pronounced in the crude, than in the washed, brain membrane homogenate.

In contrast to the results obtained with GPP(NH)P, there does not appear to be a discernible difference in the sensitivity of the binding

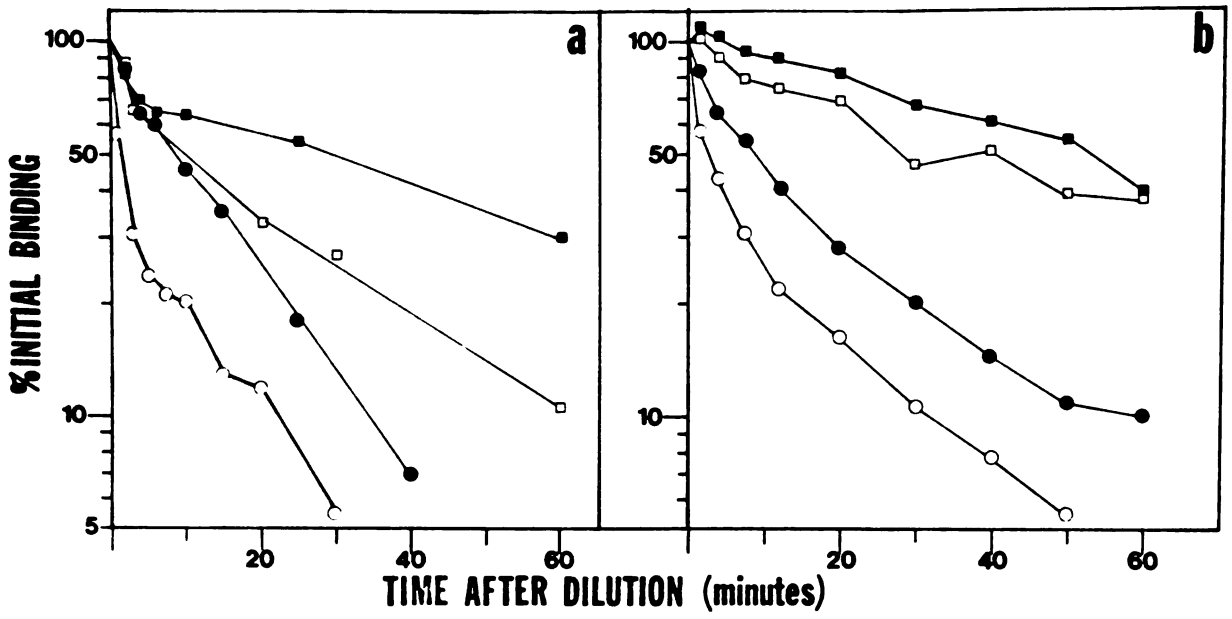

Figure I-2. Effect of NaCl (100 mM) and GPP(NH)P (50 M) on the in vitro [^{3}H]etorphine dissociation curves. Dissociation was measured in the presence of 10^{-6}M unlabeled etorphine at a 1:350 homogenate dilution (37°C). A) Untreated homogenates: labeling occurred in vivo using a 200 Ci/kg [^{3}H]etorphine dose over 20 mins. The experiment was replicated twice with similar results. B) Washed membranes: shown is the dissociation after incubation for 45 min with a tracer concentration of 1.65 x 10^{-10}M. The experiment was replicated once with similar results. Symbols for Fig. 4 A + B: Additions to dilute homogenate (■) no additions, (□), 50 M GPP(NH)P, (●) 100mM NaCl, (○) 100mM NaCl + 50 M GPP(NH)P.

of [^{3}H]etorphine to the effect of Na^+ between washed and untreated homogenates (Fig. I-2a & b). The differences detected in the sensitivity of the tracer dissociation to the combined effect of Na^+ + GPP(NH)P under the different labeling conditions appear, therefore, to be due to the observed changes in sensitivity to guanyl nucleotides. The only condition which could accurately be described by a biexponential decay is that of the most rapid dissociation (Na^+ + GPP(NH)P) (Table I-1).

5. DISCUSSION

It has been previously demonstrated that the *in vivo* receptor dissociation rate of [^{3}H]etorphine is largely monophasic with ~ 90% dissociating with a half-life of ~ 50 sec.[42] This result does not agree with the much slower dissociation rate of [^{3}H]etorphine *in vitro* in the presence of Na (initial $t_{1/2}$ = 5-8 min).[49] Knowing that both Na^+ and GTP are required for opiate activity and accelerate agonist dissociation[22,50] the [^{3}H]etorphine dissociation kinetics were determined in the washed membrane preparation in the presence of both Na^+ + GPP(NH)P. Addition of both regulators together further accelerated the off-rate but failed to reconstruct the rapid *in vivo* dissociation rate (Fig. I-1 and Table I-1).

In order to minimize any changes that may occur during membrane work-up and incubation, receptor labeling was accomplished *in vivo*, and the dissociation curve was observed *in vitro* immediately after sacrifice of the animals. Under these conditions, the dissociation of

[^{3}H]etorphine is accelerated by GPP(NH)P alone, an effect that is partially lost in the washed membrane preparation (Fig. I-2).

The *in vivo* labeling - *in vitro* off-rate approach in the presence of Na^+ + GPP(NH)P still does not yield an off-rate as fast as that which occurs *in vivo*.[42] I therefore investigated the possibility that changes in sensitivity to regulation by Na^+ and guanine nucleotides may also occur during the brief period after sacrifice and in ice-cold Tris buffer (Fig. I-1). After killing the animals, endogenous tissue GTP is instantly hydrolyzed to GMP,[51] which is not a regulator of either opiate receptor binding or activity.[47,50] Moreover, homogenization in Tris buffer causes a dilution of endogenous Na^+ ions, which means that both major regulatory factors are lost during the homogenization process. Therefore, we studied the effect of adding Na^+, GPP(NH)P, or both to the ice cold Tris buffer prior to brain homogenization. Homogenization in the presence of one or both of these regulatory factors after binding *in vivo* further accelerates the dissociation rate to a value similar to that found *in vivo*. Furthermore, both equilibrium binding and association of [^{3}H]etorphine *in vitro* in the presence of Na^+ + GPP(NH)P are more sensitive to these regulatory factors when the brain is homogenized in their presence than when the brain is homogenized in Tris buffer alone (data not shown). Therefore, changes in opiate receptor binding already occur during brain homogenization in ice-cold Tris buffer unless these regulatory factors are present to attenuate such changes.

The changes in the *in vitro* dissociation rates of [^{3}H]etorphine in the presence of Na^+ and GPP(NH)P under different labeling and homogenization conditions (Fig. I-1) appear to be largely due to changes

in dissociation half-life rather than in the relative number of labeled sites that are responsive to Na^+ + GPP(NH)P (Table I-1). This change in dissociation half-life reflects a decrease in affinity of the [^{3}H]ligand for the receptor (or receptor-cyclase coupled) complex in the in vivo system. A similar decrease in affinity of the agonist for the receptor (as measured by a higher K_D) in intact cells relative to that in cell membranes has been observed for the β-adrenergic system,[44] and may be a result of a partial receptor-effector decoupling in vitro. Moreover, the relative affinity of narcotics for the opiate receptor of the NG108-15 hybrid cells agrees with the effectiveness of these ligands as inhibitors of adenylate cyclase in cell homogenates,[23] whereas affinity does not correspond with activity in the intact cell population.[52] In another in vitro study, opiate receptor affinity in rat brain and guinea pig ileum homogenates agrees well with their pharmacological effect on the guinea pig ileum.[53] However, the opiate agonist etorphine has been shown to be pharmacologically active at a very low fractional occupancy (~ 2%) of receptors in the intact rat[42] which suggests a disparity between in vivo binding and effect. It appears, then, that the high coupling efficiency observed for opiate agonists in whole NG108-15 cells[52] or in the intact rat[42] is lost when moving from the in vivo experimental situation. This phenomenon is accompanied by a decrease in sensitivity of the tracer-receptor binding to guanine nucleotides.

CHAPTER II

DISCRIMINATION OF THREE TYPES OF OPIATE BINDING SITES IN RAT BRAIN *IN VIVO*

1. 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 non-linear regression program. Three types of binding sites could be distinguished: Site 1 (18 pmol/g brain), site 2 (15 pmol/g brain), and site 3 (20 pmol/g brain). Site 1 is bound selectively by sufentanil (the ratio of the apparent equilibrium dissociation constant at site 2 to that at site 1 (K_2/K_1) $\approx$ 1200), etorphine ($K_2/K_1 \approx 20$) and naloxone ($K_2/K_1 \approx 15$), and resembles the μ 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 δ and κ 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.

2. INTRODUCTION

The concept of opiate receptor multiplicity was originally formulated on the basis of the differing pharmacodynamic profiles of selected opiates.[8-11] *In vitro* binding studies support this concept and point to the existence of binding sites associated with the μ (morphine), δ (enkephalin), κ (benzomorphan), and σ (SKF 10,047) receptors in the rat CNS.[12,54,55] 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[38] and concentration of binding sites.[39] Earlier studies[38-42] 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.

A method has been developed for measuring opiate receptor binding after administration of the ligands to the intact animal (*in vivo*). The approach involves the subcutaneous 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.[38,39,42] The results were analyzed 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*.[56,57] The method analyzes untransformed binding data from a number of similar experiments simultaneously while allowing for the nonhomogeneity of variance among the data sets.[57,58] In contrast, the Scatchard technique[59] requires a linear transformation of experimental observations which distorts the error structure of the data and may result in biased parameter estimates.[60,61]

This chapter describes a method which allows the characterization of three opiate binding sites in the intact rat. The binding site populations identified in vivo are compared to those established by in vitro techniques.

3. MATERIALS AND METHODS

3.1 Materials

Tracers and unlabeled drugs were obtained from the following sources: [^{3}H]naloxone (21-50 Ci/mmol), [^{3}H]etorphine (33-51 Ci/mmol), etorphine HCl, and diprenorphine HCl from the NIDA (Rockville, MD); [^{3}H]sufentanil (15 Ci/mmol) 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, IL), as was [^{3}H]diprenorphine (7.5-11 Ci/mmol). All tracers were purified by HPLC using systems indicated here and in (9,11) 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, CA) or Bantin and Kingman, Inc. (Freemont, CA).

All numerical, statistical and graphical analyses were performed using the PROPHET system.[62] Model specification and parameter estimation by non-linear regression used MKMODEL[63] with an extended least squares objective function.[58]

3.2 Methods

3.2a In Vivo Binding Studies

The rapid membrane filtration technique described in detail by Perry et al.[42] was used to determine the amount of bound and unbound ligand in the cerebrum 20 minutes after subcutaneous administration to male Sprague Dawley rats. This is the time at which brain concentrations of etorphine[42] and naloxone[64] reach their peak and the time at which one can expect minimal interference with the diprenorphine-receptor equilibrium from the postulated receptor microcompartment.[64] Sufentanil binding was also determined at this time point but the pharmacokinetics 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.

3.2b Confirmation of Ligand Stability During the In Vivo Labeling Period

The potential contribution of drug metabolites (generated in vivo) to the binding results was determined using the following procedure: [^{3}H]naloxone (143 μCi/kg) was administered to 4 rats. Two of these also received 2 mg/kg diprenorphine since a binding experiment for naloxone was also determined under this condition (see Results). The animals were sacrificed after 20 min. The brains were homogenized in 25 ml .05M Tris HCl buffer (pH 7.4 at 37°C) and filtered in 250 μl aliquots through

Whatman GF/B filters (6 filters/rat). The filters were combined in groups of 3 and extracted overnight with 7.0 ml methanol in the presence of 50 µg 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°C) to near dryness. The residue was taken up in 250 µl of the HPLC 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 were injected with 300 µCi/kg [^{3}H]sufentanil and the resulting methanol extracts were obtained with 50 µg unlabeled sufentanil carrier. The HPLC system for sufentanil was 70% acetonitrile: methanol (55:45)/30% .005 M K_2HPO_4. At a flow rate of 2.5 ml/min on the same column, sufentanil has a retention time of 10.5-12 min. For both naloxone and sufentanil, when the extraction recovery is taken into account, >95% of the radioactivity on the filters can be accounted for as unchanged drug. This eliminates 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.

3.2c Estimation of Binding Parameters

A three binding site model to describe the binding data 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 dissociation constants of the ligand at these sites. U_L is the unbound concentration of ligand, determined experimentally as the difference between total and bound brain ligand concentration. The non-specific binding 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 non-specific binding. This expression is used when the labeled and unlabeled drug are the same ligand; the values of B_L are computed from the labeled ligand binding, specific activity, and the concentration of unlabeled ligand.

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

$$B_L = U_L\left[\frac{Bmax_1}{U_L + K_{1L}(1 + D/K_{1D})} + \frac{Bmax_2}{U_L + K_{2L}(1 + D/K_{2D})} + \frac{Bmax_3}{U_L + K_{3L}(1 + D/K_{3D})} + NS_L\right] \quad (2)$$

This model requires that the expression for D be proportional to the unbound concentration of the displacing ligand. 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[65] and embodied in computer programs such as LIGAND[56] and SCAFIT.[57] In contrast to the in vitro method, the total concentration of the displacing 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 II-1, Fig. II-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 II-1), was used to convert dose to brain unbound concentration units where appropriate.

Table II-1. Dose-Concentration Relationships: Values are the slope of the dose (μg/kg) vs. unbound brain concentration (pmol/g brain) curves as determinedby least squares regression obtained after a 20 min labeling period *in vivo*. Data for naloxone are illustrated in Fig. II-1.

