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Abstract

Title of Thesis: Heterogenity of Opioid Binding Sites In Guinea Pig Spinal Cord

Gary Dean Zarr MAJ/ANC, Master of Science, 1984

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High concentrations of opioid peptides derived from both proenkephalin and prodynorphin are present in spinal cord, and studies of their localization within spinal cord suggest the opioids may be involved in the regulation of both sensory and motor functions. Sensory deficits and disturbed motor functions following the local administration of opiates or opioid peptides to spinal cord also suggest a multiplicity of endogenous opioid regulatory functions in this tissue.

Studies of radiolabelled opioid ligand binding to both regional sections or whole spinal cord membrane preparations confirm that saturable high affinity binding sites in this tissue show heterogeneity of binding properties. However, many of these studies have been conducted at non-physiological temperatures and in buffers lacking major physiological cations. Past studies have shown that opioid receptor binding is significently affected the by presence of major physiologic cations as well as by varations in temperature. In this study of radiolabelled saturable high affinity stereoselective specific binding sites in whole spinal cord as well as lumbo-sacral spinal cord, conditions which parallel the *in vivo* situation were adhered to as closely as possible. The saturable binding of [^{3}H] Tyr-D-Ala-Gly-MePhe-NH $(CH)_2$ OH ([^{3}H]DAGO), [^{3}H]D-Ala^2-D-Leu^5 enkephalin ([^{3}H]DADLE), and [^{3}H]ethylketocyclazocine ([^{3}H]EKC) in spinal cord membrane preparations with Na^+ and other major physiological cations at pH of 7.4 and temperature of 37^0C were studied in the presence and the absence of selective blocking ligands. In addition, displacement studies of the above radiolabelled compounds with a variety of unlabelled compounds in the presence and

absence of site selective opioid blocking drugs were performed to establish receptor subtype selectivities for a variety of ligands and to verify the B_{MAX} values that were derived from Scatchard analyses. Hill slope analysis was carried out before and after appropriate selective opioid blocking drugs were added to the displacement assay tubes and an increase of the Hill slope value toward unity was taken as evidence that the opioid blocking drug had produced a more homogeneous receptor population. Experiments indicate that both the radiolabelled and unlabelled drugs used in the binding assay were stable under the assay conditions for up to 60 min without necessitating the addition of peptidase inhibitors. Isolated binding sites with mu (μ), delta (δ), and kappa (k) receptor type characteristics were readily identifiable.

Ligand affinities measured in the presence of major physiologic cations and temperature may give more useful indices of opiate drug binding site selectivity. Additionally, ligand receptor selectivity measured under conditions of physiological cation concentration and temperature should be more useful for comparison with pharmacologic selectivity and drug concentrations mediating pharmacological effects *in vivo*.

HETEROGENEITY OF OPIOID

BINDING SITES IN

GUINEA PIG SPINAL CORD

by

Gary Dean Zarr

Thesis submitted to the faculty of the Department of Pharmacology
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To my family and friends who have always given me support and encouragement during good times as well as times of adversity.

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Introduction

In Vitro Evidence For Opioid Receptors

The presence of stereospecific saturable opioid receptor binding sites in neural tissue was initially reported by three independent laboratories (Pert and Snyder, 1973a; Simon et al., 1973; Terenius, 1973). Pert and Snyder (1973a) used [^{3}H]naloxone as a radioligand to study binding to guinea pig ileum and brain as well as mouse and rat brain. Simon and co-workers (1973) used [^{3}H]etorphine, an opioid agonist, to study receptor binding in rat brain homogenates. Terenius (1973) used the opioid agonist [^{3}H]dihydromorphine as a radioligand to study receptor binding in cerebral cortex synaptic membrane fractions. Quantitative analysis of specifically bound drug was determined by separation from free drug in solution by centrifugation (Terenius, 1973), or filtration through glass fiber filters (Pert and Snyder, 1973a; Simon et al., 1973). The advantage of the rapid filtration technique is that a greater portion of the more rapidly dissociating non-specific binding component is washed out during filtration, resulting in improvement in the ratio of specific to non-specific binding. These three laboratories utilized radioligand binding techniques that were first described in principle by Goldstein and co-workers (1971). These investigators used [^{3}H]levorphanol to describe both saturable and non-saturable non-specific, as well as specific binding. In addition, they showed that specific binding to opioid receptors could be computed by subtracting the amount of labelled opiate bound in the presence of a large molar excess of the unlabelled form of the drug from the amount of binding in the presence of the same concentration of the inactive positive isomer of opiates. Additional opioid receptor binding studies (Pert and Snyder, 1974) showed a maximum of binding at pH 7.4. Pert and Snyder (1974) also showed that temperature and ionic composition affected specific opioid binding. The opioid antagonoist [^{3}H]naloxone in the presence of Na^+showed a 1.7 fold increase in specific binding when the temperature was increased from 5°C to 35°C. The opioid agonist [^{3}H]dihydromorphine in the presence of Na^+ showed a 7 fold increase in specific binding when

temperature was increased from 5°C to 25°C. Temperatures above 40°C resulted in a reduction of specific binding. Pharmacological relevance of *in vitro* opioid receptor binding studies was established (Pert and Snyder, 1973a,b; Simon et al., 1973) by the fact that the ability of unlabelled opiate drugs to inhibit the specific binding of [^{3}H]naloxone or [^{3}H]etorphine correlated well with their relative *in vivo* potencies as analgesic antagonists or agonists.

Isolation of Endogenous Opioid Peptides

The discovery of opioid receptors prompted the search for endogenous opioid like substances. The existence of endogenous receptors that only had high binding affinity for opiate alkaloid substances injected into the body was felt to be unlikely. The existence of endogenous opioid polypeptide substances in whole brain or from pituitary extracts was discovered by several groups (Terenius and Walstrom, 1974; Hughes, 1975a; Teshemacher et al., 1975; Cox et al., 1975). These opioid polypeptides interacted with opioid receptors in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassay preparations in a naloxone reversible fashion. The polypeptide identified by Hughes (1975a) was later established by Hughes and his co-workers (1975b) to be two closely related pentapeptides, with the sequences Tyr-Gly-Gly-Phe-Met (methionine enkephalin or [Met^5]enkephalin) and Tyr-Gly-Gly-Phe-Leu (leucine enkephalin or [Leu^5]enkephalin). It was soon shown that these pentapeptides were part of a larger polypeptide isolated from pituitary extracts (Cox et al., 1975; Teshemacher et al., 1975), ultimately leading to the discovery of and subsequent naming of the opioid active peptide β-endorphin. The larger polypeptide containing β-endorphin was isolated from pituitary extracts and shown to be β-lipotropin (β-LPH) which had been characterized by Li and co-workers (1965). The suggestion (Cox et al., 1976) that β-LPH, a pituitary hormone, with a 91 amino acid sequence was actually a prohormone that should have opioid activity in the β-LPH (61-91) fragment and no opioid

activity in the ß-LPH (1-60) fragment was proposed for two reasons. Firstly, the methionine enkephalin pentapeptide isolated by Hughes and his co-workers (1975b) was contained within the ß-LPH-(61-65) fragment, and secondly, residues 59 and 60 were Lys and Arg. It was known that in other prohormone systems a cleavage and removal of the Lys-Arg residues occurs on the carboxyl group side of the paired basic amino acids by a carboxypeptidase A mechanism. Another smaller polypeptide isolated from pituitary tissue (Cox et al., 1975; Lowney et al., 1979) had properties different from ß-endorphin. It had a lower molecular weight than ß-endorphin, was resistant to cyanogen bromide, and had a longer latency in reversal of its inhibitory effect on the GPI bioassay preparation. Futher work on this smaller pituitary peptide (Goldstein et al., 1979) resulted in the purification and identification of the sequence of the first thirteen amino acids of what this group called dynorphin due to its potency in the MVD and GPI bioassy. Dynorphin (1-13) was later shown to have full potency of the subsequently characterized heptadecapeptide now known as dynorphin A. Conclusive evidence that these three major opioid peptide groups are independently processed was provided by the isolation of mRNA coding for each of the opioid peptide precursors. Pre-proopiomelanocortin (POMC) from which ß-endorphin and adrenocorticotropin (ACTH) are derived was the first precursor to be described (Nakanishi et al., 1979). A mRNA that codes for the precursor of the enkephalins was characterized (Noda et al., 1982). A mRNA coding for porcine pre-prodynorphin has been described (Kakidani et al., 1982). Since the discovery of the enkephalins, ß-endorphin, and dynorphin, many other opioid active peptides derived from the same precursors have been discovered. However, in many cases it remains to be demonstrated whether many of these peptides are selectively processed or are fragments of dynorphin or ß-endorphin.

Heterogenity of Opioid Receptors

The discovery of multiple endogenous opioid peptides led to the hypothesis that there

would be multiple receptors for these endogenous peptides. Heterogeneity of opioid receptors was first postulated on the basis of physiologic studies in the chronic spinal dog (Martin et al., 1976; Gilbert and Martin, 1976) and studies of opioid action in *in vivo* bioassay in GPI and MVD (Lord et al., 1977). The three opioid receptor types proposed by Martin and co-workers were mu (μ), the morphine selective site, kappa (k), the ketocyclazocine selective site, and sigma, the SKF-10047 selective site. An opioid receptor site in MVD that selectively bound leucine enkephalin and methionine enkephalin was described (Lord et al., 1977) and this group named this site the ∂ receptor. This same receptor site was later identified by *in vitro* binding experiments using homogenates of MVD and [^{3}H-Tyr^1-D-Ala^2-D-Leu^5]enkephalin ([^{3}H]DADLE) a stable analog of [Leu^5]enkephalin as a radioligand (Leslie et al., 1980). In addition, evidence for the epsilon (ε) opioid receptor site, a site that has a high affinity for [^{3}H]β-endorphin has been presented (Law et al., 1979; Ferrara and Li, 1980; Akil et al., 1980). Additional evidence for opioid receptor heterogeneity has been supplied by protection of receptor sites with a selective unlabelled ligand and alkylating unprotected sites with β-chlornaltrexamine (James and Goldstein, 1984). A further technique to study receptor heterogeneity is to utilize a radioligand that is partially selective for a preferred receptor type, in the presence of high concentrations of unlabaelled ligands that are partially selective for the non-preferred receptor types (Gillan and Kosterlitz, 1982). The utilization of high concentrations of blocking drugs is applicable to binding assays but not to *in vivo* bioassay due to the blocking ligand producing opioid agonistic or antagonistic effects. In addition to the receptor sites discussed, subtypes of μ and k receptors have been proposed. Pasternak and co-workers have proposed μ_1 and μ_2 receptor subtypes based on *in vitro* binding assays which indicate heterogenity of binding sites in biphasic Scatchard plots and complex displacement studies, as well as *in vivo* analgesia and toxicity tests. In these studies pretreatment of mice with naloxazone, an opiate receptor antagonist, resulted in loss of a high affinity site which represented 10 % of the total receptor sites for [^{3}H]naloxone. The loss of these sites resulted in a marked reduction of morphine's analgesic potency, but not its lethal

effects. This would imply that the analgesic effects of morphine are mediated by the high affinity receptors that are lost with naloxazone treatment and that the lethal effects of morphine are mediated by the receptors that are resistant to naloxazone treatment. Evidence for the existence of k_1 and k_2 subtypes has been presented (Attali et al., 1982; Pfeiffer et al., 1981). Attali and co-workers (1982) utilized guinea pig lumbo-sacral spinal cord to study k subtypes. These authors were unable to identify μ binding in guinea pig spinal cord using the radioligands [^{3}H]DADLE or [^{3}H]dihydromorphine. In this work k-subtypes were defined using [^{3}H]ethylketocyclazocine ([^{3}H]EKC) as the radioligand. k_1 sites are proposed to be sites not blocked by 5 μM [D-Ala^2-Leu^5]enkephalin (DADLE) while k_2 sites are the sites labelled by [^{3}H] EKC that are blocked by 5 μM DADLE. The most potent ligand for the k_2 sites described by Attali and co-workers was β-endorphin while benzomorphan drugs had a high affinity at the k_2 site as well. However β-endorphin is not found in high concentrations in the spinal cord (Lewis et al., 1983) therefore it is unlikely that β-endorphin is the endogenous ligand for this receptor. Additional evidence for a benzomorphan site that may be a k-subtype is offered by Chang and co-workers (1981). The endogenous ligands for various opioid receptors are not clear in all cases. However, there is good evidence that "dynorphin" (1-17) is the endogenous ligand at the "k" type receptors (Cox and Chavkin, 1983). At this time the most probable endogenous ligands for the "δ" type receptor is "[Leu^5]enkephalin" and "[Met^5]enkephalin" (Lord et al., 1977; Cox et al., 1980). Recent evidence for an endogenous opioid that is immunoreactive with antibodies to morphine has been presented (Goldstein and Lowney, 1984). The possibility that this "newly discovered endogenous opioid substance" may be an endogenous ligand for the "μ" type receptor has not been thoroughly examined. The sigma opioid receptor may not be a classic opioid receptor. N-allylnorphenazocine (SKF-10047), a compound with psychotomimetic activity is a high affinity ligand for the sigma receptor. In addition SKF-10047 and cyclazocine displace [^{3}H]phencyclidine with

high affinity (Quirion et al., 1981). Not all of the actions of SKF-10047 are naloxone reversible (Zukin and Zukin, 1984; Wood, P.L., 1981). The ϵ receptor has not been well characterized at this time, therefore in this thesis only μ, δ and k opioid receptors will be considered further.

Location of Opioid Receptors

Opioid receptors are predominantly associated with neural tisssue. Opioid receptors found in tissue outside the central nervous system (CNS) have also been shown to be associated with neural tissue. Removal of the myenteric plexus from the guinea pig intestine resulted in a lack of any specific opioid receptor binding (Pert et al., 1973a). Analysis of opioid receptor binding in subcellular fractions of whole brain homogenates has shown opioid receptor binding in all fractions (Pert et al., 1974b). The crude mitochondrial fraction (P_2) contained 50% of all binding and the second supernatant (S_2) contained 17 % of all binding. Further subfractionation of P_2 revealed significant binding in all fractions with highest levels located in synaptosomes. The high concentration of opioid receptors in synaptosomes suggests that opioid receptors are associated with areas of synaptic contact. Evidence that the synaptic pattern of enkephalin staining opioid terminals on thalamic projection neurons may have a functional organization was shown in recent work by Ruda and co-workers (1984). In this work thalamic projection neurons in lamina I of spinal cord receive immunoreactive enkephalin staining contacts on the soma or proximal dendrites of the neuron. Thalamic projection neurons that receive enkephalin contacts in lamina V of the spinal cord are multipolar cells that received enkephalin contacts on their distal dendrites.