Drug	Slope (conversion factor)	Standard Error of Slope	Dosage Range (μg/kg)	r^2	# of Data Points
Etorphine	.7912	.0187	0-200	.9714	54
Sufentanil	.7061	.0555	0-900	.9152	16
Diprenorphine	.7377	.0263	0-10,000	.9764	20
Naloxone	2.724	.0603	0-20,000	.9903	21
Naloxone, in the presence of a 2 mg/kg Diprenorphine dose	1.361	.0143	0-20,000	.9980	19

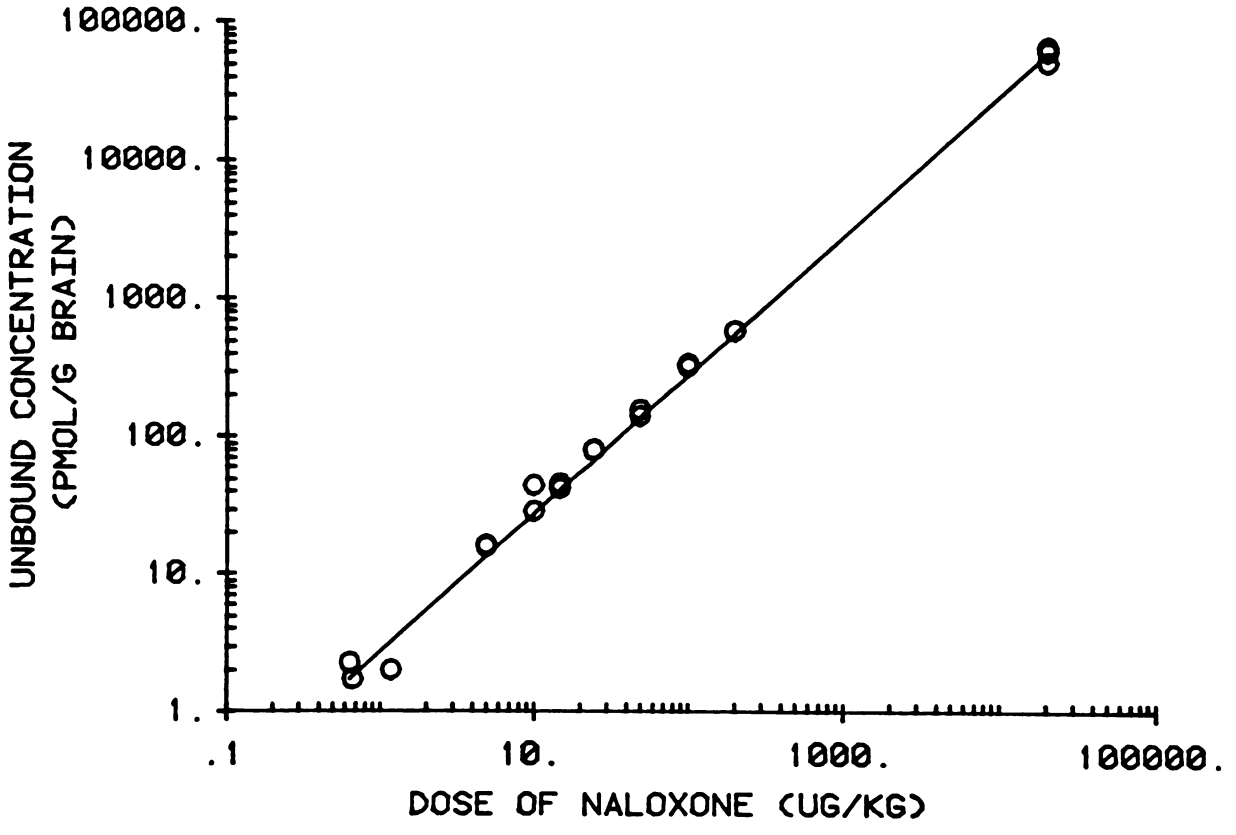

Fig. II-1. Dose-concentration relationship for naloxone. The unbound concentration was determined at each administered dose after a 20 min labeling period *in vivo*. Each point represents the mean of duplicate determinations in a single rat. Logarithmic scales are used only to aid in visualizing the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

The variance (VARi) of the ith observation in the jth data set was modeled by equation (3):

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

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 in Table II-2.

Table II-2. In Vivo Binding Ligand Combinations. The estimates of the variance scale parameters (see equation (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	.0514(54)	-----	.2140(15)	-----
Sufentanil	.0449(18)	.0072(16)	-----	.0490(23)
Diprenorphine	.0216(16)	-----	.0239(20)	-----
Naloxone	.5918(20)	.0143(15)	.1336(18)	.0199(21)
Naloxone + 2mg/kg Diprenorphine	-----	-----	-----	.0191(19)

Note: The size of the variance scale parameter is an indication of the relative goodness-of-fit of each data set.

4. RESULTS

4.1 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. II-1 for naloxone was constructed. The data are represented on logarithmic axes only to visualize the data throughout the entire concentration range. This figure 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 II-1.

4.2 Binding Site Populations Differentiated In Vivo

The estimates of B_{max} were obtained from simultaneously analyzing the data sets indicated in Table II-2, and the results of fitting the sufentanil, diprenorphine, and naloxone binding data to a three binding site model are illustrated in Fig. II-2. Sufentanil binds predominantly to a single type of binding sites, with a B_{max} of 18 pmol/g brain. Diprenorphine binds to an additional type of binding sites, whose concentration is 15.3 pmol/g 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 sites with a B_{max} of 19.8 pmol/g brain. The three binding site model, therefore, proved to be the optimum model for describing the binding data (see discussion).

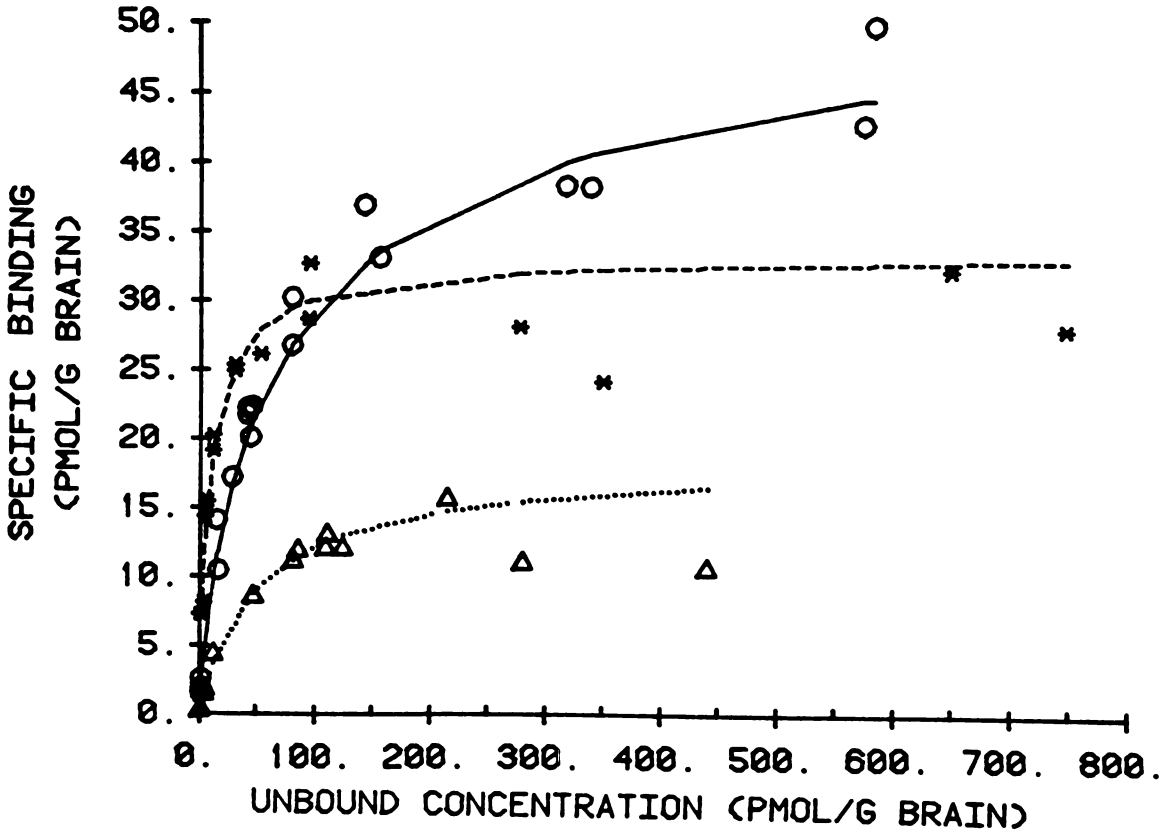

Fig. II-2. Saturation isotherms obtained in vivo after a 20 min labeling period. Each curve represents the computer generated fits of the data using the three binding site model (see methods). Each point represents the mean of duplicate determinations in a single rat: (△)sufentanil, (*)diprenorphine, and (0)naloxone binding, respectively. The lines represent the (•••••)sufentanil, (- - - -)diprenorphine, (_____) naloxone predicted values using the three binding site model. Specific binding was derived by subtracting the model predicted values for the nonspecific binding from each experimentally determined data point.

The binding of naloxone to an additional binding site with insignificant affinity for diprenorphine is illustrated in Fig. II-3 by two sets of naloxone binding observations that were obtained using naloxone in the absence or the presence of 2 mg/kg diprenorphine. No changes in tracer amounts of naloxone binding were observed with 1 mg/kg or 10 mg/kg diprenorphine. The 2 mg/kg dose of diprenorphine essentially saturates all of the diprenorphine binding sites in vivo.[38,39,this chapter] Therefore, the binding of naloxone in the presence of this diprenorphine dose indicates the existence of binding sites for which diprenorphine has negligible affinity.

Estimates of the concentration of each of the three types of binding sites and the tentative associations with the classes of opiate receptors defined in other systems are shown in Table II-3.

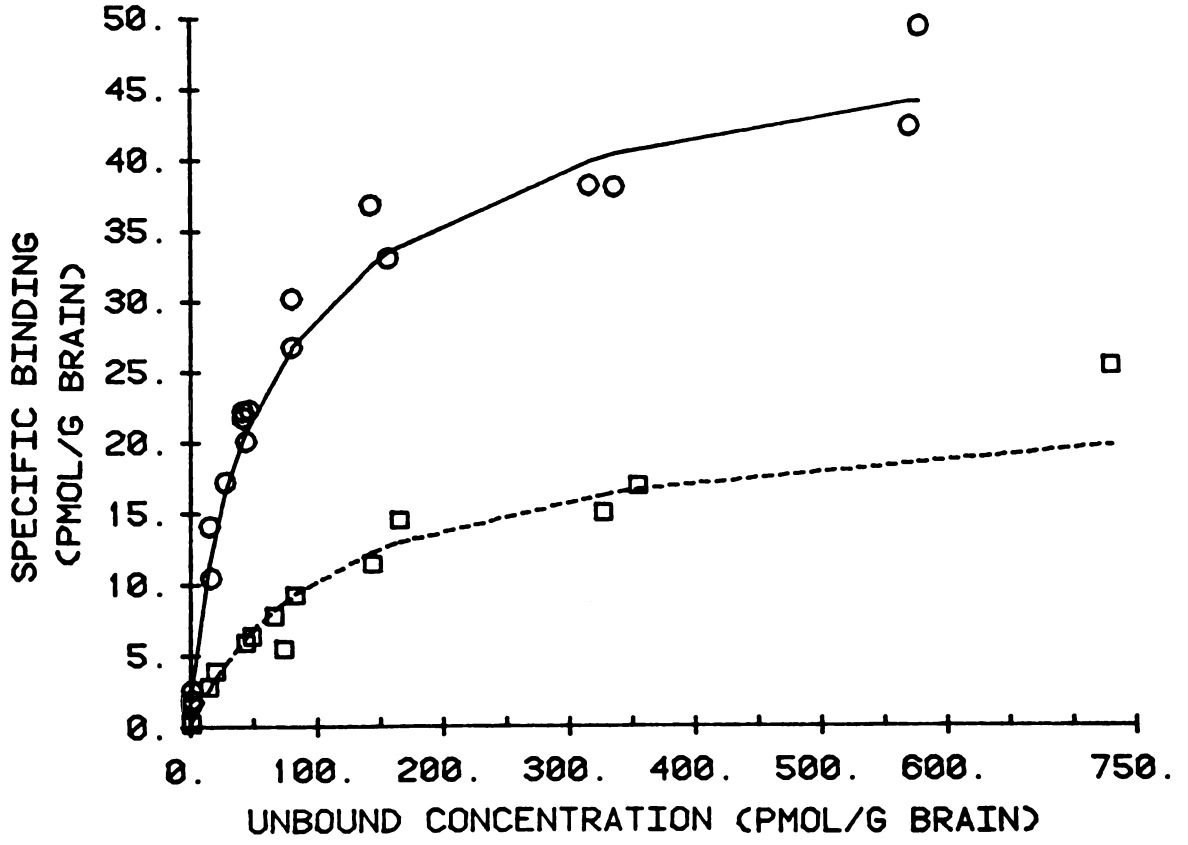

Fig. II-3. Naloxone saturation isotherms obtained in vivo after a 20 min labeling period. The lines represent the predictions by the three binding site model. Each point represents the mean of duplicate determinations in a single rat: naloxone binding alone(O,_____), naloxone binding in the presence of a 2 mg/kg diprenorphine dose (□ , - - - - - - -). Specific binding is determined as in Fig. II-2.