Regional Distribution of Opioid Receptors

Localization of opioid receptors within various sites in the CNS has been studied by four

primary techniques. Gross disection of brain and analysis of opioid receptor density by *in vitro* receptor binding assays has been used extensively (Hiller et al., 1973). Another technique is the intravenous administration of tritium labelled opiate agonists or antagonists and separation of bound drug from total drug in the tissue by washing homogenates of selected brain regions (Pert et al., 1975). Some studies employ *in vivo* autoradiographic localization of opioid receptors in brain (Pert et al., 1976), or *in vitro* autoradiographic techniques (Goodman et al., 1980; Schubert et al., 1981). In addition, immunocytochemical techniques combined with electron microscopic analysis have been utilized to isolate opioid containing neurons (Hunt et al., 1980; Ruda, 1982; Ruda et al., 1984). All these techniques of investigation show that in the brain, opioid receptors are found in highest concentration in areas associated with the limbic system, but other brain systems also have high levels of opioid receptors. Brain locations that have shown a high concentration of opioid receptors are the periaqueductal grey, locus coeruleus, amygdala, caudate nucleus, substantia nigra and hypothalmus. At the spinal cord level, autoradiographic analysis (Lamotte et al., 1976) shows high levels of opioid receptors in laminae I, II and III. The use of immunohistochemical techniques (Hunt et al., 1980) showed high levels of enkephalin staining in laminae I and II of spinal cord. In addition, Ruda (1982) was able to show enkephalin staining synapses in lamina V of thalamic projection neurons. The variable distribution pattern of opioid receptors throughout the nervous system could indicate opioid regulation or modulation of neuronal processes associated with nociceptive, motor, sensory and endrocrine functions.

The exact presynaptic or postsynaptic location of opioid receptors is an issue that is controversial in the literature. Evidence for presynaptic localization of opioid receptors on primary afferents comes from work by Lamotte and co-workers (1976). They were able to show that dorsal root rhizotomy resulted in a decrease in the concentration of opioid receptors in the spinal cord. Specific binding to dorsal roots of spinal cord was demonstrated by Fields and co-workers (1980). Other studies (Yaksh et al., 1980) showed that morphine inhibits the release of substance P from spinal cord. Substance P is one of the putative transmitters of

nociceptive impulses (Lembeck et al., 1981), and is located in primary afferents of spinal cord (Jessel et al., 1978). Demonstration of morphine's ability to inhibit the release of substance P from primary afferents is additional evidence for a presynaptic location of opioid receptors. Electrophysiological studies (Mudge et al., 1979) demonstrate enkephalin's ability to inhibit substance P release from sensory neurons in culture as well as to decrease the action potential of these neurons. In spite of all these lines of evidence for a presynaptic site for opioid receptors, morphologic support for a presynaptic location of opioid receptors has not been found at the ultrastructural level (Hunt et al., 1980; Sumal et al., 1982). The inability to identify enkephalin immunoreactive terminals forming synaptic contact with horseradish peroxidase labelled primary afferents in spinal cord is not conclusive evidence that presynaptic opioid receptors do not exist. Immunohistochemical studies (Hunt et al., 1980; Sumal et al., 1982) have utilized antibodies directed towards enkephalins. The crossreactivity of these antibodies with non-enkephalin opioid peptides is less then 2 %. If dynorphin, a substance present in high concentrations in spinal cord (Botticelli et al., 1981), was contained in presynaptic terminals of neurons that formed presynaptic contacts with primary afferents, it is unlikely that dynorphin containing terminals would have been recognized. An alternative explanation for loss of receptor binding after dorsal root rhizotomies without presynaptic changes at the ultrastructural level may be found in the recent work of Sugimoto and Gobel (1982) and Gobel (1984). These investigators have shown that removal of tooth pulp or cutting the distal radial nerve, damage which results in loss of the peripheral receptive fields of these sensory primary afferents, does not result in loss of axonal processes returning to the spinal cord. In addition, the axons of the primary afferents that have had their receptive fields removed show no morphologic change. However, primary sensory neurons that have had their peripheral receptive fields removed have been shown to become spontaneously active. Loss of peripheral receptive fields of sensory neurons does result in cavatation and eventual involution of dendritic processes of second order neurons in laminae I and II of the spinal cord. This type of damage in second order neurons certainly

would result in loss of opioid receptors from these dendrites. This damage may result from excessive release of transmitter from the primary afferents or loss of transportable proteins from primary afferents. However, Gobel (1984) states that dorsal root rhizotomy with short or long term survival times failed to produce the changes seen with peripheral non-lethal damage to sensory primary afferents. It may be possible that dorsal root rhizotomy doesn't produce morphologic changes visble at the electron microscopic level but may produce functional changes in second order neurons which results in loss of opioid receptor binding. These findings may have clinical relevance in providing a greater insight into the mechanism of chronic pain disorders such as phantom limb sensations and causalgias.

Ionic Effects On Opioid Binding

Early work showed opioid binding was regulated by monovalent and divalent cations (Pert and Snyder, 1974; Simon et al., 1973; Pasternak et al.,1975). Divalent cations have their greatest effects on modulation of opioid binding regulation by guanine nucleotides (GTP) (Childers and Snyder, 1980). Manganese was most active in decreasing the inhibitory effects of GTP on opioid binding to receptors. However, the concentration necessary for this effect is higher than the concentration present in normal neurons. Magnesium, an ion present in physiologic fluids, at a concentration that is effective in decreasing the inhibitory effects of GTP on opioid binding may have effects similar to manganese (Chang et al., 1983). Manganese has a greater effect of increasing opiate agonist binding in the presence of sodium while little effect on binding of opiate antagonists in the presence or absence of sodium is noted (Pasternak et al., 1975). Recent studies (Cox et al., 1984; Werling et al., 1984b) varied the ionic composition of the buffer, but maintained a constant ionic strength by replacing Na^+ with K^+. The replacement of Na^+ by K^+ resulted in a 2 fold increase in affinity of [^{3}H]EKC at k sites, a 2.8 fold increase in affinity of [^{3}H]DAGO at μ sites and a 1.2 fold increase in affinity of [^{3}H]DADLE a ∂ sites. The B_{MAX} of ∂ sites increased when Na^+ was replaced by K^+. This effect

was not seen with μ and k sites. The exact site of Na^+ regulation is uncertain; however, from these recent studies an intracellular site of Na^+ regulation may be suggested by the low concentrations of Na^+ necessary to decrease the affinity of [^{3}H]DADLE binding.

Effects of GTP On Opioid Receptor Binding

The effects of GTP on opioid receptor binding are variable depending upon the ionic composition of the buffer medium in which the binding assay is conducted. Childers and Snyder (1978 and 1982) reported that in rat brain homogenates GTP or GDP reduced the binding affinity of opioid agonists and to a lesser extent decreased the binding affinities of antagonists as well. However, in the presence of sodium, GTP and GDP decreased the binding affinities of opioid agonists but not of opioid antagonists. Chang and co-workers reported that GTP, GDP and non-hydrolyzable analogs reduced binding of [^{3}H]DADLE to isolated μ and ∂ receptors. The inhibitory effect was increased by addition of sodium to the incubation medium, and the inhibitory effects of GTP or GDP and sodium could be reversed by Mg^{2+}. The inhibitory effects of GTP analogs could not be reversed by addition of Mg^{2+}. The increase in high affinity sites and the reversal of inhibitory effects of GTP and GDP on opioid binding by Mg^{2+} are proposed to be due to increased hydrolysis of GDP or GTP and dissociation from the receptor (Chang et al., 1983). Recent work by Werling and co-workers (1984b) has shown GTP reduces binding affinities significntly at both isolated μ and ∂ sites with a smaller reduction in the affinity for binding at k sites, possibly indicating that fewer k receptor sites are regulated by GTP. The significence of GTP regulation of opioid binding is not totally clear at this time. GTP effects on opioid receptor binding may help to explain the marked difference between the opioid receptor affinities measured *in vitro* and the effective concentrations of opioid agonists necessary to produce physiologic effects *in vitro* (Cox et al., 1984). The variable effects of GTP, sodium and manganese on the binding of opioid agonists or antagonists may be utilized in *in vitro* binding assays to determine if the binding of an agonist/antagonist

opiate analgesic at an isolated opioid receptor site is consistant with binding of an agonist or an antagonist to each isolated opioid receptor site.

Transduction of Opioid Receptor Occupation

The initial effect of activation of opioid receptors by an opiate or opioid agonist is unclear at this time. Studies of the effects of opiates on neuroblastoma X glioma (NG-108-15) hybrid cells have demonstrated that opioid receptors of the ∂ type mediate inhibitory effects on adenylate cyclase in whole cells (Sharma et al., 1975) or homogenates of NG-108-15 hybrid cells (Blume et al., 1979). In addition, the initial report that adenylate cyclase is inhibited in homogenates of mammalian brain (Collier and Roy, 1974) has recently been verified using homogenates of rat striatal membrane homogenates (Law et al., 1981; Cooper et al., 1982). Blume and co-workers (1979) have noted that GTP and Na^+ are required in the assay in order to be able to measure opiate inhibition of adenylate cyclase. Koski and Klee (1981) have shown that opiates increase GTPase activity in NG-108-15 cells. Barchfeld and Medzihradsky (1984) have shown that opiates also stimulate GTPase activity in rat striatial membranes. Increasing GTPase activity may have a role in a reduction of the GTP concentrations necessary for the activation of adenylate cyclase, which then results in a reduction of cyclic AMP (cAMP). However, opiate induced increase in GTPase activity may not be the primary mechanism for opiate inhibition of adenylate cyclase. GTP analogues can function as a substitute for GTP and in the absence of opiates produce inhibition of adenylate cyclase (Frey and Kebabian 1984). Work by Frey and Kebabian (1984) using a PRL-secreting 7315c tumor cell further clarifies the mechanism of opiate inhibition of adenylate cyclase. This 7315c tumor cell has an opioid receptor with apparent μ characteristics. In addition, this tumor cell has two guanyl nucleotide regulatory proteins designated N_I for the inhibitory protein and N_S for the stimulatory protein. These workers suggest that the μ opiate receptor may be coupled to N_I. The binding of an opiate to the μ opiate

receptor in this system in the presence of GTP is proposed to result in the binding of GTP to N_I which results in the inhibition of adenylate cyclase. Binding of an opiate agonist to the μ opiate receptor in this system increases the binding of GTP to N_I as well as hydrolysis of GTP to GDP. However, this GTPase activity of N_I is the result of opiate binding to the μ opiate receptor site and is not a necessary component of inhibition of adenylate cyclase since GTP analogs resistant to hydrolysis can substitute for GTP in this system.

The electrophysiologic effects of opiates are receptor specific. The μ agonist normorphine and the ∂ agonist DADLE have been shown to increase the K^+ conductance of small interneurons in the substantia gelatinosa of rat spinal cord (Yoshimura and North, 1983). Werz and MacDonald (1984) have shown that in mouse dorsal root ganglion neurons μ and ∂ agonists increase K^+ conductance. However, dynorphin which acts at k receptors blocks voltage dependent calcium entry into the neuron (Werz and MacDonald, 1984). The increased K^+ conductance produced by μ and ∂ agonists is blocked by cesium (K^+ channel blocker), while the decreased calcium conductance produced by dynorphin is unaffected by cesium application. The hyperpolarizing effect of the increased K^+ conductance could inhibit depolarization and the entry of calcium into the neuron thereby inhibiting transmitter release. Therefore μ, ∂, and k opiate and opioid ligand receptor binding could serve to block transmitter release. Moreover, the effects of μ and ∂ ligands could be additive to the effects of k ligands on calcium channels. It is unclear if the electrophysiologic effects of opioid receptor binding are primary effects or secondary to reductions in cAMP. Kandel and Schwarz (1982) have proposed a model where a cAMP dependent protein kinase is responsible for phosphorylation of a K^+ channel protein that results in decreased K^+ conductance out of the cell. When the levels of cAMP are decreased by opiates there could be a reduction of the phosphorylation of the K^+ channel protein and a resulting increase in the K^+ conductance out of the cell. This model is consistent with the electrophysiologic changes changes induced by opiates in neural tissue. A possible mechanism for the effects of μ and ∂ opioid agonist binding could be that receptor occupation results in an

increase in GTP binding by N_i which would inhibit adenylate cyclase and produce a reduction in cAMP levels. The reduction of cAMP results in decreased activity of the cAMP dependent protein kinase, and reduced phosphorylation of membrane proteins which results in an increase of K^+ conductance causing hyperpolarization and decreased calcium entry into the neuron via a voltage dependent calcium channel. The mechanism for k agonist receptor binding could be the reduced cAMP levels producing a reduction of phosphorylation of membrane proteins associated with a calcium channel and a resulting reduction of calcium entry into the neuron. However, evidence for k opioid receptor activation producing a reduction in adenylate cyclase activity and cAMP has not yet been described. It is not certain at this time if the changes in adenylate cyclase activity by opiates is the primary mechanism for reducing transmitter release from neurones. However, it is well known that opioid receptor binding of agonists results in decreased transmitter release from neurones in a number of systems. Inhibition of acetylcholine release from guinea pig myenteric plexus has been shown (Paton, 1957; Cox and Weinstock, 1966). Morphine has been shown to inhibit norepinephrine release in mouse vas deferens (Henderson et al., 1972). Inhibition of norepinephrine release from cerebral cortex due to receptor binding of opiate drugs has been reported most recently by Hagan and Hughes (1984).