Table II-3. Binding Site Populations Differentiated From Simultaneous Fitting of the Naloxone, Etorphine, Sufentanil and Diprenorphine *In Vivo* Displacement Curves Indicated in Table II-2.

Binding Site	Bmax pmole/g brain	Tentative Receptor Assignments*
1	18.0	μ
2	15.3	δ + κ
3	19.8	Additional naloxone site

*See discussion.

4.3 Binding selectivity of etorphine, sufentanil, diprenorphine, and naloxone

Figure II-4 illustrates part of the data used to estimate the binding parameters of several ligands to the three sites. It shows the decrease in the binding of tracer doses of [^{3}H]sufentanil, [^{3}H]diprenorphine, and [^{3}H]naloxone when co-administered with increasing doses of unlabeled naloxone. Because the binding of the labeled ligand depends on the unbound concentrations of both the labeled and unlabeled ligands (equation 2), and the displacer often affects the fraction of the tracer entering the brain, the actual binding data is a function of both displacer dose and the amount of tracer in the brain. Both these independent variables were used to predict binding. The predicted binding cannot be readily shown in graphical form because of the separate variation in both of these dosing variables. Therefore, for the purposes of illustration, the data in Fig. II-4 have been normalized to the amount of tracer in the cerebellum. Since the cerebellum does not contain opiate receptors,[41] it can be used as a reference to account for changes of the tracer pharmacokinetics.

Compared with its ability to displace itself, naloxone is more potent in displacing sufentanil but less potent in displacing diprenorphine (Fig. II-4). Similarly, sufentanil is less potent in displacing naloxone and etorphine compared with its ability to displace itself (data not shown). A 1.5 mg/kg dose of sufentanil, while completely displacing [^{3}H]sufentanil, displaces etorphine and naloxone only to 16% and 26% of control binding respectively (data not shown).

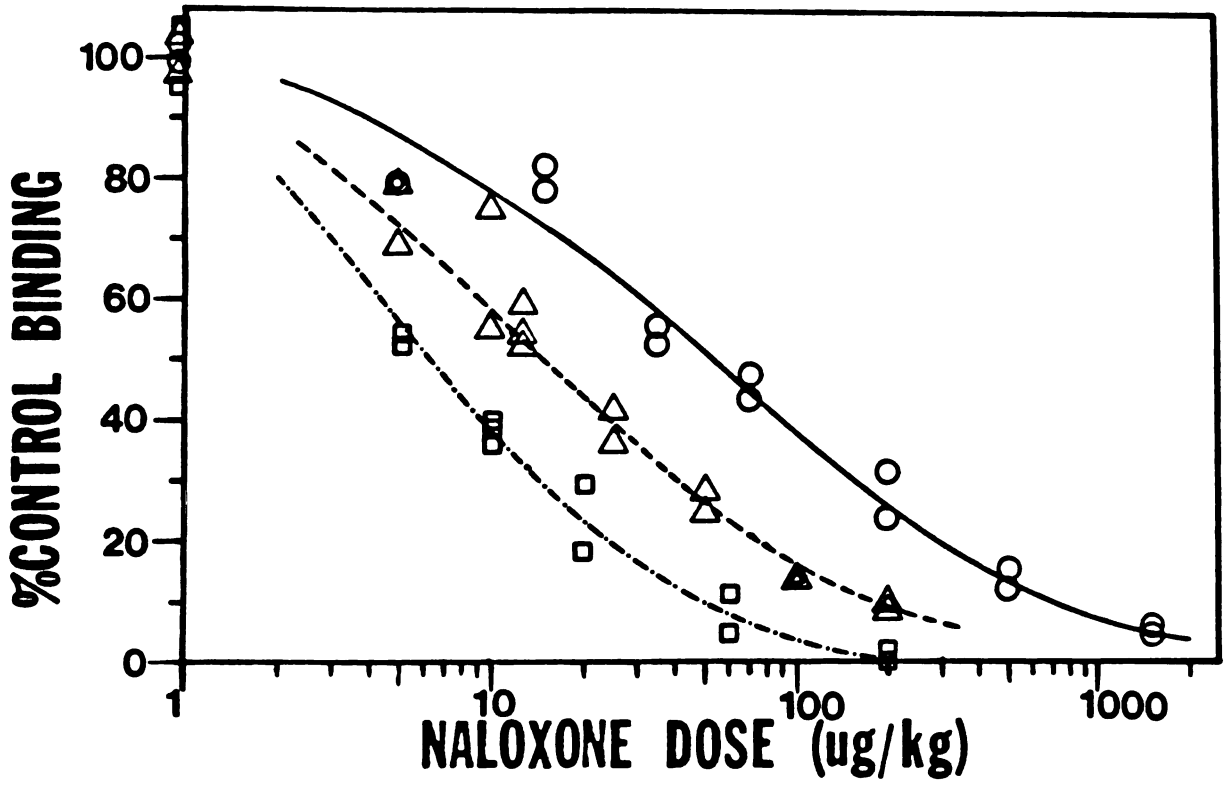

Fig. II-4. Ability of naloxone to displace various ligands in vivo. Each point represents the mean of duplicate determinations in a single rat. The lines show naloxone's ability to displace: sufentanil(□), naloxone (△), and diprenorphine(O) respectively. The displacer was coadministered with the tracer, and sacrifice occurred after a 20 min labeling period. The ordinate represents the % of control (tracer only) specific binding. The range of control values is indicated on the Y-axis. All data has been normalized to the amount of radioactivity in the cerebellum to account for the amount of radioactivity in the brain (see text). For the purpose of the figure, 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 (△ , O), was subtracted from the observed binding. The lines were drawn by hand.

Unfortunately, the rats generally do not survive sufentanil doses exceeding 2.5 mg/kg for the duration of the receptor labeling period. Therefore, complete displacement of naloxone and etorphine by sufentanil was not demonstrable.

Estimates of the apparent dissociation constants of the four ligands for the three types of sites are summarized in Table II-4. Sufentanil is highly selective for the first type of binding site, having virtually no affinity to sites 2 and 3. Etorphine is also 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 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 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 = 36 µg/kg) with a 3-fold selectivity for this site over site 2 (K_2 = 108 µg/kg).

Table II-4. Binding Selectivity for the Three Binding Sites Differentiated *In Vivo*: The dissociation constants for each drug at the three binding sites were estimated using the data sets in Table II-2 and equations (1), (2), and (3).

Drug	K_1 (μg/kg)	K_2 (μg/kg)	K_3 (μg/kg)
Sufentanil	69	>70,000	>70,000
Etorphine	28	650	>70,000
Diprenorphine	6.8	25	>70,000
Naloxone	7.1	108	36

4.4 Non-specific 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 where 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 II-2 for each ligand combination. The estimate of the power Z was 1.84.

DISCUSSION

I 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*: it has previously been shown that etorphine labels a subset of sites labeled by diprenorphine.[38] Furthermore, it has been shown that naloxone labels a binding site population distinct from that labeled by diprenorphine.[66,67] On the basis of these observations a minimum of

three types of opiate binding sites appear to exist in the rat brain. Attempts were made to fit the data from the experiments listed in Table II-2 to a four binding site model, and the fit was not significantly improved, 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 the discrimination of an additional type of binding site.

I have shown that a linear relationship between administered dose and unbound brain concentration (Fig. II-1, Table II-2) 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.

The properties of the first type of binding site population ($B_{max}1$ = 18 pmol/g brain) resemble those of the μ receptor, i.e. the high selectivity (K_2/K_1 ~ 1500) of the μ specific ligand sufentanil[68,69], and the high affinity (K_1 = 7 μg/kg) and selectivity (K_2/K_1 ~ 15) of the μ selective antagonist naloxone[70] 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 that etorphine is equipotent in displacing ligands from μ and δ binding sites in rat membrane preparations[12] and that it behaves as a universal ligand for the μ, δ, and κ sites in homogenates of guinea pig brain.[71] However, more recently Wood et al.[54] and Rothman and Westfall[72] found that etorphine is, in fact, μ-selective in rat brain membranes. On the

other hand, etorphine binds to an additional type of sites, site 2, as shown by its ability to completely displace [^{3}H]diprenorphine binding *in vivo*, at suitably high doses.[38] Thus, etorphine's behavior *in vivo* (K_2/K_1 ~ 20) is compatible with its moderately μ-selective binding properties *in vitro*.[54] Diprenorphine is thought to be a universal ligand for the opiate receptor system, binding with equal affinity to the μ, δ, and κ binding sites *in vitro*.[13] Since diprenorphine also binds to site 2 with relatively high affinity *in vivo*, it may therefore be surmised that this site represents a mixture of the δ and κ sites. With the present data it is not possible to differentiate the δ and κ sites which may comprise site 2, and additional studies with δ- and κ-selective ligands are needed.

An additional binding site for naloxone has previously been demonstrated by others *in vitro*.[73,74] Moreover, it has previously been demonstrated that tracer doses of naloxone cannot be fully displaced by doses of buprenorphine that saturate the diprenorphine binding sites *in vivo*.[67] Therefore, this site does not resemble the known μ, δ, and κ sites. The present study has identified a type of binding site *in vivo* which appears to be the same as that site shown previously to be selectively bound by naloxone. We have further characterized this site (site 3) by providing an estimate of the concentration of binding sites (20 pmol/g brain) as well as the affinity of naloxone for this site *in vivo*. Surprisingly, naloxone displays a greater affinity at site 3 than at the putative δ and κ 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.[73,74] 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 this new site are currently in progress (Grevel and Sadée, unpublished). The pharmacological significance of this third type of binding sites presently remains unknown.

Analyses such as the one described here enable the determination of the binding constants of a series of opiate ligands to three types of binding sites in the intact rat brain, circumventing any modifications that might take place *in vitro*.[38,39,Chapter I] It is now possible to relate administered dose directly to receptor occupancy at each binding site and to the resulting pharmacological effect. This is an essential step in linking observations concerning opiate binding sites to their functional significance in mediating pharmacological and physiological effects.

CHAPTER III

OPIATE RECEPTOR BINDING - EFFECT RELATIONSHIP: SUFENTANIL AND ETORPHINE PRODUCE ANALGESIA AT THE μ-SITE WITH LOW FRACTIONAL RECEPTOR OCCUPANCY

1. SUMMARY

The analgesic activities of the opiate agonists etorphine and sufentanil and the antagonistic effects of diprenorphine and naloxone have been related to the occupancy of three classes of opiate binding sites previously defined *in vivo*[Chapter II] in order to establish their pharmacological significance. Sufentanil binds specifically *in vivo* to the first type of sites (site 1), exhibiting ~ 1100-fold selectivity over site 2, whereas etorphine displays ~ 20-fold selectivity for site 1 over site 2. Neither agonist has a measurable affinity to the third type of binding site. The binding data suggest that site 1 is analogous to the μ site previously identified *in vitro*.[Chapter II] Both agonists produce analgesia in the rat tail flick test at the same low fractional occupancy of site 1 (~ 2% at the ED_{50}) while they display much lower and quite different occupancies at site 2. Both of the opiate antagonists naloxone and diprenorphine reduce the potency of sufentanil and etorphine by a factor of 2 at 50% occupancy of site 1 alone. These results provide strong evidence that these four drugs exert their effects by interaction with site 1 (μ sites) which therefore may be regarded as the receptor responsible for analgesic action in this test. There appears to be a direct relationship between antagonistic

effect and fractional occupancy for naloxone and diprenorphine at site 1, while the agonists exert their action at a very low fractional occupancy implying a non-linear binding-effect process.

2. INTRODUCTION

The term "receptor" as used in the pharmacological literature, refers to "that.....molecular constituent of the cell with which an active drug combines to produce a response. [As such it] is, therefore, something more than a binding site."[75] The concept of opiate receptor multiplicity originated from the pharmacological investigations of Martin et al.[8] using the chronic spinal dog, and has been supported by other pharmacological studies.[10,11] Martin[8] has classified the opiate receptor system into the μ, κ, and σ subtypes based on the pharmacological syndromes produced by a series of morphine congeners; he has proposed that the μ receptor mediates analgesia and respiratory depression, the κ receptor sedation, and the σ receptor is responsible for the dysphoric effects of some opiates.[31] According to this scheme, morphine, ketocyclazocine, and N-allylnormetazocine (SKF-10,047) are the prototypic agonists at the μ, κ, and σ opiate receptors, respectively.[8] A somewhat different classification scheme has emerged from in vitro binding studies such that the μ site is selective for opiate alkaloids, the δ-binding site is selective for opiate peptides, the κ binding site preferentially binds the benzomorphans, and the σ site binds N-allylnormetazocine and phencyclidine.[13,17] Attempts to determine the functional significance of these sites have been indirect and are based on a comparison of rank order affinities for the binding

sites with the rank order potencies of opiates in a variety of pharmacological tests. These results suggest that it is primarily the μ binding site that mediates analgesia.[32,33,76] Magnan et al[77] have proposed a further classification of narcotic analgesic drugs into four groups based upon their agonistic and antagonistic activity in three bioassays. However, these authors were unable to propose a model linking *in vitro* binding at multiple sites with the observable differences in pharmacological activity.

There are a number of factors which compromise the conclusions drawn from *in vitro* binding assays alone, such as disturbances of the receptor and its environment *in vitro*.[38,39,Chapter I] Furthermore, it has previously been demonstrated that the opiate agonist etorphine elicits analgesia at a very low fractional occupancy of receptors *in vivo*.[42] A large discrepancy between potency (ED_{50}) in the guinea pig ileum assay and binding affinity *in vitro* was also noted for sufentanil, fentanyl, and etorphine by Magnan et al.[77] Similarly, Fantozzi et al[52] have noted a discrepancy between the binding affinity and activity for the opioid peptide [D-Ala^2-Met^5]enkephalinamide in whole NG108-15 cells. This phenomenon of low fractional receptor occupancy for agonistic effect complicates the assignment of functional significance to the opiate binding sites since there may be a large discrepancy between the dose needed to occupy 50% of the receptor sites and that required for 50% response. One explanation for such a discrepancy includes the concept of spare receptors,[78] where occupancy < 50% at the ED_{50}. The obvious lack of a linear relationship between binding and effect requires that the occupancy of multiple drugs be studied at various binding sites in order to establish the connection between the

binding site and the receptor. Both the pharmacological activity and receptor binding of antagonists are also needed for identification of a binding site as a bona fide receptor.

I have described a method for estimating opiate binding after administration of the ligands in vivo which establishes the existence of three opiate binding sites in the intact rat brain. Site 1 has characteristics similar to those of the μ sites identified in other systems, site 2 has been tentatively identified as a composite of δ and κ sites and site 3 is a novel naloxone binding site.Chapter II I demonstrate here, using a direct comparison of in vivo receptor occupancy and pharmacodynamic parameters of etorphine, sufentanil, naloxone and diprenorphine in the rat tail flick test, that the two opiate agonists exert their analgesic actions via interaction with the μ site at a low fractional receptor occupancy.

3. MATERIALS AND METHODS

3.1 Materials

All drugs used were generous donations from the following sources: naloxone HCl, Endo Laboratories (Garden City, NY); sufentanil citrate, Janssen Pharmaceutica (New Brunswick, NJ); etorphine HCl, the NIDA (Rockville, MD). Male Sprague Dawley rats (120-200 g) were purchased from either Simonsen Laboratories (Gilroy, CA) or Bantin and Kingman, Inc. (Freemont CA).

All numerical, statistical, and graphical analyses were performed using the PROPHET system.[62] Parameter estimation was done by MKMODEL[63] using an extended least squares objective function.[58]

3.2 Methods

3.2a In Vivo Receptor Binding Analysis

The binding parameters were estimated by simultaneously fitting binding data of naloxone, diprenorphine, etorphine, and sufentanil using a three binding site model as described in detail elsewhere.Chapter II Binding to rat cerebrum was measured after a 20 min *in vivo* labeling period following simultaneous subcutaneous administration of labeled and unlabeled drug.

3.2b Determination of the ED_{50} for Etorphine, Sufentanil and Naloxone

Analgesia was assessed using a modification of the radiant heat tail flick test.[79,80] In order to obtain a uniform absorption of light, the tails were first painted with a black felt tip pen and then allowed to dry. Control latencies were between 2.0 and 5.0 sec; animals not responding within this time period were discarded. Control latencies were determined 1-2 hrs prior to use. The animals were injected subcutaneously with increasing doses of etorphine or sufentanil concurrently with varying naloxone doses. The latencies were measured 20 min after injection as this is the time of peak brain concentration for etorphine[42] and naloxone;[64] this is also the time at which the

binding analysis was performed. A 12 sec cutoff time was chosen to prevent damage to the tail. Both control and test latencies were determined in triplicate. Animals not responding at 12 sec were said to be analgesic. The dose response curves were then analyzed in a quantal fashion. Three to five rats were used at each dose level, 15-30 rats for each dose-response curve. Rats were used only once in a one week period. All doses are expressed as μg free base/kg.

3.2c Estimation of Pharmacodynamic Parameters

Dose-response curves for etorphine and sufentanil analgesia in the presence of increasing naloxone doses were analyzed simultaneously using the following model which is based on the law of mass action and describes competitive antagonism:

$$E = \frac{E_{max} \cdot A^N}{A^N + (ED_{50}(1 + \frac{B}{ID_{50}}))^N},$$

where E is the percent of animals showing analgesia (latency > 12 sec) following a dose, A, of agonist, and E_{max} is the maximum possible effect (100% of the animals are analgesic). Thus, the ED_{50} is that dose of agonist which produces analgesia in 50% of the animals. B represents the dose of antagonist (naloxone) and is held constant for a given agonist dose-response curve. ID_{50} is that dose of antagonist which doubles the agonist dose required to produce analgesia in 50% of the animals.

The parameter N permits differences in the the steepness of the dose-response curves from the simple law of mass action prediction. N is a parameter estimated separately for the sufentanil and etorphine dose-response curve series.

The response variable, E, may be thought of as the probability of analgesia at any given dose. The steepness of the dose response curve is thus in part determined by the homogeneity of the rats used in the analgesic test. If all rats were identical then the response would appear to be a step function changing from no analgesia to analgesia at the ED_{50}.

The variance (VAR_i) of the ith observation in the jth data set was modeled by the following equation:

$$VAR_{ij} = V_j E^Z{}_{ij}$$

where V_i is the variance scale parameter for data in jth data set, and E_{ij} is the predicted effect raised to the power Z.

3.2d Naloxone Activity Against Etorphine and Sufentanil

In order to determine whether naloxone exhibited the same ID_{50} when antagonizing both etorphine and sufentanil analgesia, we compared two different models describing the dose response curves. The first model allowed the ID_{50} of naloxone to be different when sufentanil or etorphine was the agonist; the second model constrained ID_{50} to be the same against both etorphine and sufentanil.

4. RESULTS

4.1 Determination of the Analgesic Activity of Etorphine and Sufentanil in the Rat Tail Flick Test

The dose-response curves for etorphine and sufentanil in the presence of increasing naloxone doses are illustrated in Fig. III-1 and Fig. III-2, respectively. Simultaneous analysis of these curves results in an ED_{50} for etorphine of .59 µ/kg (Fig. 1), and of 1.5 µg/kg for sufentanil (Fig. III-2).

4.2 Determination of Naloxone Activity Against Etorphine and Sufentanil

The dose-response curves for etorphine and sufentanil in the presence of increasing doses of naloxone were analyzed according to the method of Arunlakshana and Schild[81] which gave a K_B (i.e. ID_{50}) of 10.8 µg/kg and 11.1 µg/kg for naloxone against etorphine and sufentanil analgesia, respectively. The slopes of the Schild plots were .9994 and 1.220, respectively. However, the interpretation of Schild plots is problematic when the slopes deviate from one,[82] and when the dosage ratios are estimated from linear regression lines of the often seemingly non-parallel agonist dose-response curves. Therefore, the data were also analyzed simultaneously employing the equation for competitive antagonism (see Methods). The estimates using non-linear regression of the dose-response curves in Figs III-1 and III-2 results in an ID_{50} for naloxone = 6.51µg/kg.

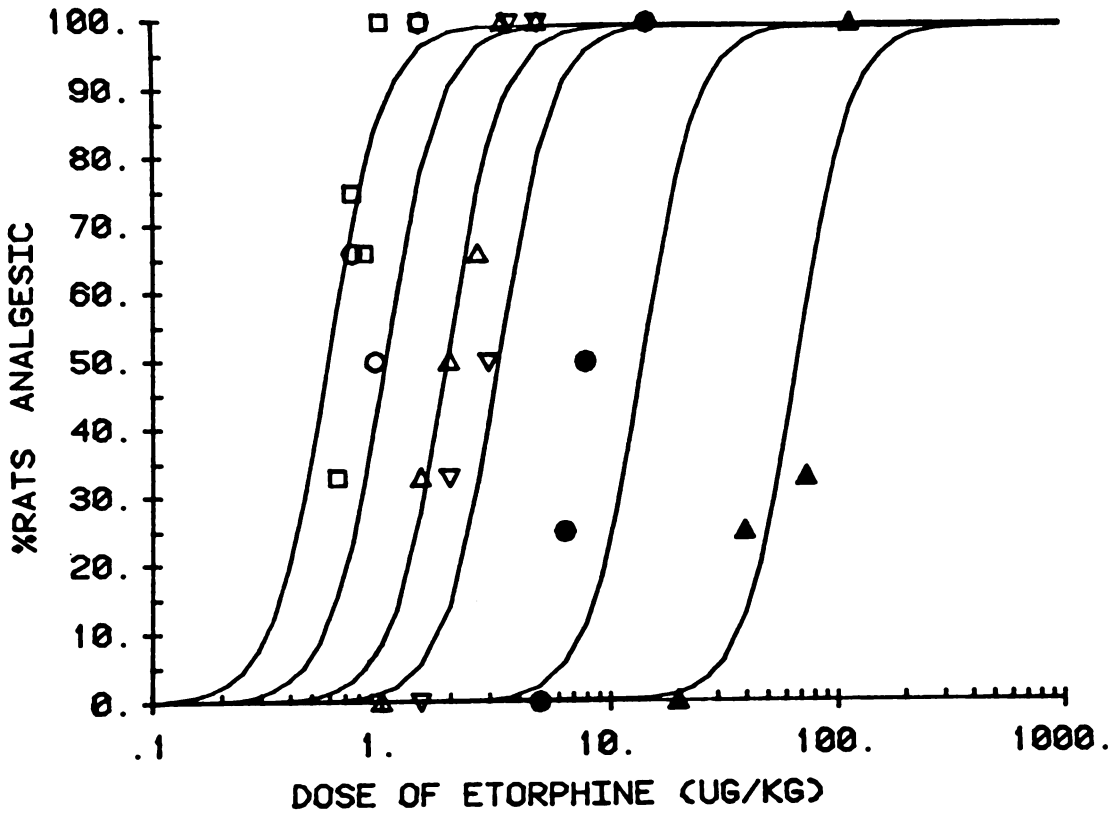

Fig. III-1. Dose-Response curves for etorphine (rat tail-flick test) in the presence of increasing naloxone doses. (□)etorphine alone, (○) + 5 µg/kg naloxone, (△) + 15 µg/kg naloxone, (▽) + 30 µg/kg naloxone, (●) + 150 µg/kg naloxone, (▲) + 750 µg/kg naloxone. Each point represents the percentage of the rats at that dose which did not flick their tails by cutoff (3-5 rats/dose). The curves represent the computer generated predictions for the competitive equation where the ED_{50} of etorphine = .59 µg/kg, the ID_{50} of naloxone = 6.5 µg/kg and N = 3.6. The dose-response curves were constructed in three sets of experiments with different batches of rats from the same lot (i.e. different days) which may account for the relatively poor fit for some of the response data.

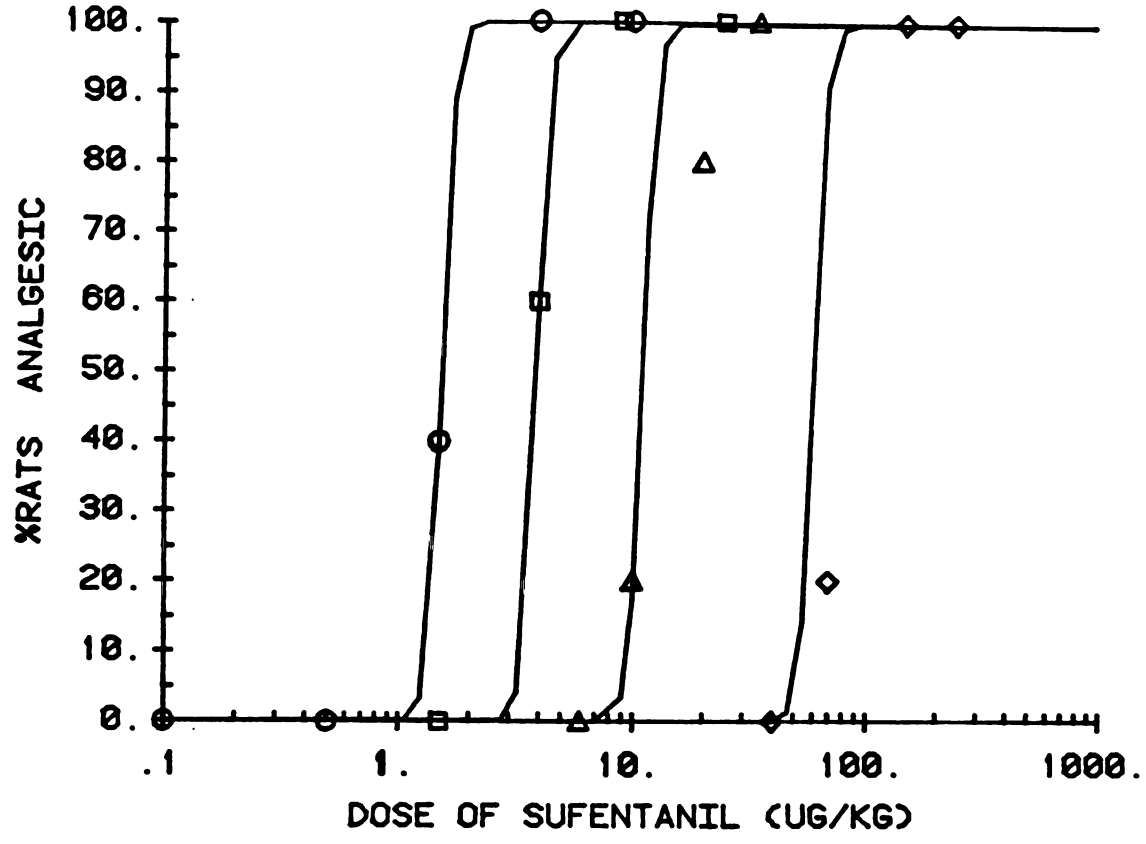

Fig. III-2. Dose-response curves for sufentanil (rat tail-flick test) in the presence of increasing naloxone doses. (O)sufentanil alone, or in the presence of the following naloxone doses:(□) 10 µg/kg, (△) 40 µg/kg, (◇) 250 µg/kg. Each point represents the percentage of rats at that dose not flicking their tails by the time of cutoff (5 rats/dose). The curves represent the computer generated predictions for the competitive equation where the ED_{50} of sufentanil = 1.5 µg/kg, the ID_{50} of naloxone = 6.5 µg/kg, and N = 16.3. Because of the steepness of the response slopes, typically only one dosage level fell in between no response and full response.