Intrathecal and Epidural Opiate Analgesia

The idea for the use of intrathecal and epidural opiate injections for selective analgesia evolved from the work of four groups. The initial discovery of immunoreactive leucine enkephalin in laminae I and II of spinal cord (Elde et al., 1976), indicated that endogenous opioids could have a role in modulation of nociceptive input to higher brain centers. Lamotte and co-workers (1976) showed that dorsal root rhizotomy decreased opiate receptor binding in spinal cord. Work by Jessell and Iversen (1978) showed capsaicin produced a depletion of substance P from small diameter primary afferent C-type fibers. Mudge and co-workers

(1979), using cell culture, and Yaksh and co-workers (1980), using *in vivo* studies, were able to show decreased release of substance P from primary afferents after administration of enkephalin or morphine. This set of observations implied that substance P is the transmitter of nociceptive input from primary afferent C-type fibers to the dorsal horn of spinal cord and that morphine or opioid peptides acting via opioid receptors located pre- or postsynaptically can modulate nociceptive input by inhibiting the release of substance P. Yaksh and Rudy (1976a) developed a technique for chronic cannulation of the intrathecal space and were able to show analgesia mediated by a direct spinal action of opiates in animals (Yaksh and Rudy, 1976b). Futher work by Yaksh (1978) showed that the analgesia from intrathecal opiates had a somatotopic distribution, with the cord dermatomes nearest the catheter tip the earliest to be affected. In no case did the analgesia extend above the shoulders, implying that the analgesia was due to opioid receptor occupation at the spinal cord level and not to activation of descending pathways to the dorsal horn of spinal cord. The use of intrathecal morphine for analgesia in man was first utilized by J.K. Wang (1977). Later Wang and co-workers reported on the use of intrathecal morphine to relieve severe intractable pain in lower back and legs secondary to malignancies of the genitourinary tract. The analgesia achieved with intrathecal opiates in man or animals is not associated with any motor or autonomic impairment. Likewise, sensation to temperature and light touch are unaffected. Since the early use of intrathecal opiates by Wang, the use of intrathecal or epidural (extradural) opiates has appeared in a variety of clinical settings. Epidural and intrathecal opiates have been utilized for relief of post-operative pain from upper and lower abdominal procedures (Bromage et al., 1980; Behar et al., 1979; Cousins et al., 1979), postoperative thoracotomies (Magora et al., 1980), and for analgesia during labor and delivery (Scott and Mc Clure, 1979). The concentrations of opiate utilized in these studies were from 0.5 - 3 mg morphine, or 10 - 30 mg meperidine, which resulted in analgesia from 12 - 24 hrs. As noted earlier, the site of action of intrathecal or epidural opiate administration is proposed to be at the spinal cord level.

To futher investigate the site of action of intrathecally administered opiates, Wood and co-workers (1981) studied the effects of EKC and morphine in the chronic spinal animal. EKC had a marked analgesic activity when administered intrathecally but not when administered to the periaqueductal gray (PAG). Morphine had a greater analgesic effect when injected to the PAG then when injected to the intrathecal space. The ability of EKC to produce analgesia in the chronic spinal animal without any central effect is taken as evidence by these workers that the actions of EKC are due to direct effects at the spinal cord level. However, if the dose of opiate administered to the intrathecal or epidural space is high enough to allow for absorption and redistribution to the systemic circulation or for rostral redistribution to higher brain centers, then analgesia will be mediated at the cord level as well as by activation of descending analgesia pathways to the dorsal horn of spinal cord. The central endogenous decending analgesic pathways are thought to consist of opioid receptor activated excitatory neuronal projections from the PAG to decending serotinergic neurons that have their origin in the nucleus reticularis magnocellularis, medullary nucleus raphe magnus, and dorsal nucleus reticularis gigantocellularis. These serotinergic neurons form synaptic contacts with immunoreactive enkephalin containing interneurons in laminae I and II of dorsal horn of spinal cord (Basbaum and Fields, 1978). In addition, the medullary nucleus raphe magnus sends excitatory projections to the locus coeruleus, which in turn sends decending excitatory noradrenergic projections to small immunoreactive enkephalin containing interneurons in laminae I and II of the dorsal spinal cord (Gobel et al., 1980). The decending serotinergic and noradrenergic synaptic contacts with immunoreactive enkephalin interneurons have been shown to take up [^{3}H]serotonin (Ruda and Gobel, 1980) and [^{3}H]norepinephrine (Ruda et al., 1979). Activation of the serotinergic and noradrenergic synaptic contacts in laminae I and II of the dorsal horn of spinal cord may provide an additional mechanism to increase analgesia at the spinal cord level. Yaksh (1979) showed that intrathecal administration of serotonin or norepinephrine produced analgesia similar to that seen with stimulation of the decending serotinergic and noradrenergic pathways by the application of opiates to the PAG. In addition,

intrathecal administration of methysergide and phentolamine significently reversed the analgesia seen with opiate administration to the PAG.

The opioid receptor sites in the spinal cord that are responsible for antinociception are not totally clear at this time. The work of Martin and co-workers (1976) established evidence for μ, k and sigma receptors in spinal cord as well as brain based on pharmacologic studies. Further evidence of receptor heterogeneity in spinal cord has been established by radioligand binding studies. Previous radioligand binding studies in spinal cord from various species have all been carried out in Tris HCl buffer at a wide variety of temperatures from 4°C to 37°C (Kelly et al., 1980; Czlonkowski et al., 1983; Mack et al., 1984; Attali et al., 1982; Gouarderes et al., 1982). Opioid receptor heterogeneity with binding sites that have μ, ∂ and k characteristics have been identified in human, rat and guinea pig spinal cord (Czlonkowski et al., 1983) and rat spinal cord (Mack et al., 1984). However, the total reported receptor densities as well as the ratios of receptor subtypes vary widely even within the same species. Czlonkowski and co-workers (1983) showed that postmortem whole human spinal cord had a total opiate receptor density of 22 fmols/mg protein and rat lumbo-sacral spinal cord had a total densily of 8 fmol/mg protein. Mack and co-workers found that whole rat spinal cord had a total opiate receptor density of 143 fmol/mg protein. Two research groups working with rat lumbo-sacral spinal cord (Gouarderes et al., 1982) and guinea pig lumbo-sacral spinal cord (Attali et al., 1982) have claimed absence of any specific μ or ∂ binding in these tissues based upon the lack of specific binding of [^{3}H]dihydromorphine, [^{3}H]morphine, or [^{3}H]DADLE. The apparent absence of μ or ∂ binding in rat or guinea pig lumbo-sacral spinal cord prompted Gouarderes and co-workers (1982) to believe that all specific opioid receptor binding in rat lumbo-sacral spinal cord was of a k receptor type. However, the low affinity of DADLE and morphine at this site in rat lumbo-sacral spinal cord lead Gouarderes to conclude that all specific binding of [^{3}H]EKC and [^{3}H]etorphine in rat lumbo-sacral spinal cord was to a benzomorphan binding site similar to a site characterized

by other workers in mouse brain homogenates (Chang et al., 1981). Attali and co-workers proposed the existence of two k receptor types in guinea pig lumbo-sacral spinal cord on the basis of the absence of observed μ or ∂ binding and the differential effects of a 5μM DADLE block on [^{3}H]EKC and [^{3}H]etorphine binding. When 5μM DADLE block was added to the binding assay there was a loss of a component of the high affinity binding of [^{3}H]EKC and no change in the low affinity binding of [^{3}H]EKC and loss of specific binding for [^{3}H]etorphine. The portion of the high affinity binding of [^{3}H]EKC that was lost was identified as the DADLE sensitive site (DAL_s) and the high affinity binding of [^{3}H]EKC that remained was identified as the DADLE insensitive site (DAL_i). The DAL_i site was proposed to be a k opioid receptor of the type previously described by Kosterlitz and co-workers (1981). The DAL_s site was proposed to be a benzomorphan site. The absence of μ or ∂ binding in lumbo-sacral spinal cord (Attali et al., 1982; Gouarderes et al.,1982) is difficult to resolve since morphine and DADLE administered intrathecally to rats produce analgesia with a somatatopic distribution that can be selectively limited to the lumbo-sacral spinal cord segments (Yaksh, 1984). In addition, animals rendered tolerant to intrathecal morphine do not show cross-tolerance to intrathecal injections of DADLE. However, there is an assymetric cross-tolerance to morphine in animals rendered tolerant to intrathecal injections of DADLE (Yaksh, 1984). These pharmacologic findings are consistant with morphine producing tolerance at μ receptors but not at ∂ receptors. DADLE, an opioid agonist with affinity for both μ and ∂ receptors (Werling et al., 1984a) produces tolerance at both μ and ∂ receptors. This type of *in vivo* pharmacologic evidence strongly supports the existance of μ and ∂ opioid receptors in lumbo-sacral spinal cord.

It was the purpose of this study to attempt to quantify μ,∂, and k type opioid receptors in guinea pig whole spinal cord under conditions of physiologic temperature and cation concentrations. It has been shown early in the introduction of this thesis that sodium and other major physiologic cations as well as temperature have significent effects on radioligand

binding. Therefore we feel it is important to characterize opioid receptor binding in this tissue under conditions close to physiological conditions. Due to the difficulties associated with maintaining a stable pH using bicarbonate buffers in a binding assay 25 mM HEPES buffer was substituted for bicarbonate in this system. In addition, DAGO, a μ site selective ligand (Kosterlitz et al., 1981), has been utilized to identify μ receptor sites in guinea pig lumbo-sacral spinal cord. Severe delayed respiratory depression is a rare but potentially fatal complication of intrathecal or epidural opiate analgesia that has only been reported in postoperative patients (Yaksh, 1981). It is because of this complication that nalbuphine HCl, an opiate agonist antagonist analgesic with a ceiling effect on respiratory depression equal to 30 mg of morphine, (Romagnoli and Keats, 1980) and morphine have been included in this study. Nalbuphine HCl given intrathecally to rats produces analgesia when the intraperitoneal acetic acid writhing test is used to measure analgesia. Nalbuphine is not an effective analgesic when fast pain tests such as the paw press or shock titration test are used to measure analgesia (Schmauss et al., 1983). Co-administration of low doses of morphine and nalbuphine intrathecally produce analgesia equal to that seen with high doses of morphine when the acetic acid writhing test is employed. Co-administration of high doses of morphine and nalbuphine results in a marked reduction in the analgesia seen with co-administration of low doses of nalbuphine and morphine. Nalbuphine HCl is one of the few agonist antagonist opiate analgesics that has been found to have a significent intrathecal analgesic effect. Knowledge of nalbuphine's affinity at isolated spinal cord opioid receptor types may help to understand this compound's intrathecal analgesic activity *in vivo*.

Materials.

[^{3}H]Ethylketocyclazocine ([^{3}H]EKC) 19.9 Ci/mmole, was purchased from New England Nuclear Corporation (Boston, MA.). [^{3}H-Tyr^1- D-Ala^2-MePhe^4-Glyol^5]enkephalin ([^{3}H]DAGO), 60 Ci/mmole, was purchased from Amersham Corporation (Arlington Heights, IL). [^{3}H-Tyr^1- D-Ala^2-D-Leu^5]enkephalin ([^{3}H]DADLE), 36.5 Ci/mmole, was purchased from Amersham Corporation (Arlington Heights, IL). [D-Ala^2-MePhe^4-Glyol^5]enkephalin (DAGO) was purchased from Cambridge Research Biochemicals LTD. (Beach NY). [D-Ser^2-Leu^5] enkephalin Thr^6 (DSTLE) was purchased from Sigma Chemical Company (St. Louis, MO). Ethylketocyclazocine methane sulfonate (EKC) was supplied by Sterling Winthrop Labs (Rensselaer, NY), naloxone hydrochloride from Endo Labs. (Garden City, NY), and U50488H was a gift from Upjohn Labs. The following ligands were generous gifts: morphine sulfate, Dr. L. Aronow; nalbuphine hydrochloride Dr. S. Muldoon, and WIN 44,401-2 as well as WIN 44,401-3 Dr. A.I. Faden. HEPES and HEPES NaCl were purchased from Sigma Chemical Corp. (St. Louis, MO). Fresh dilutions of drugs were prepared for each experiment and 25mM HEPES salt buffer was used to dilute stock drugs.

Methods

Preparation of Spinal Cord Membranes

The method of guinea pig brain membrane preparation (Dunlap et al., 1979) was modified in the following manner for spinal cord membrane preparation. Male Hartley guinea pigs (400-500 gm) were purchased from Controlled Animal Management and Marketing (Wayne, NJ). Guinea pigs were killed by decapitation, the vertebral column was dissected in block, divided into four segments, and placed on ice. Spinal cord segments were rapidly removed from each vertebral column segment by application of a high pressure air line to the distal end. Spinal cord segments removed in this manner were rapidly placed in ice-cold HEPES salt buffer (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, pH adjusted to 7.4).

Tissue from 4-6 animals was pooled, weighed, and 10 volumes of HEPES salt buffer was added to the wet weight of the tissue. Tissue was homogenized using a teflon glass homogenizer from Thomas Scientific (Philadelphia, PA) driven by a T-Line laboratory stirrer (Emerson, NY) setting of 70, using ten full strokes. The crude membrane homogenate was divided into 10-15 ml portions, placed into 50 ml centrifuge tubes, and centrifuged at 27,000 x g for 15 min at 4^0C in a Sorvall RC-5B refrigerated centrifuge made by Dupont Instruments (Newton, CT). The supernatant was discarded and the pellet resuspended in 20 volumes of HEPES salt buffer with a Vortex mixer and incubated at 0^0C for 60 min to remove any endogenous ligand. At the end of 60 min, the membrane suspension was centrifuged at 27,000 x g for 15 min at 4^0C. The resulting supernatant was discarded, and the pellet was resuspended in 10 volumes of HEPES salt buffer. After 2 additional centrifigation steps at 27,000 x g for 15 min at 4^0C, discarding the supernatant, and resuspending the residual pellet in ten volumes of HEPES salt buffer, the final pellet was resuspended to a concentration of 2% w/v in 25 mM HEPES salt buffer and frozen at -70^0C for not more then three weeks prior to use in receptor binding assays. In seven independent membrane preparations, the protein content ranged from 290-320µg protein/250µl membrane suspension. There was no significent differences ($P > 0.05$) between the mean protein contents of each membrane preparation when compared using post-hoc Scheffe analysis. Therefore the grand mean of 300µg protein/250µl membrane suspension was used to adjust all B_{MAX} values to B_{MAX} in pmols/mg protein.

Protein Assay

Protein concentrations were determined by a modification of the Lowry procedure (Peterson, 1977). This technique includes a precipitation step with 0.1ml of 0.15% sodium deoxycholate (DOC) and 0.1ml of 72% trichloracetic acid (TCA), and employs bovine serum albumin as standard.

Measurement of Radioligand Binding

Radioligand binding techniques of Dunlap et al., (1979) were modified as follows for this series of experiments. Aliquots of spinal cord membrane suspension were incubated at a final concentration of 1% (W/V) and in a final volume adjusted to 500 µl with 25 mM HEPES salt buffer in 12 X 75 mm disposable plastic test tubes from Chase Instruments Corp. (Poultney, VT). Displacing drugs or blocking drugs were added in volumes of 50 µl. The reaction was started by adding 100 µl radiolabelled drug to samples after both the radiolabelled drug and the triplicate samples were prewarmed for 5 min to assure a 37^{0}C temperature for the entire incubation period. Tubes were incubated an additional 20 min in a Dubnoff metabolic shaking unit from Precision Scientific Group (Chicago, IL). The incubation was terminated by addition of 4 ml ice-cold 25 mM HEPES salt buffer to the incubation tube and filtered through #32 Glass Fiber Fillers from Schleicher and Schuell Inc. (Keene, NH) in an Amicon 12 position filter box (Amicon, Lexington, MA) followed by 2 additional 4 ml washes per filter. Total termination time for each incubation tube was less then 15 seconds. After filtration, filters were placed in 20 ml plastic scintillation vials and soaked in 1 ml of 100% ethanol overnight. Radioactivity retained on the filters was measured by liquid scintillation counting after addition of 8 ml of Ready-Solv EP (Beckman Instruments Inc., Fullerton CA). The samples were counted for 5 min at an efficency of about 39% in a Beckman LS 7800 Liquid Scintillation Counter (Beckman Instruments Inc., Irvine, CA). Specific binding was determined by subtracting from total binding the amount of binding that remained when 1 µM final concentration of the appropriate unlabelled drug was added to the incubation triplicates (non-specific binding). For [^{3}H]EKC experiments nonspecific binding was measured by inclusion of unlabelled EKC, for [^{3}H]DADLE, DSTLE, and for [^{3}H]DAGO experiments, unlabelled DAGO was added to incubation triplicates. When 1 µM naloxone was included in assay to measure non-specific binding there was no significent difference in the level of non-specific binding measured by naloxone or the previously mentioned unlabelled compounds. Pseudo first order conditions were assumed, i.e.

that radioligand concentration was much higher than receptor concentration. This was a valid assumption since in our experiments specific binding of guinea pig spinal cord membrane suspension was always less then 4% of the total added radiolabelled drug. In addition, saturation binding experiments were performed to establish the K_D of the radioligand to allow displacement studies of the radiolabelled compound with unlabelled ligands below the K_D of the radiolabel at its high affinity site. This allowed the use of the Cheng /Prusoff formula (Cheng and Prusoff, 1973) to calculate an inhibition constant for the unlabelled ligand as follows: $K_I = IC_{50}/1 + [L]/K_D$ where K_D is the K_D of the radiolabel and [L] is the concentration of the radiolabel. This formula allows for normalization of various displacement IC_{50}'s from displacement studies performed at various concentrations of radiolabelled ligand. Inhibition constants (K_I's) derived by this formula are comprable to K_D's derived from saturation curve experiments.

Stability Check of Radioligands

Radioligands were incubated with 25 mM HEPES salt buffer or guinea pig spinal cord membrane suspension for 60 min at 37^0C. TLC of radiolabelled ligands was performed using techniques described by (Leslie et al.,1980) except where otherwise noted. After incubation, reaction tubes were placed on ice, 500 µl of 1-butanol added to each tube. The tubes were mixed by inversion, and centrifuged at 4^0C and 5,000 x g for 15 min. This butanol extraction step was included to separate the drug from the salts and membranes, which we had previously found to interfere with the migration of the drug on the TLC. The upper 400 µl of the butanol phase was aspirated from each reaction tube and placed in fresh 12 X 75 mm test tubes and dried under a nitrogen dryer, 30 µl of methanol was added to each tube to allow spotting of the extracted radiolabel on chromagram sheets. Eastman Silica Gel Chromagram sheets 20 X20 cm sheets (Eastman Kodak Comp., Rodchester, NY) were divided into 5 cm strips and each of these portions

marked into three TLC lanes. In lane 1, authentic drug in distilled water was spotted, in lane 2, drug incubated with HEPES salt buffer was spotted, and in lane 3, drug incubated with spinal cord membrane suspension was spotted. TLC strips spotted with [^{3}H]EKC were developed in a mixture of $CHCl_3$-CH_3OH-28% NH_4OH (6 ml:4 ml:1drop). [^{3}H]DAGO or [^{3}H]DADLE TLC sheets were developed in a mixture of $CHCl_3$-CH_3OH-CH_3COOH-H_2O (10:10:2:3). The TLC strip was cut horizontally into 0.5 cm bands starting 0.25 cm before the origin and ending 0.25 cm after the solvent front, placed in 20 ml plastic liquid scintillation vials, 1 ml of 100% ethanol and 8 ml of Beckman Ready-Solv EP liquid scintillation fluid added, and vials counted at an efficency of aproximately 39%.

Stability Determination of Unlabelled Ligands

Stability of the unlabelled ligands was assessed by extending the assay incubation time of 20 min to 60 min at 37^{0}C. Preincubation with displacer was extended to 45 min. Labelled compound was then added to the incubation mixture and incubation continued for an additional 20 min. The reaction was terminated as described in the "Measurement of Radiolabel Binding" section. Specific binding to membranes with incubation times of 20 min and 60 min with the same concentration of cold displacer were compared. If significent degradation of unlabelled displacing ligand had occured, inhibition of specific binding at 60 min would be less then after 20 min incubation.

Results

TLC of Radiolabelled Ligands

Recovery of [^{3}H]EKC, [^{3}H]DAGO, and [^{3}H]DADLE, from 1-butanol and aqueous phases were 84%, 91%,and 91% respectively. All the radiolabelled coumpounds used in these experiments showed preferential affinity for the butanol phase with the following butanol/HEPES salt buffer partition coefficents: [^{3}H]EKC, 123; [^{3}H]DAGO, 2.2; and [^{3}H]DADLE, 4.4. Both [^{3}H]DAGO and [^{3}H]DADLE incubated in the presence of salt buffer as well as in the presence of membrane suspension displayed the same pattern of migration as authentic drug spotted directly onto the chromatogram (Fig.1). The TLC pattern for [^{3}H]EKC incubated with HEPES salt buffer or guinea pig spinal cord membrane suspension showed a distinctive peak that migrated the same distance as the unincubated drug peak (Fig. 2). However, in both cases the TLC pattern showed two additional small peaks. The additional small peaks could be due to salt from buffer partitioning into the butanol phase during the extraction step and inhibiting normal [^{3}H]EKC migration pattern on TLC sheets.

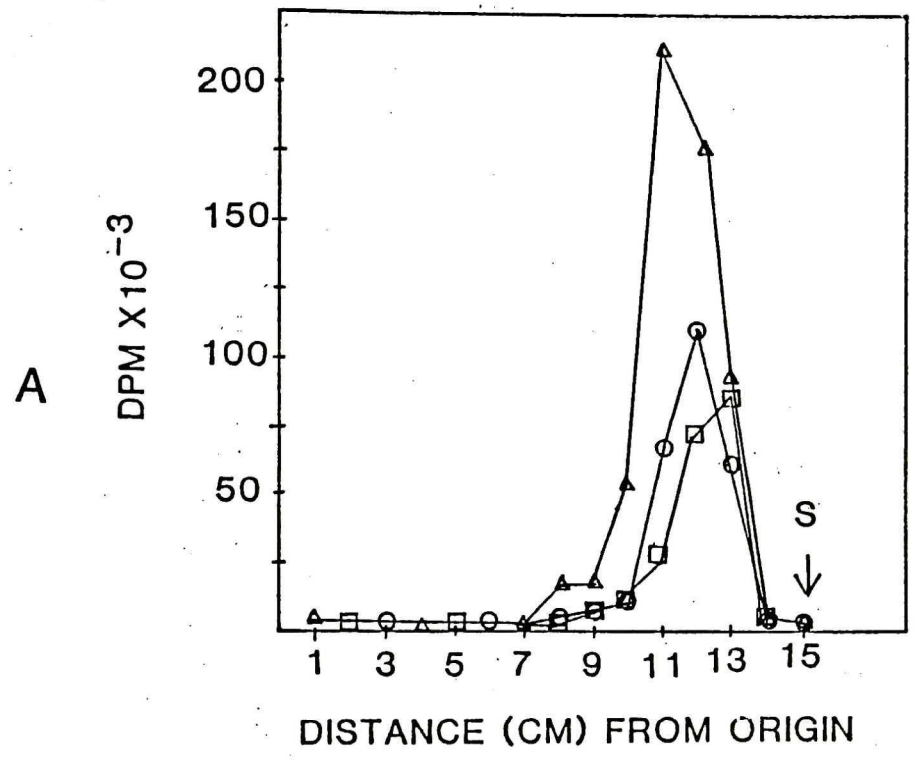
Stability of Unlabelled Coumpounds

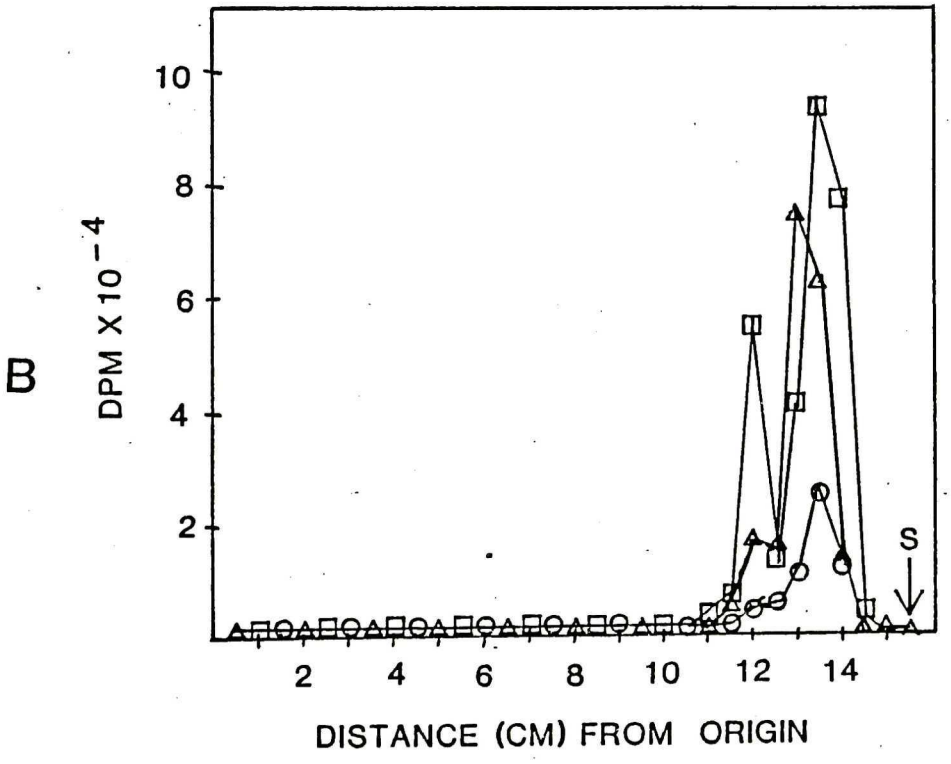
In comparing the amount of displacement of specific binding at 20 and 60 min time points for all unlabelled drugs used in these experiments, 90-97% of all displacement seen at 20 min was still present at 60 min. These findings were taken as evidence that unlabelled coumpounds were stable under our assay conditions.

Characterization of Specific Binding of Labelled Ligands

To maintain all binding conditions as close to physiologic conditions as possible all binding assays were carried out at 37^{0}C, pH 7.4, and using HEPES salt buffer. The kinetic properties of radiolabelled ligand binding in guinea pig spinal cord membranes was tested for

FIGURE 1. TLC OF [^{3}H]DADLE (A) AND [^{3}H]DAGO (B). TLC carried out as described in Methods section, control drug (Δ), drug incubated with HEPES salt buffer (□), drug incubated with guinea pig spinal cord membranes in HEPES salt buffer (0). N = 1.