The estimates using a model allowing different ID_{50}'s for naloxone against etorphine and sufentanil results in ID_{50} values of 6.68 and 6.50 μg/kg, respectively. The difference in these values is ~ 2 1/2 % indicating that naloxone exhibits the same activity against these two agonists.

4.3 Examination of the Slope Factor of the Etorphine and Sufentanil Dose-Response Curves.

The series of etorphine and sufentanil dose-response curves were modeled such that each series consists of a set of parallel curves; each set of curves has a separate steepness factor N. This parameter, was estimated to be 3.55 and 16.25 for the etorphine and sufentanil dose-response curves, respectively. The difference in these two slope factors is illustrated in Fig. III-3 for etorphine (open squares) and sufentanil (open circles) in the absence of naloxone.

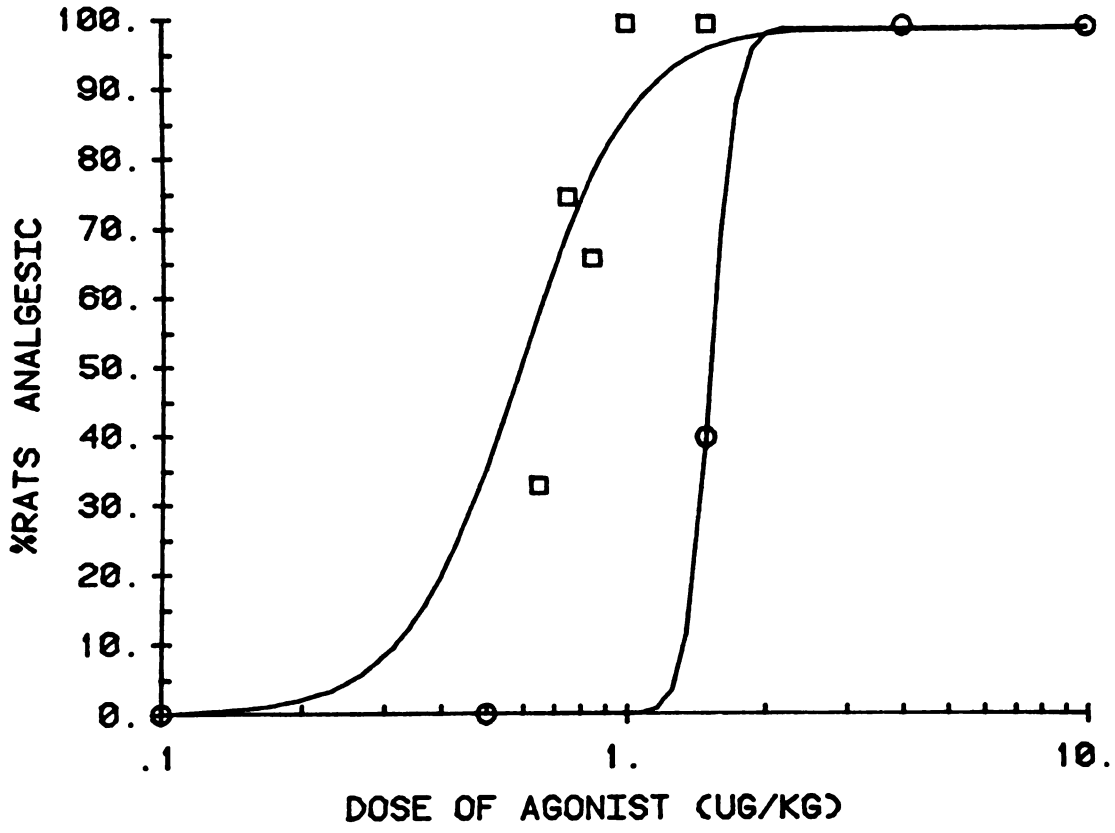

Fig. III-3 Comparison of the etorphine and sufentanil dose-response curves. Illustrated are the dose response-curves for etorphine (□) and sufentanil (O) in the absence of naloxone, replotted from Fig. III-1 and III-2, respectively. Note that the steepness factors have been calculated from all dose response curves in the absence and presence of naloxone (see Fig. III-1 and III-2).

4.4 Comparison of the Receptor Binding and Pharmacodynamic Parameters

The dissociation constants of sufentanil, etorphine, diprenorphine, and naloxone for the two binding sites after a 20 min labeling period *in vivo*[Chapter II] are listed in Table III-1. Figure III-4 illustrates the difference in specificity for the μ receptor (site 1) exhibited by etorphine and sufentanil. Sufentanil exhibits ~ 1200 fold selectivity for site 1 as compared to site 2, whereas etorphine only exhibits a 20 fold selectivity (Table III-1). Site 1 has a B_{max} of 18 pmol/g brain; this is the level at which sufentanil binding saturates.[Chapter II] Over the entire dosage range etorphine labels more receptors than that of sufentanil (Fig III-4). Therefore, even though both ligands are selective for site 1 only etorphine binds to site 2 (the putative δ and κ sites) to any appreciable extent, in the pharmacological dose range, and saturates these sites at high doses.[38]

The ED_{50} and ID_{50} values for these drugs are listed in Table III-1. The ED_{50} value for diprenorphine was not determined in this laboratory, but is an average of values taken from the literature.[83,84] Receptor occupancy, or the fraction of the binding site that is bound at a specified degree of effect, is calculated using the relationship occupancy = $ED_{50}/(K_D + ED_{50})$; an occupancy of 50% at the ED_{50} is expected if binding is directly proportional to effect. Both etorphine and sufentanil show low fractional occupancy in the rat tail-flick test at both binding sites; however, the occupancy is the same (2%) for these two drugs at site 1 only.

Table III-I. Relationship Between the In Vivo Binding (K_D) and Pharmacological Potency (ED_{50}, ID_{50})

Drug	K_D(μg/kg) Site 1 (μ)	K_D(μg/kg) Site 2 (δ + κ)	Pharmacological D_{50}** (μg/kg)	Occupancy at D_{50}		Analgesic Test
				Site 1	Site 2	
Sufentanil	68.8	80201	1.5	.022	2.0×10^{-5}	Tail flick
Etorphine	27.5	650	0.59	.022	9.0×10^{-4}	Tail flick
Diprenorphine	6.8	25.3	6.0	.470	.20	Tail pressure
Naloxone	7.1	108	6.5	.480	.06	Tail flick

*Average of literature values (versus morphine analgesia).[83,84]

**D_{50} = ED_{50} for agonists, ID_{50} for antagonists.

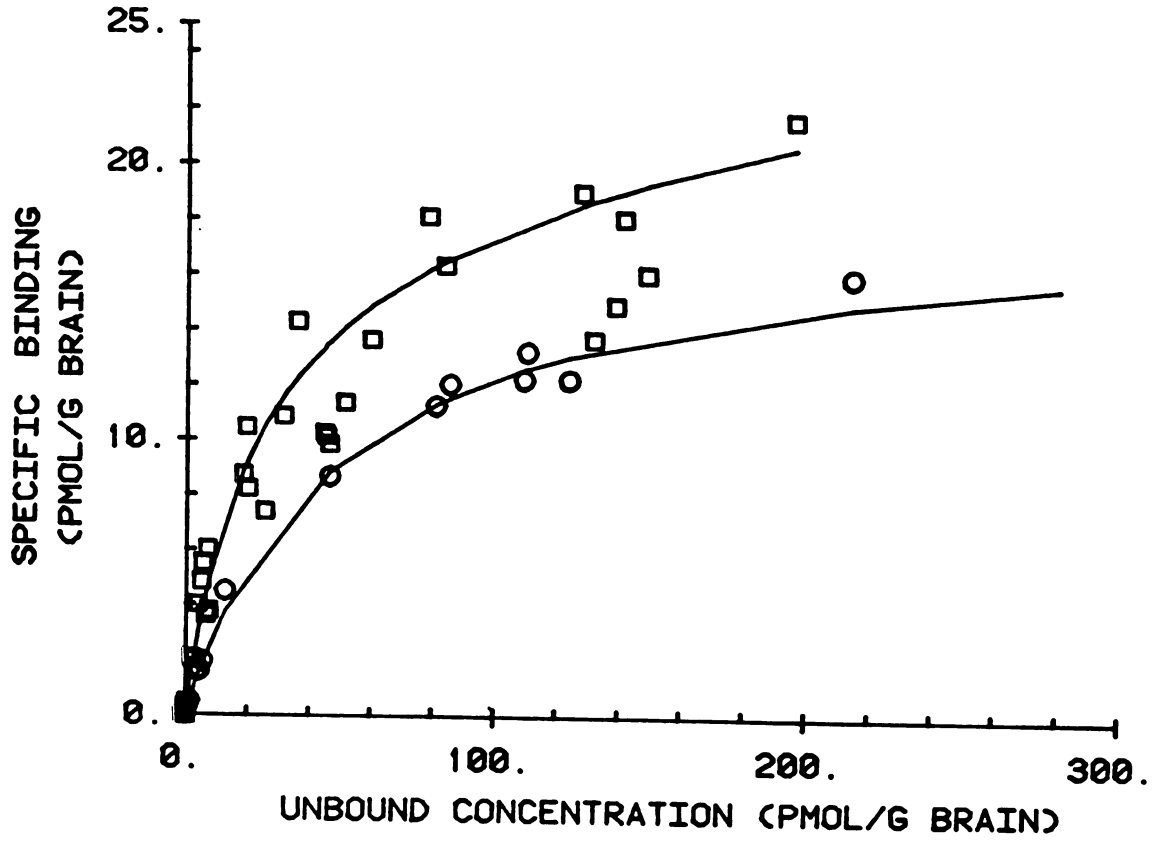

Fig. III-4. Comparison of the etorphine and sufentanil in vivo saturation isotherms. The specific binding of etorphine (□) and sufentanil (O) is plotted against the unbound brain drug concentration. The curves represent the computer generated predicted values for a three binding site model where the $B_{max}1$ = 18 pmol/brain, $B_{max}2$ = 15 pmol/g brain, $B_{max}3$ =20 pmol/g brain; the K_D for etorphine at the three sites is 21.8, 514.2, and 118037 pmol/g brain respectively; the K_D for sufentanil at the three sites is 48.6, 56630, and 52702 pmol/g brain, respectively. The nonspecific binding for etorphine and sufentanil is .033 and .072 x the unbound concentration for each tracer, respectively. Nonspecific binding has been subtracted from all points. Each point represents the average of duplicate determinations in one rat.

The occupancy is approximately 50% for both antagonists (naloxone and diprenorphine) at site 1 but not at site 2.

5. DISCUSSION

I have previously demonstrated the existence of three binding sites by the use of an *in vivo* receptor binding technique; site 1 ($B_{max}1$ = 18 pmol/g brain) resembles the σ sites and site 2 ($B_{max}2$ = 15 pmol/g brain) appears to be a mixture of the δ + κ binding sites. Etorphine, sufentanil and diprenorphine do not bind to the third site, whereas naloxone exhibits appreciable affinity at site 3.[Chapter II] Since the agonists we have studied do not appear to interact with the novel naloxone binding site,[Chapter II] this discussion will be restricted to the occupancy of sites 1 and 2 at the ED_{50} of these four drugs.

In order to establish the pharmacological role of the opiate binding sites I determined the occupancy of these four drugs at site 1 and site 2 at the ED_{50} for eliciting (or inhibiting) opiate analgesia in the rat tail flick test. The pharmacodynamic parameters were estimated using the mass action model for competitive antagonism. The deviation between some of the observed and predicted values in the dose-response curves (Fig. III-1) may be due to the experimental design, i.e., the large number of animals involved required that some dose-response curves be obtained on different days. Furthermore, the use of the logistic function assumes continuous (i.e. graded) data, whereas the data was of quantal nature because of the all-or-none nature of the tail-flick response.[85]

In an earlier study, it was demonstrated that the opiate agonist etorphine exerts its analgesic action at a low fractional occupancy of receptors[42] in intact rats. In order to investigate further the relationship between opiate binding in the brain and binding site function, sufentanil was selected as a potentially μ specific agonist with sufficiently high affinity for in vivo receptor binding studies. Indeed, sufentanil was found to bind specifically to site 1 (μ) and, similar to etorphine, the fractional occupancy of site 1 was exceedingly low at the ED_{50}. The fractional receptor occupancy of etorphine and sufentanil at site 1 (~ 2%) was identical at the ED_{50}. This suggests that these two agonists act via the same receptor, represented here by site 1. However, etorphine binds to two distinct binding site populations in vivo (Table III-1) Chapter II. The fractional occupancy of site 2 at the ED_{50} for sufentanil and etorphine is 1.9×10^{-5} and 9.1×10^{-4}, respectively. The 50-fold difference in occupancy and the extremely small fraction of receptors occupied at the ED_{50} make it unlikely that binding to site 2 results in the analgesic activity of these drugs.