A
DPM X10^{-3}
200
150
100
50
S
1 3 5 7 9 11 13 15
DISTANCE (CM) FROM ORIGIN


B
DPM X10^{-4}
10
8
6
4
2
S
2 4 6 8 10 12 14
DISTANCE (CM) FROM ORIGIN

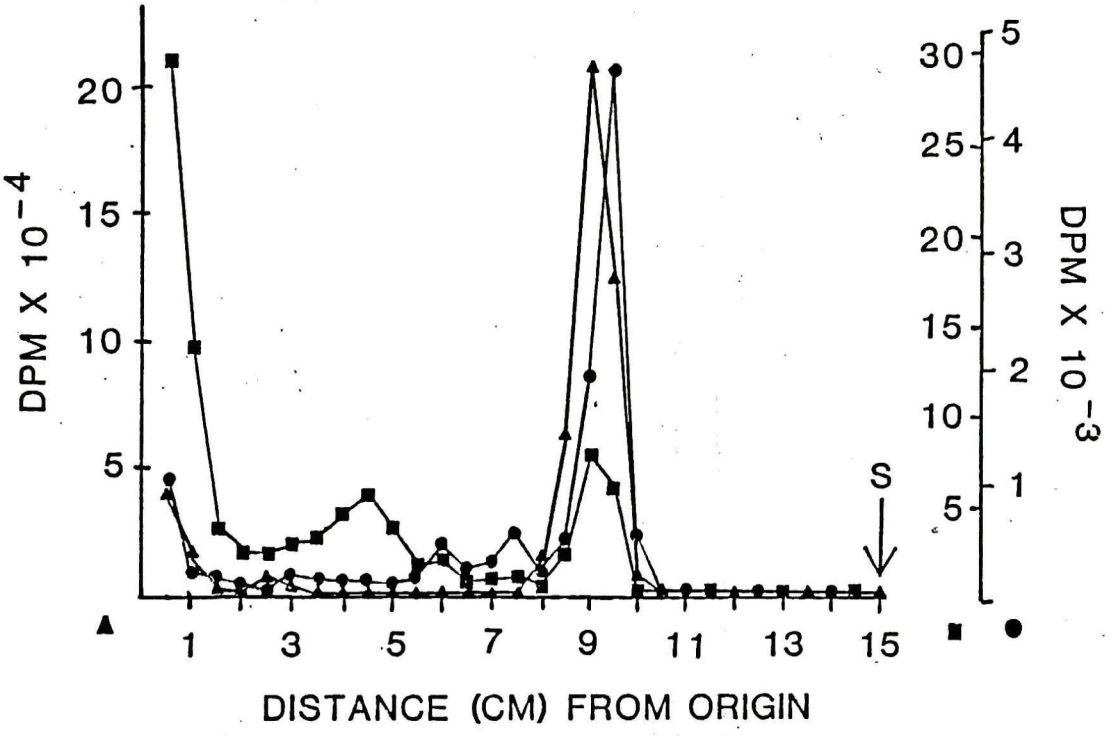


FIGURE 2. TLC OF [^{3}H]EKC. TLC carried out as described in methods section. Control drug (▲), drug incubated with HEPES salt buffer(■), drug incubated with spinal cord membranes in HEPES salt buffer (●). N = 2.

all ligands used in this study by doing both time course of association and dissociation experiments. A plateau of specific binding was reached within the first five min and showed a slight increase over the next fifteen min (Fig 3a.). Specific binding was at equilibrium 20 min after the reaction was started. For all further experiments assay tubes were incubated with labelled compound for 20 min. Dissociation time curve experiments were carried out for all radioligands by addition of 12 ml ice-cold HEPES salt buffer. Due to a shortage of guinea pig spinal cord tissue guinea pig cerebral cortex was used for dissociation experiments. The dissociation $t_{1/2}$ time for [^{3}H]EKC was 19 min (Fig. 3b.). In comparable experiments for [^{3}H]DAGO and [^{3}H]DADLE, 70 % of specific control binding of both [^{3}H]DAGO and [^{3}H]DADLE remained bound to membranes after 10 min dissociation in ice-cold buffer. This dissociation rate was not a hinderence to measurement of maximum specific binding or B_{MAX}, since reaction tubes were filtered rapidly in a time span of approximately 15 sec. Specific binding was linear for all three radioligands with membrane protein over a range of approximately 150-650 µg protein (Fig 4). As noted under Methods section 300 µg protein in a total volume of 500 µl was added to each reaction tube, thus insuring that all measurements of binding were made in the region of linearity of protein concentration versus binding. Maintaining the membrane suspension, salt buffer, and unlabelled ligands on ice for 1-2 hr prior to use had no effect on specific binding.

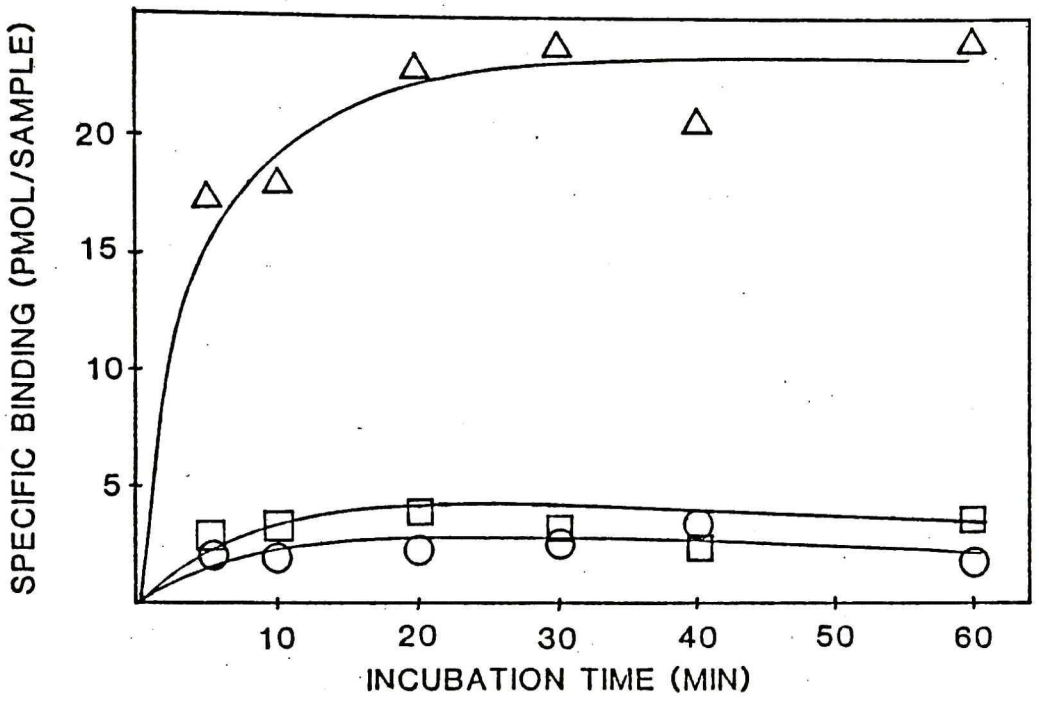
Characterization of Specific Binding of [^{3}H]DAGO

Specific binding was found to be saturable when membrane suspension was incubated with increasing concentrations of [^{3}H]DAGO (Fig. 5a.). Increasing concentrations of [^{3}H]DAGO were added up to 10 nM. Higher concentrations were achieved by adding non-radioactive DAGO. Computer analysis of the binding isotherm was used for quantitative analysis of the binding data and to resolve the Scatchard plots (Munson and Rodbard, 1980). This program utilizes non-linear regression to analyze the binding isotherm and assumes the simultaneous

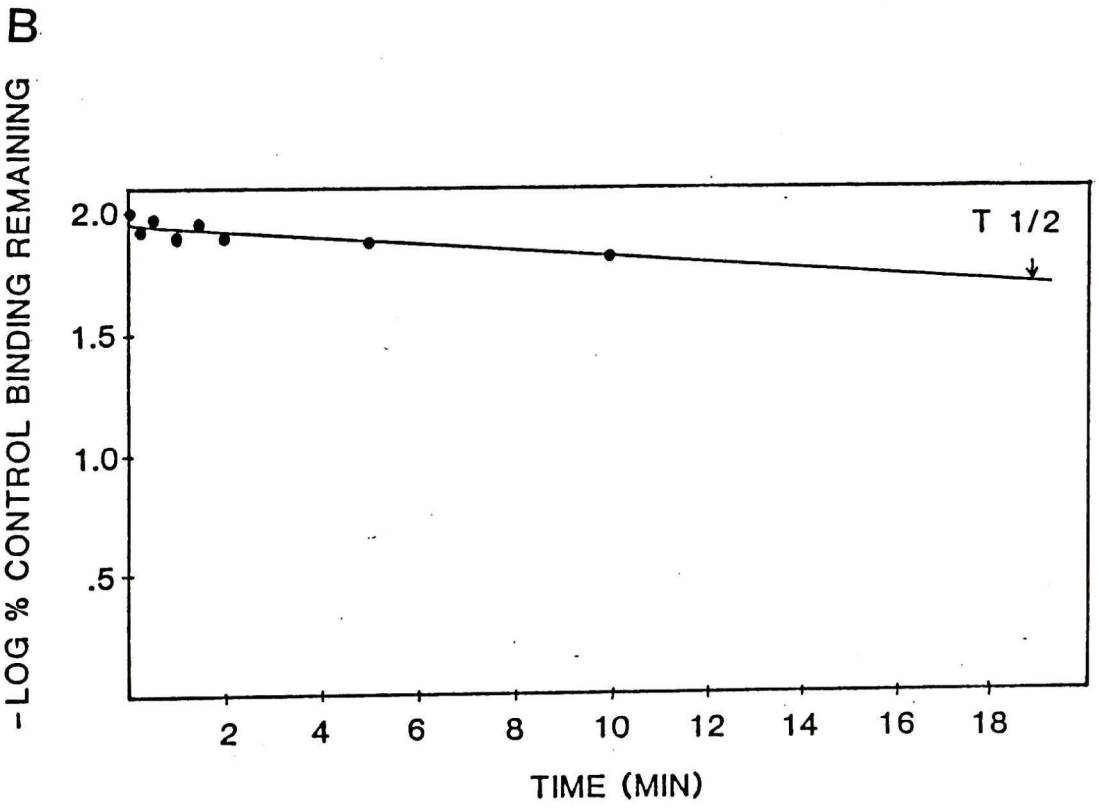
FIGURE 3a. SPECIFIC BINDING OF RADIOLIGANDS (2 nM FINAL CONCENTRATION) TO GUINEA PIG SPINAL CORD MEMBRANES AS A FUNCTION OF INCUBATION TIME. Data are shown for [^{3}H]EKC (Δ), [^{3}H]DADLE (□), AND [^{3}H]DAGO (o). N = 1.

FIGURE 3b. LOSS OF [^{3}H]EKC SPECIFIC BINDING IN GUINEA PIG BRAIN CORTEX MEMBRANES AS A FUNCTION OF TIME. Data shown for one of two similar experiments.

A


SPECIFIC BINDING (PMOL/SAMPLE)
20
15
10
5
10
20
30
40
50
60
INCUBATION TIME (MIN)

B


-LOG % CONTROL BINDING REMAINING
2.0
1.5
1.0
.5
T 1/2
2
4
6
8
10
12
14
16
18
TIME (MIN)

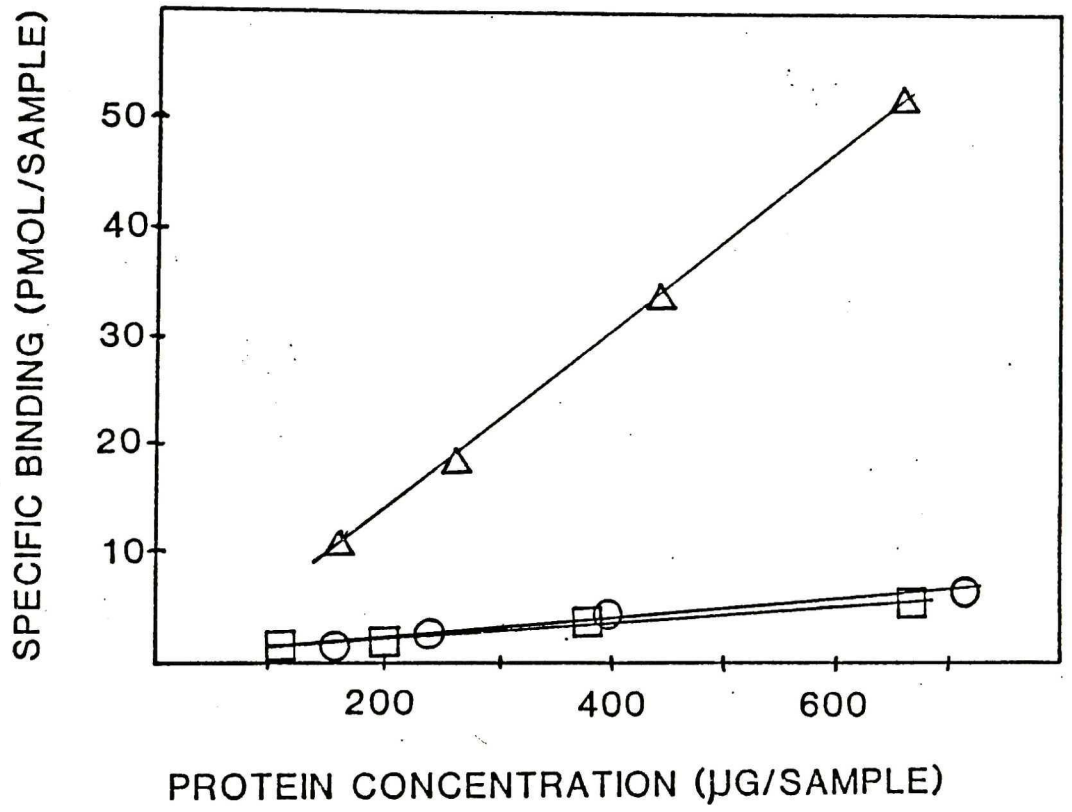
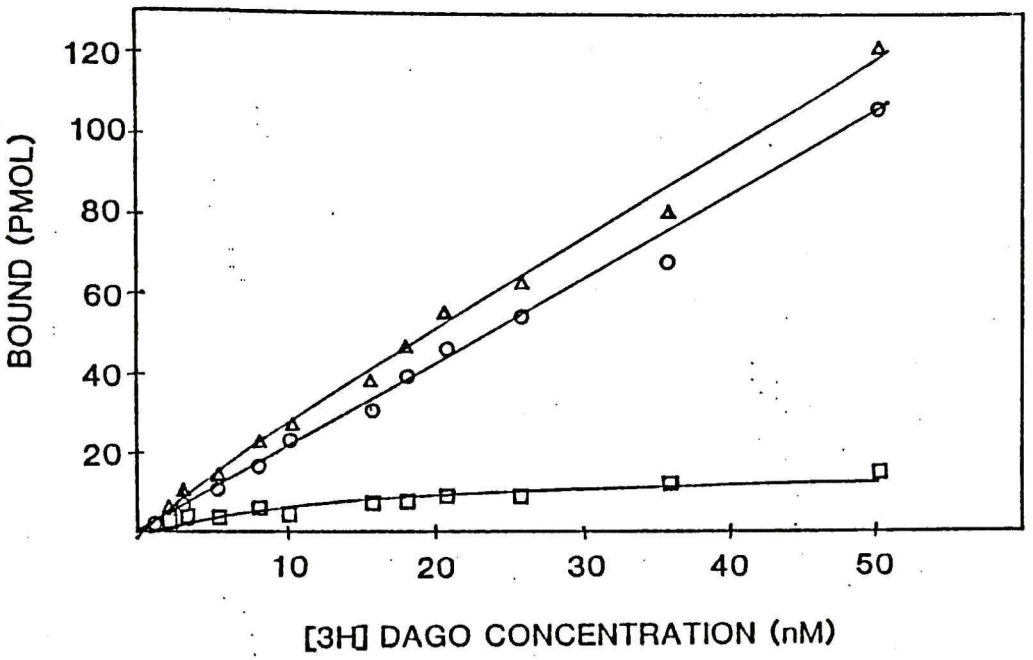


FIGURE 4. SPECIFIC BINDING OF RADIOLIGANDS (2nM FINAL CONCENTRATION) TO GUINEA PIG SPINAL CORD MEMBRANES AS A FUNCTION OF PROTEIN CONCENTRATION. Data points for each ligand were obtained using radioligand binding techniques described in Methods section. Data shown for [^{3}H]EKC (Δ), [^{3}H]DADLE (□), and [^{3}H]DAGO (o). N = 1.

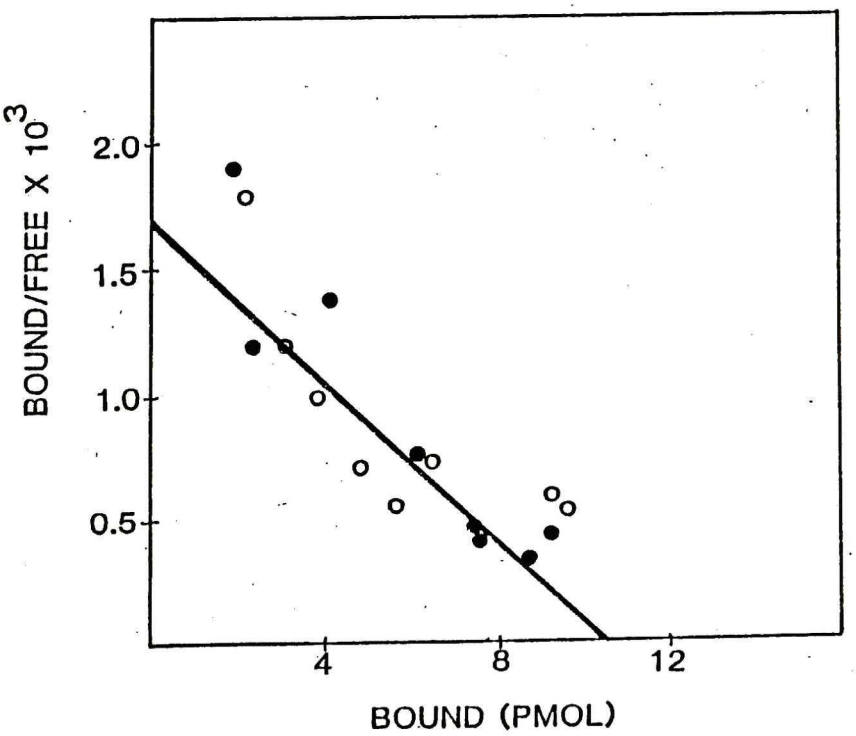
FIGURE 5a. BINDING OF [^{3}H]DAGO TO GUINEA PIG SPINAL CORD MEMBRANES AT INCREASING RADIOLIGAND CONCENTRATIONS. Membranes were incubated with 1-50 nM concentrations of [^{3}H]DAGO as described in methods. Increasing quantities of [^{3}H]DAGO were added up to 10 nM. Higher concentrations achieved by adding non-radioactive DAGO. Total (Δ), non-specific (□), and specific(o). One of two represenative experiments are shown.

FIGURE 5b. SCATCHARD PLOT OF SAME DATA. Solid line is computer fit of data from two similar curves (o) and (●). N = 2.

A


BOUND (PMOL)
120
100
80
60
40
20
10
20
30
40
50
[3H] DAGO CONCENTRATION (nM)

B


BOUND/FREE X 10^3
2.0
1.5
1.0
0.5
4
8
12
BOUND (PMOL)

contribution of one or more independent binding sites. An F-statistic and runs-test is utilized to establish the best statistical fit for different models computed for the same data. The F-statistic compares the residual variances of each model and calculates a P-value (the probability that the improvement of fit of the more complex model is due to chance), a P-value of less then $P < .05$ was required for us to choose the more complex model over the simpler model. The runs-test assumes that the order of the signs of the residuals (differences between the data point and the fitted curve) should be random. The level of significence of the pattern is calculated by the program. A $P > .05$ for a runs-test for a single data set was judged as being an accurate computer model to fit the data. When multiple data sets were modeled together the runs-test was not utilized to evaluate "the goodness of fit" of the computer model for the data. Only a single site model could be fit to the [^{3}H]DAGO saturation curve data (Fig. 5b). Computer analysis reveals a K_D of 6.0 nM and a B_{MAX} of 18 fmol/mg protein for this site. DAGO has been shown to be a highly μ selective ligand (James and Goldstein, 1984; Hiller et al., 1984). Displacement of 5 nM [^{3}H]DAGO with DAGO showed a K_I of 10 nM and a B_{MAX} of 14 fmol/mg protein (FIG. 6.). In addition, a Hill plot of the data showed a slope of 0.74 with R^2 equal to 0.99 (inset Fig. 6.). Hill slope values of less then one may indicate complex binding to more than one receptor site. However, the previously reported μ site receptor selectivity of DAGO would indicate that [^{3}H]DAGO is only binding to μ type receptor sites. Additional displacement studies of 5 nM [^{3}H]DAGO binding were carried out to further characterize the receptor population labelled by [^{3}H]DAGO (Fig. 7.). Nalbuphine HCl had a K_I of 11 nM and a B_{MAX} of 15 fmol/mg protein. Morphine sulfate had a K_I of 16 nM and a B_{MAX} of 13 fmol/mg protein. Therefore DAGO, morphine sulfate, and nalbuphine are three ligands with nearly equal affinity and B_{MAX} for μ sites labelled by [^{3}H]DAGO. U50488H, a putative k selective ligand had a K_I of 2.0 μM and a B_{MAX} of 14 fmol/mg protein, while DSTLE, a putative

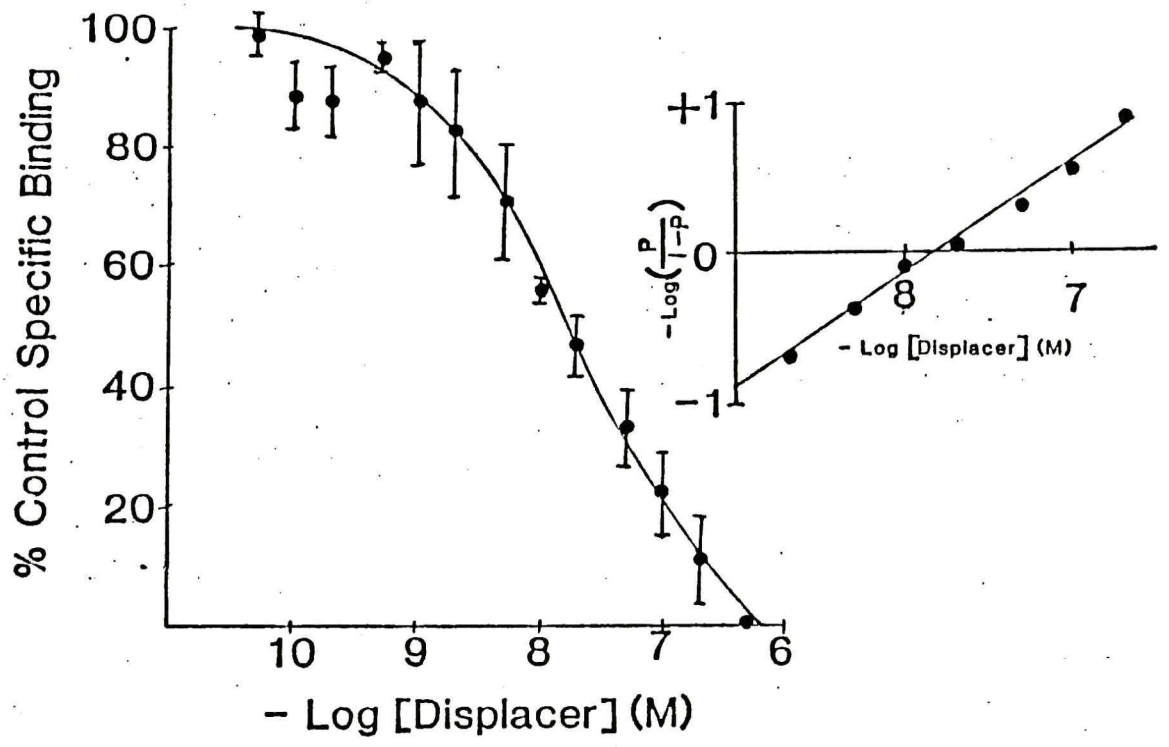
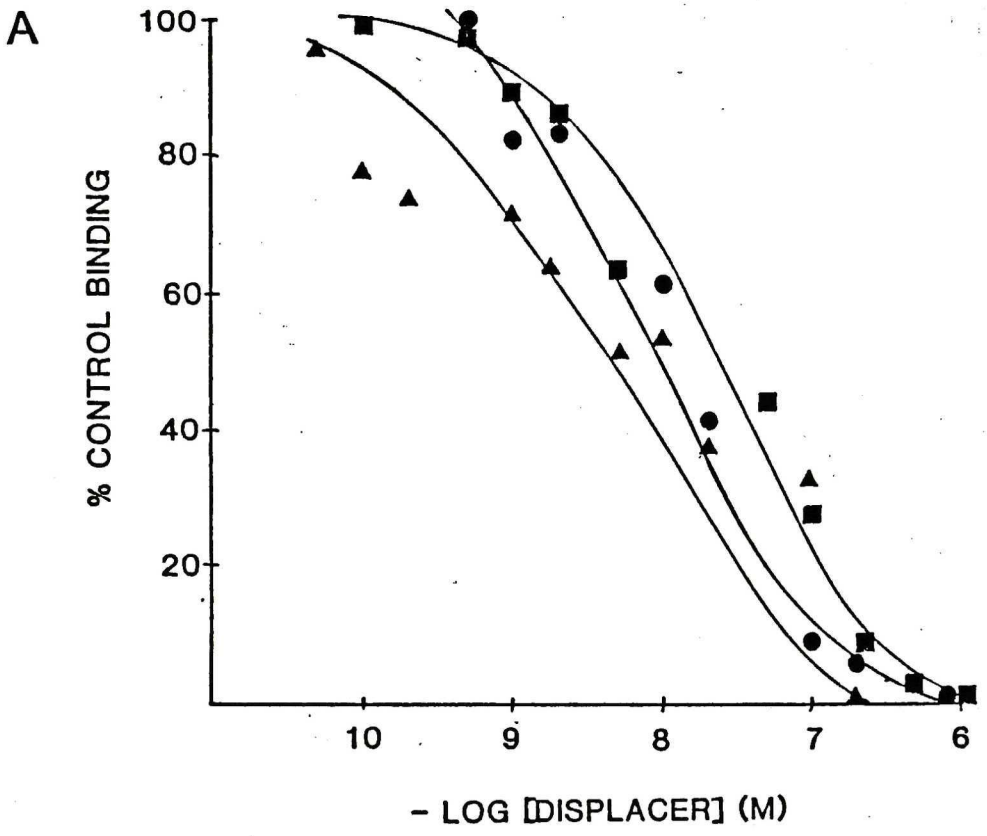
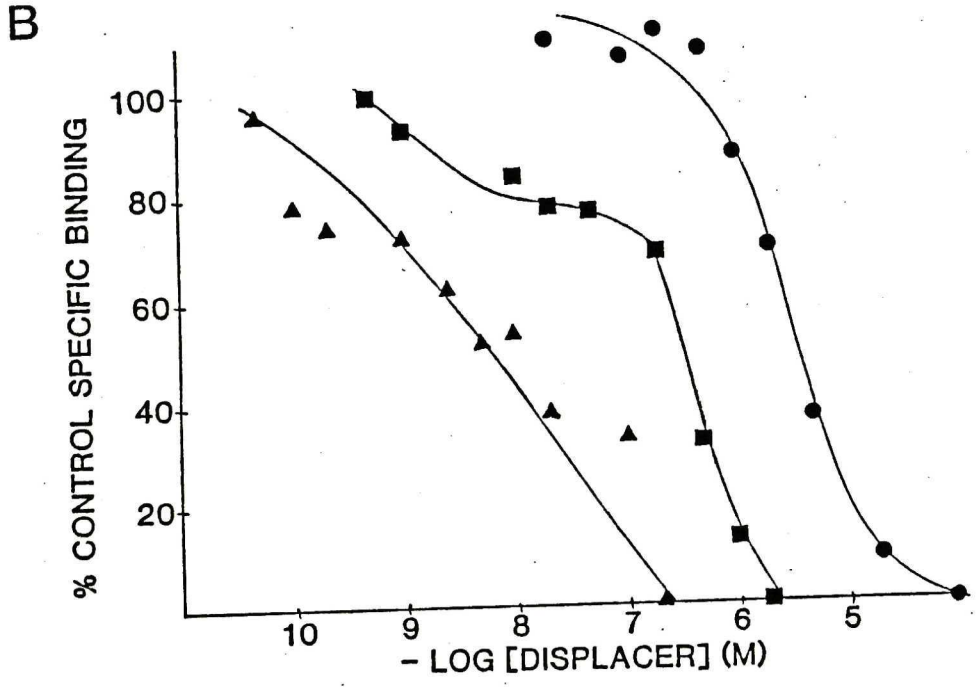


FIGURE 6. SPECIFIC BINDING OF 5 nM [^{3}H]DAGO IN THE PRESENCE OF VARYING CONCENTRATIONS OF DAGO. Values are the means ± standard error of the mean for three seprate experiments performed in triplicate. Hill plot (insert) of the same data is shown. Y axis= Log (P/1-P and P= % displacement of control binding.

FIGURE 7. SPECIFIC BINDING OF 5 nM [^{3}H]DAGO IN PRESENCE OF INCREASING CONCENTRATIONS OF UNLABELLED DISPLACERS. A: Data are shown for DAGO (▲), Nalbuphine HCl (●), morphine sulfate (■). B: Data are shown for DAGO (▲), U50488H (●), and DSTLE (■). N = 2-3 for each curve shown.