The finding of low fractional receptor occupancy at the ED_{50} for both etorphine and sufentanil indicates that the "occupancy assumption"[86] does not hold for these agonists; the concept of a linear relationship between fractional occupancy and degree of analgesia must therefore be rejected. In systems where there is a large "receptor reserve" (spare receptors or equivalent concepts[86]) the ED_{50} is not expected to coincide with the K_D.[87] Such discrepancies between ED_{50} and 50% receptor occupancy are not unique to the opiate receptor system, and have been observed in a variety of receptor systems,[24,43,88,89]

particularly with the use of binding techniques using intact cells or tissues[43,52,89] such as employed in this chapter.

In order to directly confirm the association between μ site binding and analgesia[32,33,76], the pharmacodynamic and in vivo receptor binding parameters of the antagonists naloxone and diprenorphine were studied. Since a competitive antagonist, by definition, does not elicit any pharmacological effect as a result of binding to a receptor, but rather prevents agonist action by blocking the receptors, a discrepancy between the K_D and ID_{50} would not be expected for an antagonist. This is observed here for both naloxone and diprenorphine for site 1 (μ site), whereas the antagonists display much lower and quite different occupancies at site 2 (6% and 19%, respectively) (Table III-1). The agreement of the naloxone and diprenorphine ID_{50} and K_D values at the μ binding site strongly supports the hypothesis that these agonists exert their analgesic activity (in the rat tail flick test) via interaction with the μ site. Moreover, the ID_{50} dose of the antagonists reduces the μ site binding at analgesic doses of both etorphine and sufentanil by 50%, even though only ~ 2% of these sites are occupied at the ED_{50}. This result argues against the possibility that the apparent low fractional occupancy of the agonists is merely a result of high affinity binding to a rather small as yet unidentified subtype. Therefore, in contrast to the agonists, the occupancy assumption appears to be valid for the antagonists for reversing opiate analgesia as measured by the rat tail flick test.

Although analgesia appears to be produced as a result of interaction with site 1 (μ site), the possibility that interaction with the other opiate binding sites (e.g. δ and κ) may modify analgesia must

also be considered. The dose-response curves of sufentanil, which is a highly selective μ ligand *in vivo*[Chapter II] and *in vitro*[68,69] are much steeper than those of etorphine, which unlike sufentanil also binds to site 2.[38] It has been proposed, by studying the *in vivo* binding and pharmacology of the opiate mixed agonist-antagonist buprenorphine, that opiate action at one receptor site may antagonize or modify that at another.[90] Such action was previously postulated by Martin, who proposed the concept of receptor dualism[91] (noncompetitive autoinhibition involving two different receptor types[90]). It is possible, therefore, that the differences in the steepness of the etorphine and sufentanil dose-response curves results from the interaction of etorphine with an additional receptor which modifies analgesia.

In conclusion, direct evidence is presented that binding to the μ site (site 1 as defined *in vivo*[Chapter II]) results in the analgesic actions of etorphine and sufentanil in the rat tail flick test. The μ site, therefore, corresponds to the μ *receptor* as defined by Martin,[8] i.e. the receptor mediating analgesia.

The conclusion that etorphine and sufentanil analgesia is mediated *via* the μ site must be qualified to analgesia in the rat tail flick test. Indeed, drugs which are classified as "μ agonists" and "κ agonists" from *in vitro* binding studies[77] exhibit quite different analgesic activity against heat, pressure, and chemical nociceptive stimuli.[92] It remains to be seen whether the analgesic activity of agonists in other pharmacological tests can be explained on the basis of the *in vivo* binding to the μ site alone, or whether interaction with other binding sites contributes to opiate analgesia.

CHAPTER IV

OPIATE MIXED AGONIST-ANTAGONISTS: THE CORRELATION BETWEEN *IN VIVO* RECEPTOR BINDING AND PHARMACOLOGICAL EFFECT

1. SUMMARY

The apparent equilibrium dissociation constants of a series of opiate mixed agonists at the μ binding site were determined *in vivo* by observing the displacement of [^{3}H]etorphine in rat brain. The following drugs were studied: ethylketocyclazocine, ketocyclazocine, cyclazocine, pentazocine, nalorphine, levallorphan, and buprenorphine. The μ binding site occupancy *in vivo* was determined at the analgesic ED_{50} of these drugs in three pharmacological test systems, i.e. rat tail flick, anti-writhing, and paw pressure tests in order to determine whether the occupancy data is consistent with the hypothesis that the action of these drugs results from interaction with only the μ sites. The following data argue against this hypothesis: i) μ site occupancy is >> 95% at the analgesic ED_{50} of some analogs, ii) except for naloxone, diprenorphine, or levallorphan the antagonistic AD_{50} (rat tail-flick test) occurred at μ site occupancies either above or below the expected 50% occupancy level, and iii) buprenorphine antagonizes morphine analgesia at an AD_{50} which results in a 99% occupancy of the μ sites. This is consistent with an earlier finding[67] that buprenorphine occupies an additional binding site population *in vivo* at antagonistic doses that is distinct from the μ site occupied at doses producing analgesia. Taken together, these data support the hypothesis that the analgesia and

antagonistic actions of the mixed agonists cannot be explained by interaction with the μ type of opiate binding sites alone.

2. INTRODUCTION

The major pharmacological effect of the opiates is their ability to produce analgesia against severe and continuous pain.[31] However, different pathways for pain perception appear to exist in the CNS,[93] and the neural mechanisms underlying morphine analgesia are different in different types of pain.[94] The pharmacodynamic experiments of Martin[8,9] support the concept of opiate receptor multiplicity, and heterogeneous opiate binding sites have been demonstrated in the CNS.[95] However, it has not been established which of these binding sites (μ, δ, κ, σ) mediates opiate analgesia.

Evidence that opiate analgesia is mediated *via* interaction with the μ binding site is indirect, and is based on a comparison of rank order affinities of a variety of opiates for different binding sites *in vitro* and the pharmacological potencies of these drugs in different pharmacological tests.[32,33] Ethylketocyclazocine, a purported κ-receptor agonist[34,35] has been claimed to exert analgesia in the rat tail flick test *via* interaction with the μ[96] as well as the κ[34] receptor types. Both the μ and κ receptors have been implicated in mediating the analgesic activity of drugs binding selectively to μ- and κ-binding sites;[35] however, the "μ receptor agonists" are effective against heat, whereas "κ-agonists" are more effective against chemical and pressure induced pain.[35,97] Additionally, the δ type binding site has been

implicated in the mediation of the analgesic behavior of metkephamid.[36] It therefore appears that there is no single receptor which mediates all types of opiate analgesia. In contrast, Pasternak and colleagues have published a series of reports[37,76,98,99] which state that the analgesic actions of a variety of opiates are primarily mediated by a single subtype of the opiate receptor system, i.e., μ_1. Therefore, the functional significance of the different opiate binding sites remains unclear.

There are a number of factors which compromise earlier conclusions based on *in vitro* binding experiments alone, such as disturbances of the binding sites and their environment *in vitro*.[38,39,Chapter I] A direct comparison of the *in vivo* binding behavior of a drug and its pharmacological activity circumvents such problems and is a useful tool for determining which actions are mediated *via* the various opiate binding sites. A method has been described for measuring opiate binding in the intact rat brain.[42,Chapter II] Using this technique I have established the existence of 3 distinct binding sites: Site 1 resembles the μ sites identified in other systems, site 2 has been tentatively identified as a hybrid of the δ and κ sites, and site 3 is a novel naloxone binding site.[Chapter II] A comparison of the *in vivo* binding and pharmacological parameters for the agonists etorphine and sufentanil and antagonists naloxone and diprenorphine demonstrates that the agonists exert their analgesic effects in the rat tail flick test *via* interaction with the μ binding sites at a low (~ 2%) fractional receptor occupancy.[Chapter III]

This chapter investigates the *in vivo* binding of a series of mixed agonists (levallorphan, nalorphine, buprenorphine, pentazocine, cyclazocine, ketocyclazocine, and ethylketocyclazocine) to site 1

(μ sites). The binding data are compared to the pharmacological parameters for these drugs as analgesic agonists and antagonists in a variety of pharmacological tests in order to determine if their actions can be explained solely on the basis of interaction with the μ site *in vivo*.

3. MATERIALS AND METHODS

3.1 Materials

[^{3}H]Etorphine (33-51 Ci/mmol) was donated by the NIDA (Rockville, MD) and was purchased from Amersham (Arlington Heights, IL). The tracer was purified by HPLC when purity was below 90%.[42] Unlabeled compounds were donated by: Etorphine HCl, diprenorphine HCl, buprenorphine HCl, nalorphine HCl: NIDA (Rockville, MD); ethylketocyclazocine methanesulfonate, ketocyclazocine, cyclazocine, pentazocine HCl: Sterling Winthrop (Rensselaer, NY); levallorphan tartrate: Hoffman La-Roche, Inc. (Nutley, NJ); naloxone HCl: Endo Laboratories (Garden City, NY). All doses are expressed as mg free base/kg. Female Sprague Dawley rats (100-200 g) were purchased from Simonsen Laboratories (Gilroy, CA).

3.2 Methods

3.2a In Vivo Displacement of [^{3}H]Etorphine by Unlabeled Drugs

Female Sprague Dawley rats were subcutaneously injected with [^{3}H]etorphine (15-80 μCi/kg) 20 mins prior to sacrifice, and the amount of bound and unbound labeled ligand determined using the rapid membrane filtration technique described by Perry et al.[42] Unlabeled ligands were injected simultaneously with the tracer, except for cyclazocine, ketocyclazocine, and ethylketocyclazocine which were administered 10 min prior to the tracer so that the *in vivo* labeling time would coincide with the time at which the pharmacological tests were performed (A.K. Pierson, personal communication). For the same reason, buprenorphine was injected 40 min prior to the tracer because of its slow receptor equilibration.[67]

3.2b Binding Parameter Estimation

Etorphine displacement curves were analyzed simultaneously using the following model, derived from the law of mass action, for 2 binding sites

$$B_L = U_L\left[\frac{Bmax_1}{U_L + K_{1L}(1+D/K_{1D})} + \frac{Bmax_2}{U_L + K_{2L}(1+D/K_{2D})} + NS_L\right] \qquad (1)$$

where B_L is the concentration of bound etorphine, $Bmax_1$ and $Bmax_2$ represent the concentration of binding sites for these binding populations; K_{1L} and K_{2L} represent the etorphine dissociation constants

at these two sites and K_{1D} and K_{2D} represent the dissociation constants at site 1 and site 2 of the competing ligand. D is the dose of competing ligand. U_L is the unbound concentration of labeled ligand and is defined as the difference between the total radioactivity in the brain and the amount of drug bound (specifically and nonspecifically) to the membranes on the filter. The non-specific binding is defined by the parameter NS_L.

I have previously determined the in vivo binding parameters for etorphine at site 1 and site 2.[Chapter II] Therefore, the following parameters were held constant: $Bmax_1$ = 18 pmol/g brain, $Bmax_2$ = 15 pmol/g brain, K_{1L} = 22 pmol/g brain, K_{2L} = 514 pmol/g brain. Therefore, the only binding parameters estimated are K_{1D} and K_{2D} for each competing ligand, and the non-specific binding parameter for etorphine in these experiments.

The variance (VARi) of the ith observation in the jth data set was modeled by equation (2):

$$VAR_{ij} = V_j B_j^{\,z} \qquad (2)$$

where V_j is the variance scale parameter for the jth data set. Each prediction of binding B_{ij}, regardless of the data set from which it originates, is raised to the common power Z.

Receptor occupancy at a given receptor site can be calculated using the following equation:

$$OCC = \frac{D}{K_j + D} \qquad (3)$$

where D is the dose at which the receptor occupancy is determined, and K_j is the dissociation constant of the drug at the jth binding site. Occupancy is then expressed as a percentage.

All numerical, statistical and graphical analyses were performed using the PROPHET system.[62] Parameter estimation was done by MKMODEL[63] using an extended least squares objective function.[58]

3.2c Determination of the "Antagonistic Character" of an Opiate Mixed Agonist

In order to qualitatively describe the degree of antagonistic character of a given drug the following arbitrary scale was constructed:

$$\text{A.C.} = \frac{ED_{50}\ \text{Agonist}}{AD_{50}\ \text{Antagonist (rat tail flick test)}}, \qquad (4)$$

where ED_{50} is the agonist dose that produces analgesia in 50% of the animals. The AD_{50} is a measure of antagonist potency. It is the antagonist dose that reduces the fraction of animals responding to an ED_{80}-ED_{90} analgesic dose by 50%. All of the mixed agonists studied here are active as antagonists against morphine, meperidine, and phenazocine antinociception in the rat tail flick test. Estimates of the AD_{50} were taken from the literature[35,100-116,Pierson personal communication] and averaged in order to estimate the typical AD_{50}. Similarly, the literature AD_{50} values (see above for references) for these drugs as agonists were averaged for a given pharmacological test in order to estimate a typical ED_{50}. Using such a scale, a drug which acts as a pure antagonist has a very low value for the antagonistic AD_{50} and is

not active as an agonist; the "antagonistic character" approaches infinity. Conversely, a pure agonist has an "antagonistic character" approaching zero.