A
100
80
60
40
20
% CONTROL BINDING
10
9
8
7
6
– LOG [DISPLACER] (M)


B
100
80
60
40
20
% CONTROL SPECIFIC BINDING
10
9
8
7
6
5
– LOG [DISPLACER] (M)

∂ selective ligand had a K_I of 161 nM and a B_{MAX} of 20 fmol/mg protein. Results of [^{3}H]DAGO binding at μ site are summarized in table 1. Thus [^{3}H]DAGO binding is readily displaceable only by ligands having previously characterized high affinity at μ type binding sites; ∂ and k selective ligands have relatively low potency. Thus [^{3}H]DAGO binding appears to be almost exclusively to μ type binding sites. However, Hill plots for displacing ligands have a low slope, and a shoulder may be apparent in some displacement curves. It is possible that DAGO has comparable affinity at two subtypes of the μ receptor. This is discussed further in the discussion section.

An earlier report on opioid receptor binding in guinea pig lumbo-sacral spinal cord (Attali et al., 1982) claimed a lack of μ binding as defined by the absence of specific binding of [^{3}H]dihydromorphine or [^{3}H]DADLE. These findings are not in agreement with our results in which saturation experiments with [^{3}H]DAGO in both cervical-thoracic or lumbo-sacral guinea pig spinal cord showed binding to sites with μ characteristics (Fig. 8a.). Scatchard plots for both tissue preparations were linear. Computer analysis produced a one site model with a K_D of 7 nM and a B_{MAX} of 18 fmol/mg protein for lumbo-sacral membranes and a single site model with a K_D of 9 nM and a B_{MAX} of 20 fmol/mg protein in cervical-thoracic membranes. These saturation experiments were not significantly different from one another at the $P < 0.05$ level. Displacement of 5 nM [^{3}H]DAGO binding in lumbo-sacral spinal cord membranes with morphine sulfate showed a K_I of 12 nM and a B_{MAX} of 21 fmol/mg protein while U50488H had an $IC_{50} > 10$ μM (Fig. 8b.). The site labelled by [^{3}H]DAGO in lumbo-sacral spinal cord has similar K_D and B_{MAX} values as the site labelled by [^{3}H]DAGO in whole spinal cord membrane saturation experiments. In addition the K_I for morphine in whole spinal cord and lumbo-sacral spinal cord are similar. Our results for binding studies in guinea pig lumbo-sacral spinal cord with [^{3}H]DAGO indicate that the receptor population labelled has characteristics consistent with μ opioid receptor site binding..

Table 1.

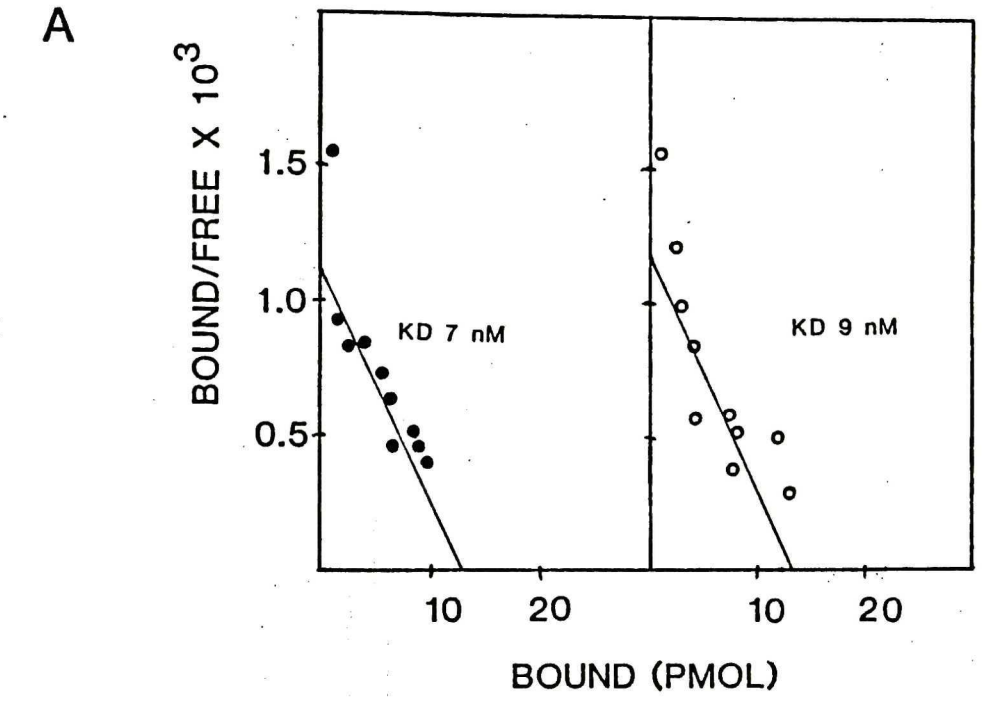
Parameters for μ site binding of [^{3}H]DAGO in guinea pig spinal cord measured by saturation and displacement studies.

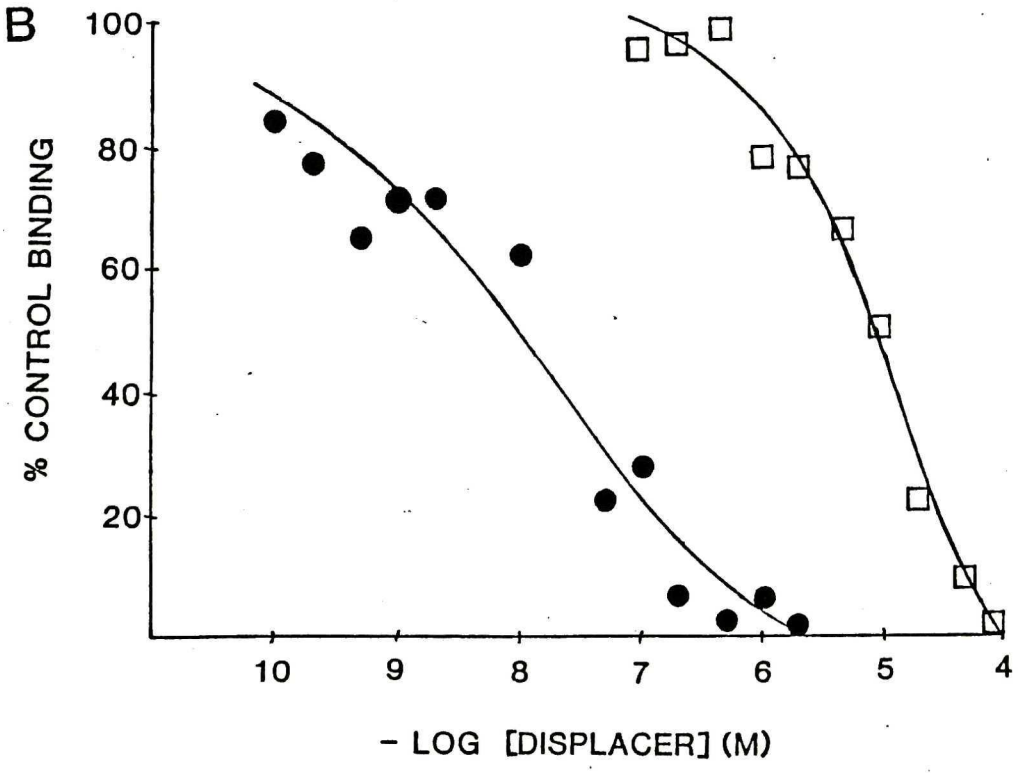
Parameters were derived by the LIGAND computer program for analysis of saturation or displacement data. For displacement experiments increasing concentrations of unlabelled ligand were used to compete against the binding of 5nM [^{3}H]DAGO. Saturation experiments were performed as described in methods. K_D and K_I values are expressed as nM, and B_{MAX} as fmol/mg protein. N = 2-3 for each value listed.

Experimental Condition	K_D	K_I	B_{MAX}
[^{3}H] DAGO Scatchard	6.0	---	18
[^{3}H] DAGO Displaced With DAGO	---	10.0	14
[^{3}H] DAGO Displaced With Nalbuphine HCl	---	11	15
[^{3}H] DAGO Displaced With Morphine	---	15	13
[^{3}H] DAGO Displaced With DSTLE	---	161	20
[^{3}H] DAGO Displaced With U50488H	---	1900	14

FIGURE 8a. SPECIFIC BINDING OF [^{3}H]DAGO TO GUINEA PIG LUMBO-SACRAL (o) AND CERVICAL-THORACIC (o) GUINEA PIG SPINAL CORD MEMBRANES. Membranes were incubated with 1-50 nM [^{3}H]DAGO. Increasing quantities of [^{3}H]DAGO were added up to 10 nM [^{3}H]DAGO, higher concentrations were achieved by adding non-radioactive DAGO. Data presented is one of two similar experiments. Solid line for each curve is the computer fitted parameter.

FIGURE 8b. SPECIFIC BINDING OF 5 nM [^{3}H] DAGO IN PRESENCE OF VARYING CONCENTRATIONS OF MORPHINE (●) AND U50488H (□) IN GUINEA PIG LUMBO-SACRAL SPINAL CORD. N = 1 for each value listed.


A
BOUND/FREE X 10^3
1.5
1.0
0.5
KD 7 nM
KD 9 nM
10
20
10
20
BOUND (PMOL)


B
100
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60
40
20
% CONTROL BINDING
10
9
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5
4
- LOG [DISPLACER] (M)

Characterization of Specific Binding of [^{3}H]EKC

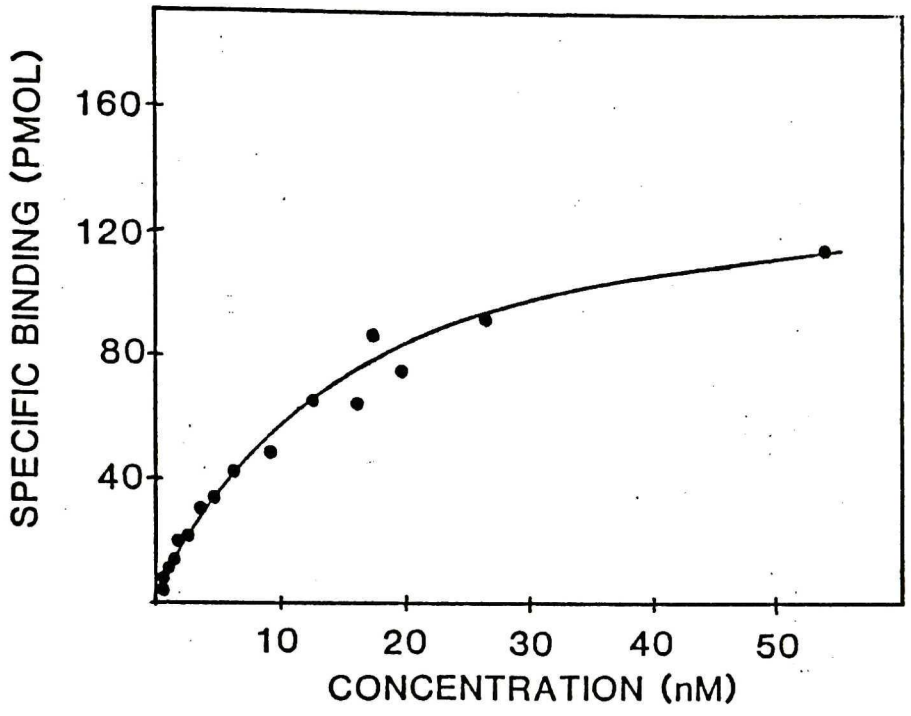
Specific binding was found to be saturable when membrane suspension was incubated with [^{3}H]EKC at concentrations of 0.1-50 nM (Fig 9a.). A curvilinear Scatchard plot was obtained from saturation curves for [^{3}H]EKC (Fig 9b.). A model of two independent binding sites fit the data better than a one-site model ($F=18$ $P=.001$). The computer program could not fit a model with three sites. Assuming the existence of 2 independent binding sites, computer analysis reveals a high affinity site with a K_D of 1.0 nM and a B_{MAX} of 41 fmol/mg protein and a low affinity site with a K_D of 42 nM and a B_{MAX} of 158 fmol/mg protein. In order to examine the two components of [^{3}H]EKC binding, displacement studies with DAGO were carried out. From earlier results it can be seen that in our system DAGO was μ site selective ligand. Displacement of 2.0 nM [^{3}H]EKC by increasing concentrations of DAGO revealed a high affinity site for DAGO displacement with a K_I of 5.5 nM, and a low affinity site that would have a K_I greater than 10 μM (Fig 10a.). The high affinity displacement of 30 % of the 2 nM [^{3}H]EKC specific binding by DAGO is consistent with displacement of [^{3}H]EKC from μ opioid receptor sites. Therefore a 1 μM concentration of DAGO was selected as a concentration that could be utilized to block μ receptor sites without blocking k receptor sites in this system.

Displacement of 2.0 nM [^{3}H]EKC with U50488H revealed a high affinity site with a K_I of 2.3 nM, and a low affinity site with a K_I of 1.9 μM (Fig. 10b) according to computer analysis. A slight shoulder can be seen at 1μM concentration U50488H in (Fig. 10b). A more pronounced shoulder could be seen in displacement studies of [^{3}H]EKC with U50488H in guinea pig cortex studies (Werling et al., 1984a). Based on this data a 1μM final concentration was selected to block k binding and leave μ receptors free. To be certain that [^{3}H]EKC was not binding to ∂ receptors in our system, DSTLE a putative ∂ selective ligand, was

FIGURE 9a. SPECIFIC BINDING OF [^{3}H]EKC TO GUINEA PIG SPINAL CORD MEMBRANES AT INCREASING RADIOLIGAND CONCENTRATIONS. Membranes were incubated with 0.1-50 nM concentration [^{3}H]EKC as described in materials and methods. Increasing quantities of [^{3}H]EKC were added up to 25 nM. Higher concentrations were achieved by adding non-radioactive EKC. Results of one of two similar experiments are shown.