RESULTS

4.1 Determination of Dissociation Constants and μ Binding Site Occupancy

The dissociation constants of the ligands for the μ site are listed in Table IV-1, along with the standard error of the parameter estimates. A summary of the pharmacological data obtained from the literature for these drugs as either agonists or antagonists is indicated in Table IV-2. All drugs exhibit quite different potencies in the three different groups of pharmacological tests; they are least potent in the tail flick, immersion, and pressure test group (Test group I).

Table IV-1. Dissociation Constants at Site 1 Determined by Displacement of [^{3}H]Etorphine *In Vivo*.

Drug	K_1 (µg/kg)	S.E.M. (µg/kg)
Etorphine	28	.819
Ethylketocyclazocine	342	157
Ketocyclazocine	909	332
Cyclazocine	52	7.8
Levallorphan	40	6.6
Nalorphine	152	16.6
Pentazocine	2201	227
Buprenorphine	26	8.6

Table IV-2. Summary of Pharmacological Data Obtained From Literature Values. The standard error of the mean (SEM) is indicated where appropriate. The number in parentheses represents the number of observations.

Drug	Test Group I*	SEM	Test Group II**	SEM	Test Group III***	SEM	Test Group IV****	SEM
Ethylketo-cyclazocine	8233	3974(3)	53	---(1)	110	--- (2)	---	---
Keto-cyclazocine	---	---	70	---(1)	245	--- (2)	4775	533 (4)
Cyclazocine	3325	---(2)	108	19(3)	---		24.86	1.1(7)
Levallorphan	---	---	41850	---(2)	---		39.4	3.9(4)
Nalorphine	25000	---(1)	1700	---(2)	190	--- (2)	104.5	3.6(8)
Pentazocine	11120	904(5)	3000	363(5)	5625	1160 (4)	5775	607 (4)
Buprenorphine	22	2(7)	26	10(3)	8.75	2.3(4)	110 (against phenazocine) 5500 (against morphine)	---(1) ---(1)

*Average ED_{50} Agonist (μg/kg), rat tail flick, tail immersion, tail pressure tests
**Average ED_{50} Agonist (μg/kg), bradykinin, other writhing tests
***Average ED_{50} Agonist (μg/kg), paw pressure (inflamed and normal) tests
****Average AD_{50}, Antagonist (μg/kg) vs. morphine, meperidine, phenazocine analgesia, rat tail flick test

The μ binding site occupancy at the agonistic ED_{50} in the three test groups is calculated using equation (3) and indicated in Table IV-3. Since values exist for all the drugs in the anti-writhing test group, (group II) the relationship between fractional receptor occupancy and antagonistic character (equation 4) for this test group is illustrated in Fig. IV-1. In general, it appears that the greater the degree of antagonistic character (or the lower the agonistic efficacy) the greater is the fraction of μ binding sites that needs to be occupied in order to exert the ED_{50} effect. In contrast, a very large fractional receptor occupancy is required for those mixed agonists active in the tail flick tests irrespective of the antagonistic character; over 80% of the μ binding sites are occupied at the ED_{50} for pentazocine, nalorphine, cyclazocine and ethylketocyclazocine for agonistic activity in this test group (Table IV-3).

Table IV-3. Percent occupancy at the Agonist ED_{50} at Site 1 for Mixed Agonists in Three Different Pharmacological Tests.

Drug	Rat Tail Flick Tail Immersion Rat Tail Pressure (Test Group I)	Anti-Bradykinin, Writhing (Test Group II)	Paw Pressure, (Inflamed and Normal) (Test Group III)
Ethyl-Ketocyclazocine	96	13	24
Ketocyclazocine	--	7	21
Cyclazocine	98	67	--
Levallorphan	--	99	--
Nalorphine	99	91	55
Pentazocine	83	57	71
Buprenorphine	46	50	25

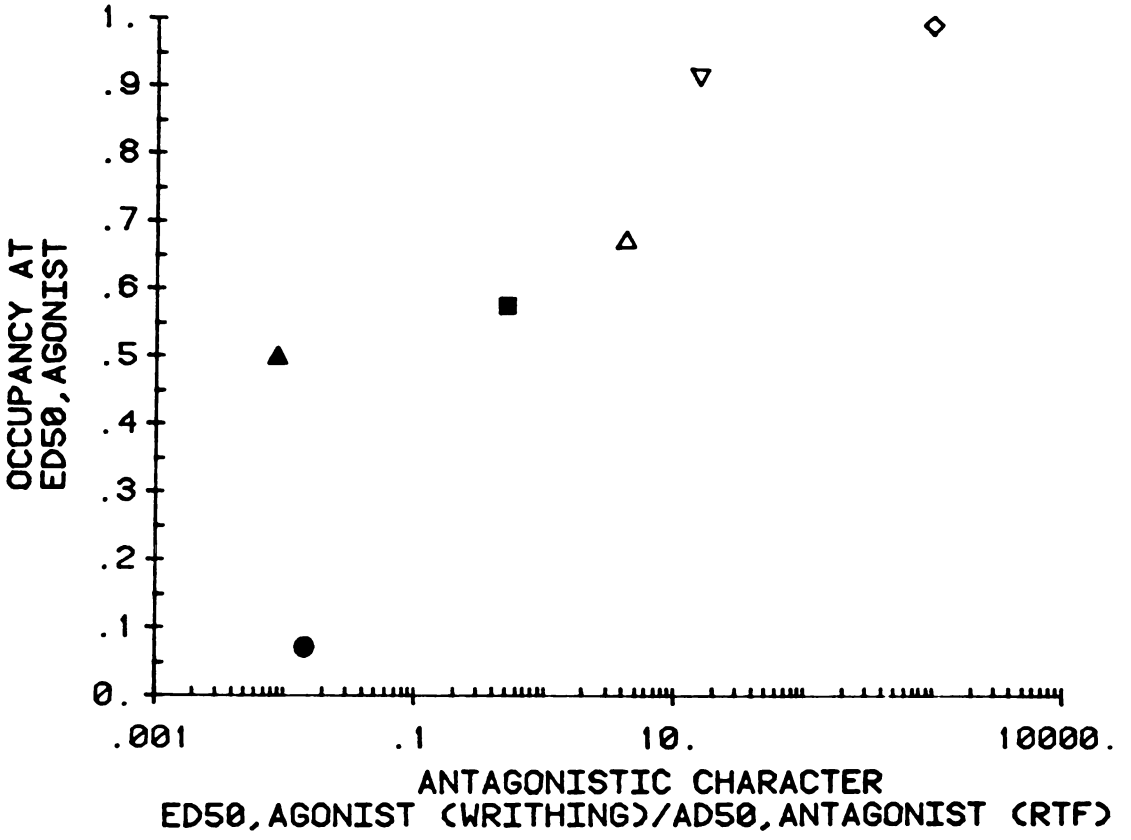

Fig. IV-1. In vivo -site occupancy for a series of opiate mixed agonists computed at the average analgesic ED_{50} in the anti-bradykinin, anti-NaCl, and anti-acetylcholine induced writhing tests. Occupancy was computed using equation (3); antagonistic character was computed according to equation (4) in order to express the relative agonistic:antagonistic potency. The drugs for which these values were computed are as follows: (●) ketocyclazocine, (▲) buprenorphine, (■) pentazocine, (△) cyclazocine, (▽) nalorphine, (◇) levallorphan.

The relationship between fractional receptor occupancy at the μ binding site and the antagonist AD_{50} (rat tail flick test) for these drugs is illustrated in Fig. IV-2. Except for ethylketocyclazocine, all drugs exhibit antagonistic actions in the rat tail flick test against morphine, meperidine, and phenazocine analgesia. Only buprenorphine exhibits clearly different antagonistic potency against phenazocine in comparison to morphine. The data for the pure antagonists naloxone and diprenorphine[Chapter III] are included for comparison. The line represents the prediction that 50% of the receptors are occupied (blocked) at the antagonist AD_{50}, or that a linear binding-effect relationship exists for the antagonists (see Chapter III). Diprenorphine, levallorphan and naloxone are the only drugs studied here which occupy 50% of the μ sites at their pharmacological AD_{50}.

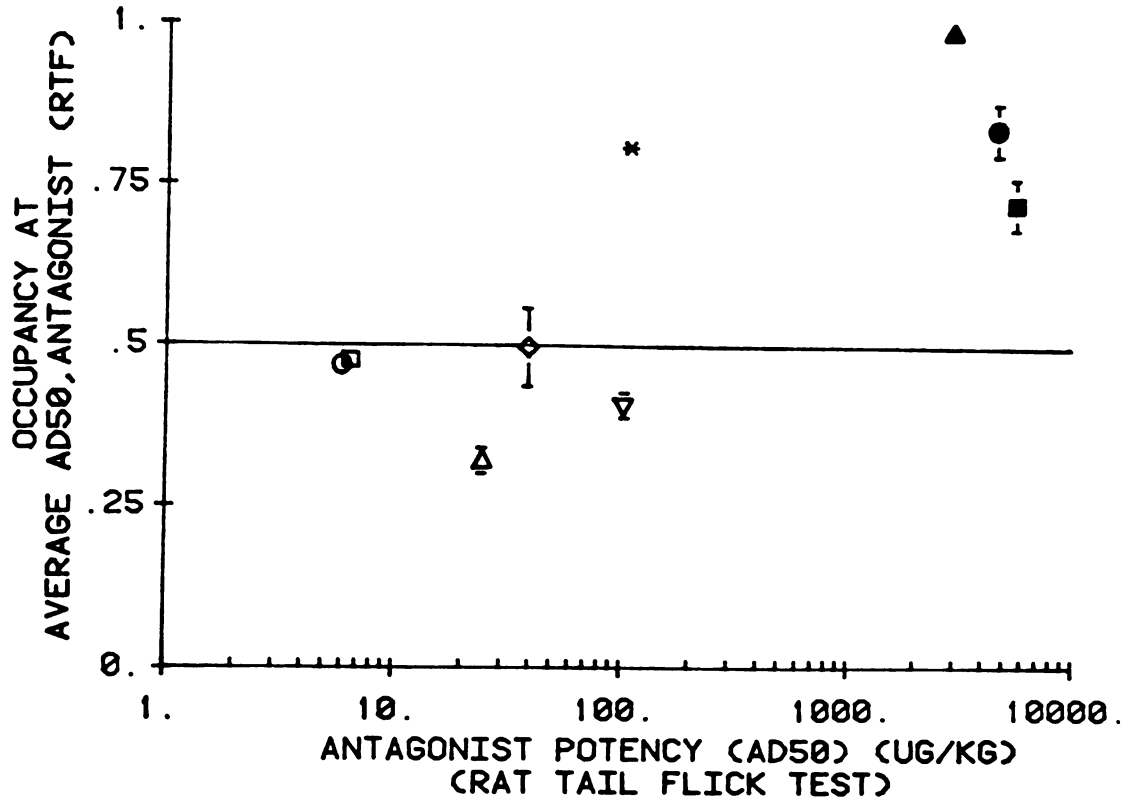

Fig. IV-2. In vivo **-site occupancy for a series of opiate mixed agonists at the average AD_{50} for antagonism of morphine, meperidine, and phenazocine analgesia in the rat tail-flick test.** Occupancy was computed using equation (3). Since the AD_{50} values used are averages of those values obtained from the literature (see Methods), the error bars are included to show the SEM for the computed occupancy values. The drugs for which the values were obtained are as follows: (◇) levallorphan, (△) cyclazocine, (▽) nalorphine, (●) ketocyclazocine, (■) pentazocine, (□) naloxone, (○) diprenorphine, (*) buprenorphine: against phenazocine analgesia, (▲) buprenorphine: against morphine analgesia.

5. DISCUSSION

5.1 Estimation of the Dissociation Constants

I have previously demonstrated that etorphine labels two binding site populations *in vivo*, the first (site 1) resembles the μ binding sites and the second (site 2) appears to be a mixture of the δ + κ sites (Rosenbaum, et al., submitted). Although etorphine exhibits considerable (~ 20 fold) selectively for site 1 over site 2, a two binding site model is, nevertheless, necessary to describe the binding data as etorphine does have access to site 2.[38,Chapter II] The standard errors of the estimate for the K_D of the mixed agonists at site 2 were so large, however, (50-100%) that a reliable estimate of these values cannot be obtained when using etorphine as the labeled ligand. Moreover, these ligands did not discriminate between the putative δ and κ sites as no significant improvement in the fit of the data was observed with a three binding site model.

The mixed agonists that were chosen for this study are representatives from the morphinan, oripavine, and benzomorphan structural classes. They were chosen because they exhibit a wide range of efficacies in various pharmacological tests.

5.2 Agonistic Effects of the Mixed Agonists

It has previously been demonstrated that the pure opiate agonists etorphine and sufentanil exert their analgesic actions at the μ receptor at a low (2%) fractional receptor occupancy.[42,Chapter III] In systems

where maximum effects can be produced by agonists occupying a very small percentage of the receptor population, it follows that agonists with different intrinsic activities will need to occupy varying proportions of receptors in order to elicit the same level of effect.[78] Consequently, partial agonists may occupy a much greater proportion of receptors than do pure agonists,[78] and the receptor occupancy of such drugs should increase with decreasing intrinsic activity if the agonistic effect is mediated *via* interaction with only one receptor population.

Since I did not have a quantitative measure for the intrinsic activities of the opiate mixed agonists studied here, I constructed an arbitrary scale of agonistic-antagonistic character. Figure IV-1 illustrates the relationship between this arbitrary "antagonistic character" and μ site occupancy for the same level of effect (ED_{50}) in the bradykinin and acetylcholine or NaCl-induced writhing test. It appears that a relationship does exist at least for buprenorphine, ketocyclazocine, pentazocine, and cyclazocine ($r^2 = .36$). These results provide suggestive evidence for the hypothesis that fractional receptor occupancy at the agonistic ED_{50} increases with increasing "antagonistic character" of the mixed agonist. However, it is less likely that nalorphine and levallorphan exert their analgesic effects in this test *via* interaction with the μ site alone, as 90-99% of the μ binding sites are occupied at the ED_{50}. Occupancy of all the μ sites, therefore, will result in not much more than 50% of maximum possible effect; yet maximal analgesia is obtainable with these drugs. No relationship exists between fractional receptor occupancy at the agonistic ED_{50} and antagonistic character for these drugs in the thermal stimulus tests (Table IV-3); all drugs studied except ketocyclazocine and levallorphan

exhibit activity in this test. In this test group, ethylketocyclazocine, cyclazocine, and pentazocine require a very large (80-100%) fractional receptor occupancy at their agonistic ED_{50}, irrespective of their antagonistic character. Fractional receptor occupancies >50% at the ED_{50} can be explained by allosteric ligand-receptor interactions[124] and/or the concept of partial agonism. Therefore, occupancy measurements which differ from 50% at the ED_{50} do not rule out the possibility that agonistic action is mediated by only one binding site. However, data such as that obtained for levallorphan are more difficult to explain by the above concepts alone. In contrast, the analgesic activity of the pure agonists sufentanil and etorphine is explainable by interaction with the μ site alone.Chapter III

It has been shown that drugs with rather high affinity for κ sites in vitro are less potent in analgesic tests employing thermal nociceptive stimuli than in the paw pressure or writhing tests.[35] Tyres has concluded that μ receptor interaction is therefore necessary in order for a drug to exhibit antinociceptive behavior against thermal stimuli. While μ site interaction may be necessary for such activity, the data presented here indicate that interaction with another site must be involved in order to explain the action of the "κ drugs" (ethylketocyclazocine, cyclazocine, pentazocine) in the thermal stimulus test group. Furthermore, although "κ agonists" may be more potent in the writhing and paw pressure tests,[35] this does not mean that analgesic activity in these tests results from κ site interaction. Whereas the action of levallorphan and nalorphine in the anti-writhing tests cannot be explained on the basis of site 1 interaction alone, action in the paw pressure tests might possibly be mediated via this site (Table IV-3).

5.3 Antagonistic Actions of the Mixed Agonists

Since a competitive antagonist, by definition, does not elicit any pharmacological effect as a result of binding to a receptor (i.e. intrinsic activity = 0),[78] but rather inhibits agonist action by blocking the receptors, it is expected that the extent of antagonism will be directly proportional to the degree of receptor occupancy (occupancy assumption).[86] Figure IV-2 illustrates the deviation from this assumption (i.e. 50% of the receptors are occupied at the antagonistic AD_{50}) for this series of opiates acting as antagonists. I have already shown that the antagonists naloxone and diprenorphine conform with the occupancy assumption;[Chapter III] these drugs are included in the figure for comparison purposes. Of the other drugs investigated, only the antagonistic action of levallorphan can be explained by occupancy of the μ site alone.

Drugs which lie below the predicted line (Fig. IV-2) occupy less than 50% of the μ sites at the antagonistic AD_{50}. It is conceivable that these drugs (nalorphine and cyclazocine), which are thought to be "κ agonists"[9,31,35] interact at another site, and it is this activity which inhibits analgesia.

Drugs which lie above the predicted line (Fig. IV-2) occupy more than 50% of the μ sites at the antagonistic AD_{50}. This behavior is explicable by the allozyme hypothesis: the opiate receptor is thought to be coupled to adenylate cyclase,[24] and it has been proposed that antagonists have an effect which is less than their receptor occupancy (allozyme hypothesis) in adenylate cyclase coupled systems.[117] However, this deviation from the predicted line can also be explained on the

basis of binding to another receptor population which is required for the antagonistic effect. Indeed, it has been shown that at antagonistic doses buprenorphine occupies an additional binding site population in vivo distinct from those occupied at doses producing analgesia in the rat tail flick test.[67] Figure IV-2 illustrates that buprenorphine occupies 99% of the μ sites at the AD_{50} required to antagonize morphine analgesia (5.5 mg/kg,).[116] This leaves only 1% of the receptors available for buprenorphine to reverse analgesia completely and suggests that an additional site is required for the antagonistic actions of buprenorphine. On the other hand, it should be pointed out that buprenorphine antagonizes phenazocine analgesia at a much lower fractional occupancy of the μ site (81% at 110 μg/kg), suggesting that its mechanism of antagonism may differ depending upon the agonist.

5.4 The Concept of Mixed Agonism-Antagonism

Martin[91] has proposed that the action of the opiate mixed agonist-antagonists is explained by the concepts of partial agonism and interaction with multiple receptor types. The data presented here support this hypothesis that both the agonistic and antagonistic actions of many of these drugs can be explained by interaction with multiple receptors.

DISCUSSION AND PERSPECTIVES

A. Differences in Opiate Receptor Binding In Vivo and In Vitro

Sadée and co-workers have previously demonstrated that a number of differences exist in the binding characteristics of opiates *in vivo* and *in vitro*: The *in vitro* receptor binding capacity for diprenorphine is less after extensive homogenate dilution or membrane washing procedures (13-22 pmol/g brain) than it is in the intact rat (~30 pmol/g brain).[39] While the agonist etorphine and the antagonist diprenorphine exhibit similar affinities *in vitro*, etorphine has considerably less affinity than does diprenorphine *in vivo*.[38] It was concluded that this difference is due to the combined action of Na^+ and guanine nucleotide on the agonist, but not the antagonist, binding.[38] Furthermore, the *in vivo* dissociation rate of etorphine was shown to be much faster than that obtained *in vitro*, even in the presence of the regulatory factors Na^+ and guanylyl imidodiphosphate (GPP(NH)P).[42]

Chapter I expands on the latter finding. Chapter I indicates that the loss in sensitivity of the etorphine dissociation rate to regulation by Na^+ and GPP(NH)P is actually a loss in sensitivity to GPP(NH)P rather than Na^+. Koski et al.[118] have suggested that the opiate receptor exists in a precoupled form to the guanine nucleotide regulatory protein (N unit) of the adenylate cyclase system. The decrease in sensitivity to the regulation by guanine nucleotide *in vitro* in comparison to that observed after *in vivo* binding may therefore be an indication that the coupling of the receptor to the N unit is partially destroyed by *in*

in vitro work-up procedures. Whatever the mechanism, the data in Chapter I indicate that the receptor environment is partially destroyed *in vitro*.

B. Multiplicity of Opiate Binding Sites In Vivo

In view of the differences in the binding characteristics *in vitro* and *in vivo* it is necessary to address the question of opiate receptor multiplicity in the intact animal to see which of the opiate binding sites previously demonstrated *in vitro*[18] actually exist *in vivo*.

Not all of the binding sites that have been demonstrated *in vitro* (μ, δ, κ, σ, ε) were demonstrable in this study. This is probably due to the fact that the types of binding sites that are described are dependent on the ligands employed in the investigation, and the ligands employed in this study only allowed the discrimination of three types of binding sites. Chapter II discusses the three types of binding sites which were discriminated in the intact rat brain. I have concluded that site 1 resembles the μ sites previously characterized in *in vitro* studies owing to the specificity of sufentanil for this site. The selectivity of etorphine and naloxone for site 1 supports this conclusion. I have also suggested that site 2 is a mixture of the δ and κ sites. Differentiation of these sites *in vivo* requires the use of ligands not employed in this study.

The technique described in Chapter II permits the demonstration of the existence of a novel naloxone binding site *in vivo* not characterized by *in vitro* methods. Squires and Braestrup[72] have described a binding site for naloxone *in vitro* which is not demonstrable at temperatures $>20^{o}C$. This site has escaped further characterization due to its

extreme lability *in vitro*. This finding underscores the importance of the *in vivo* binding technique. Whether this binding site represents a distinct entity or is another conformation of one of the known opiate binding sites (Grevel and Sadée, unpublished) bears further investigation. However, the existence of this site *in vivo* leads to the speculation that it may mediate some of the unusual effects of naloxone.[119-121]

C. Which Binding Site Represents the Receptor Mediating Opiate Analgesia?

Chapter III represents the first direct data implicating the μ sites as the mediators of the analgesic activities of etorphine and sufentanil in the rat tail flick test. The conclusion that the μ site mediates opiate analgesia is supported by other studies;[32,33] however, these authors have arrived at this conclusion indirectly by comparing rank order potencies *in vivo* with binding site affinity *in vitro*. Chapter III indicates that, since there are a number of differences in the *in vitro* and *in vivo* binding characteristics, such a comparison is not necessarily valid. This chapter also establishes the criteria necessary for associating analgesic activity with a given binding site; such criteria are applicable for all receptor systems which exhibit a non-linear relationship between drug binding and effect.

The conclusion that is drawn in Chapter III (μ receptor interaction results in opiate analgesia) may at first seem to contradict that drawn in Chapter IV, i.e. μ receptor interaction alone cannot explain the analgesic and antagonistic effects of a series of opiate mixed agonist-

antagonists. Two concepts are necessary in order to resolve this apparent discrepancy: 1) Only those drugs having a very high efficacy (i.e. pure agonists)[78] at the μ site as well as selectivity for this site will produce analgesia *via* interaction with the μ site alone. The opiate mixed agonist-antagonists do not achieve the maximum possible pharmacological effect in many test systems,[8,9,92] and have therefore been defined as partial agonists[91] with lower efficacy[78] at the μ site. Furthermore, many of these drugs do not bind selectively to the μ sites.[77] 2) The μ binding site is not the only receptor capable of producing opiate analgesia. Indeed, a variety of binding sites have been implicated in the mediation of opiate analgesia.[34,35,36]

D. Limitations to the In Vivo Approach

The *in vivo* approach requires the performance of a number of control experiments which are not required in *in vitro* systems: It is highly important to understand the nature of the radioactivity adhering to the membranes on the glass fiber filter, as this radioactivity is converted (through the use of the specific activity value) to the amount of drug that is receptor bound. For this reason, experiments were designed to analyze the radioactivity on the filters, and for all labeled ligands investigated >95% of the radioactivity could be accounted for as unchanged drug, ruling out the possibility of interference from metabolites.[39,42,Chapter II] It is also important to minimize the degree of reassociation and dissociation of the ligand-receptor complex during the time period between sacrifice of the animal and the time of filtration of the brain homogenate (~ 1 1/2 min). For

this reason, the brains were homogenized in 0° Tris buffer and filtered immediately; furthermore, it was determined that there is no significant reassociation or dissociation of the labeled ligands during this time period.[39,42] These issues have been addressed in earlier papers from Sadée's laboratory which describe the in vivo approach.[38,39,42]

The major criticism of the in vivo approach has been the issue of equilibrium. As it is not possible to demonstrate, under the conditions employed in these studies, that the binding reaction between the ligand and the receptor is truly at equilibrium, I have performed all experiments at the time of peak binding of the tracer. This is the time at which a "pseudo-equilibrium" is reached, as at this time there is no net association or dissociation of the ligand-receptor complex, or the rate of change in the amount of complex formed is equal to zero. (The rationale for picking the 20 min time point is discussed in Chapter II). Since the issue of true equilibrium cannot be resolved, the apparent dissociation constants that I have determined using the mass action model (Chapter II) may be somewhat in error. The value of the apparent equilibrium dissociation constant, as defined in the model, represents that concentration of ligand which occupies 50% of the binding sites.

In addition, the assumption has been made that the cold competitor (displacing ligand) does not alter the in vivo time course of the labeled ligand in a dose dependent fashion. However, this may not be the case, and it should be noted that this assumption has not been tested. Deviations in the time of peak binding would introduce errors in the estimates of the apparent equilibrium dissociation constants as all experiments were performed at the same time (20 min).

Finally, as the free concentrations are not known using the in vivo approach, the data have been modeled using the mass action equation in terms of the unbound concentrations (Chapter II). Therefore, the apparent equilibrium dissociation constants determined in this study are not directly comparable to those determined in in vitro studies. However, these constants are directly comparable to the pharmacodynamic parameters ED_{50} and ID_{50}.

E. Ramifications of This Dissertation

The in vivo binding method has been developed, and by using the established criteria, it is now possible to relate drug dosage, binding to the different sites, and pharmacological effect. It is now possible to relate the other effects of opiates (tolerance and physical dependence, respiratory depression, inhibition of GI motility)[31] to binding at the various opiate binding sites in vivo. After the determination of the identity of binding site 2 (tentatively identified as a mixture of the δ and κ sites), it will be possible to determine the functions of all the various opiate binding sites demonstrated in vivo, and which ligands are specific for these sites. Thereby, it will become possible to design new drugs, specific for one receptor, which will have predictable pharmacological effects.

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EPILOGUE

Thus if we trace the history of opium from its earliest beginnings to the brilliant researches of recent years, if we but compare the analytic and synthetic, chemical, physiologic and pharmacologic studies of the same old drug with the fantastic and puerile effusions on the subject of our medical predecessors, we cannot help being impressed with the long strides forward which medicine has made; yet, on the other hand, our very recent studies on opium and its alkaloids serve but to emphasize the more our meager knowledge of the subject and the still greater task before us.

- David I. Macht, 1915

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