FIGURE 9b. SCATCHARD PLOT OF SAME DATA. This plot was resolved into two components by a computerized non-linear regression program that assumes two independent binding sites. Dashed lines are computer fitted model of each independent binding site. Data from one of two represenative experiments are shown.

A


SPECIFIC BINDING (PMOL)
160
120
80
40
10
20
30
40
50
CONCENTRATION (nM)

B

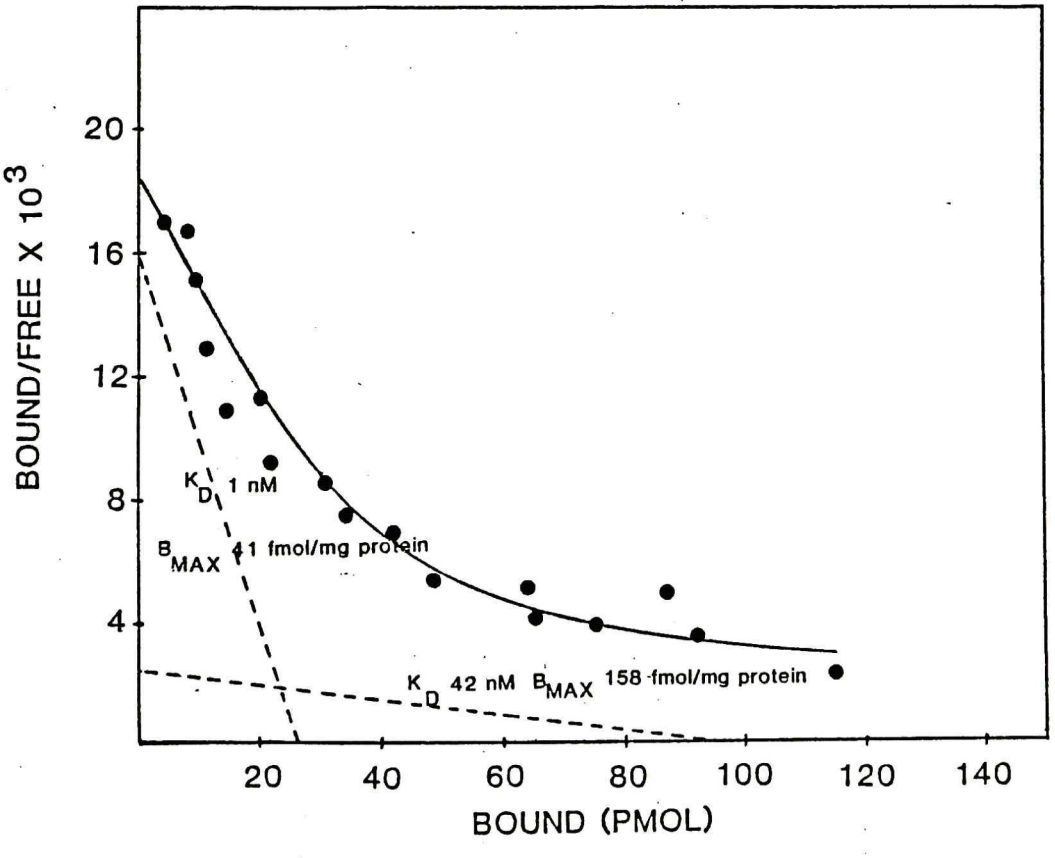
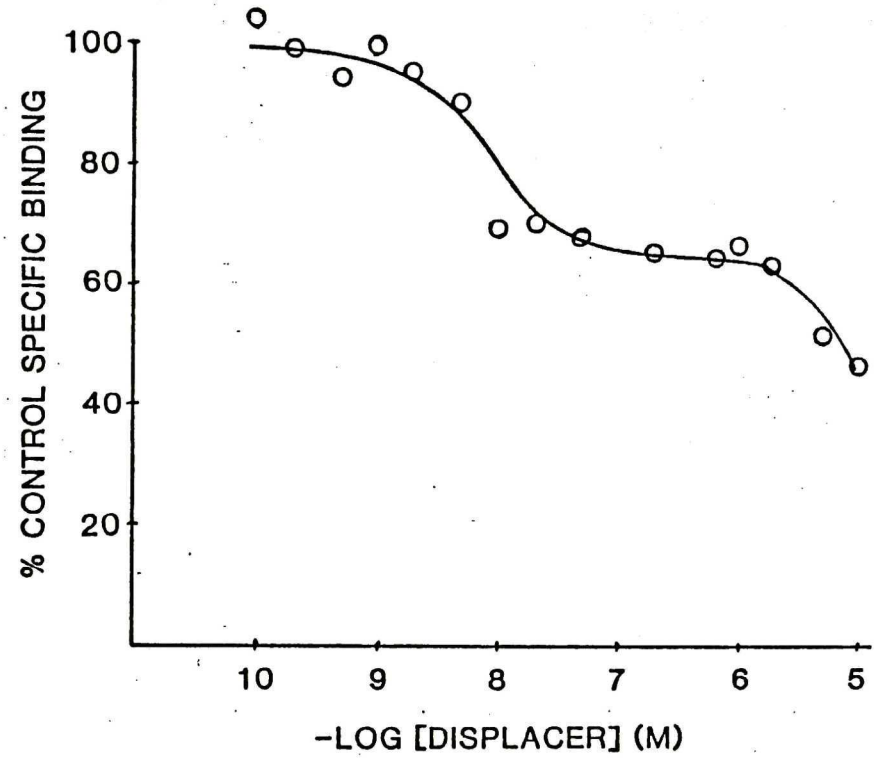

BOUND/FREE X 10^3
20
16
12
8
4
K_D 1 nM
B_{MAX} 41 fmol/mg protein
K_D 42 nM B_{MAX} 158 fmol/mg protein
20
40
60
80
100
120
140
BOUND (PMOL)

FIGURE 10. SPECIFIC BINDING OF 2nM [^{3}H]EKC IN PRESENCE OF VARYING CONCENTRATIONS OF DAGO AND U50488H. Each curve presented is a representative curve from two similar experiments. Data are shown for displacement with DAGO (A), and U50488H (B).

A


100
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% CONTROL SPECIFIC BINDING
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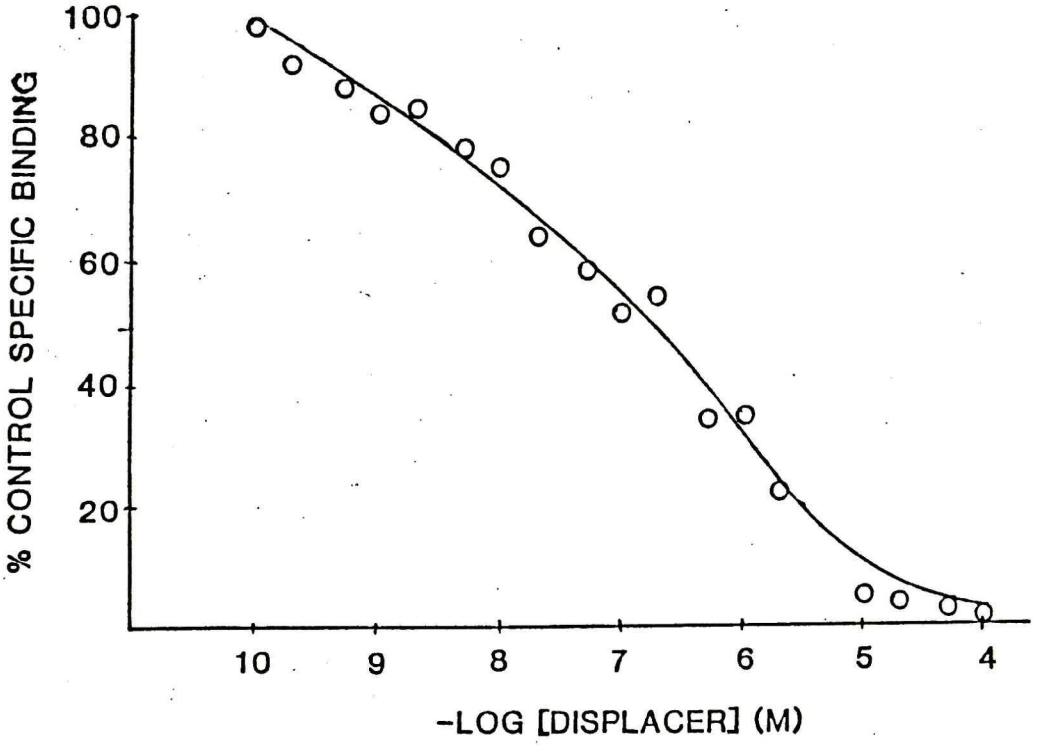
-LOG [DISPLACER] (M)

B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-LOG [DISPLACER] (M)

used to displace 2.0 nM [^{3}H] EKC in both unblocked and 1 μM DAGO blocked displacement studies (Fig. 11). DSTLE had a K_I of 45 nM when no DAGO was included. When 1 μM DAGO was added to block [^{3}H]EKC binding to μ sites, DSTLE was unable to displace any specific binding even at 50 μM final concentration. From these DSTLE displacement experiments one of two conclusions can be reached, either [^{3}H]EKC has no measurable affinity at ∂ receptors or 1 μM DAGO block is a sufficent concentration to block [^{3}H]EKC binding to ∂ receptors if they are recognized by the labelled ligand. From these initial displacement studies it appeared possible to isolate opioid receptor subtypes in guinea pig spinal cord membranes. To assess if a 1μM DAGO block of [^{3}H]EKC binding would be able to isolate a population of k receptors, displacement of approximately 2 nM [^{3}H]EKC with unlabelled EKC and U50488H was carried out with and without 1 μM DAGO block. Displacement of [^{3}H]EKC with EKC in an unblocked experiment had a IC_{50} of 63 nM; when the same experiment was repeated with 1 μM DAGO block the IC_{50} was reduced to 1.2 nM with a K_I of 0.47 nM and a B_{MAX} of 30 fmol/mg protein (Fig. 12a.). In addition the Hill slope without a block was 0.56 and with a block the Hill slope increased to 0.73 (Inset Fig. 12a.). Displacement of [^{3}H]EKC with U50488H in an unblocked experiment had an IC_{50} of 200 nM. When the same experiment was repeated with a 1μM DAGO block the IC_{50} was reduced to 31 nM with a K_I of 14 nM and a B_{MAX} of 29 fmol/mg protein (Fig. 12b.). Again the Hill Slope was increased from 0.36 without a block to 0.51 with a block (Inset Fig. 12b.). An attempt to study the μ binding of [^{3}H]EKC by displacement of [^{3}H]EKC in the presence of 1μM U50488H block was not possible due to the very low residual specific binding. The small number of counts did not allow us to accurately construct a displacement curve.

Saturation analysis of 0.12-50 nM [^{3}H]EKC binding was repeated under three conditions in parallel. These were: no blocking drugs, 1μM DAGO (μ site block), and 1μM U50488H (k site block). Without blocking concentrations of either U50488H or DAGO,

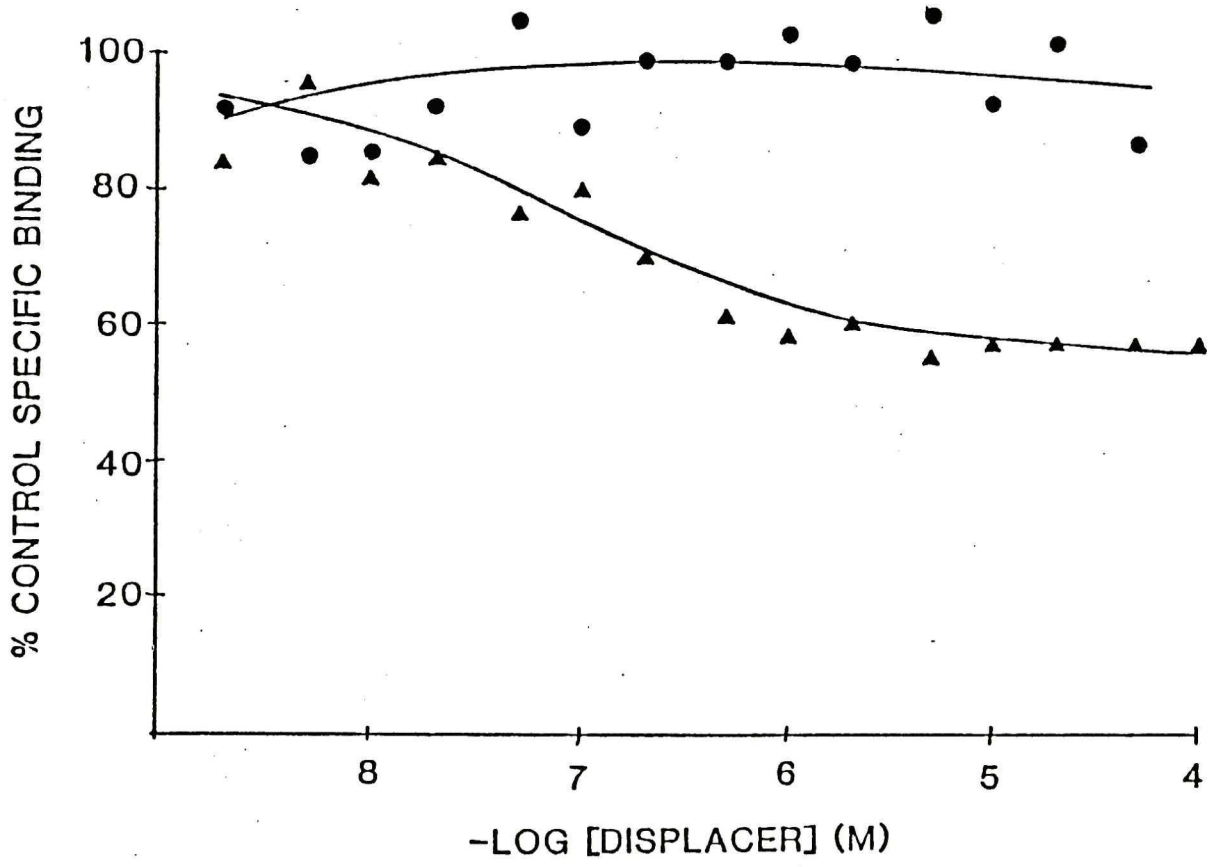
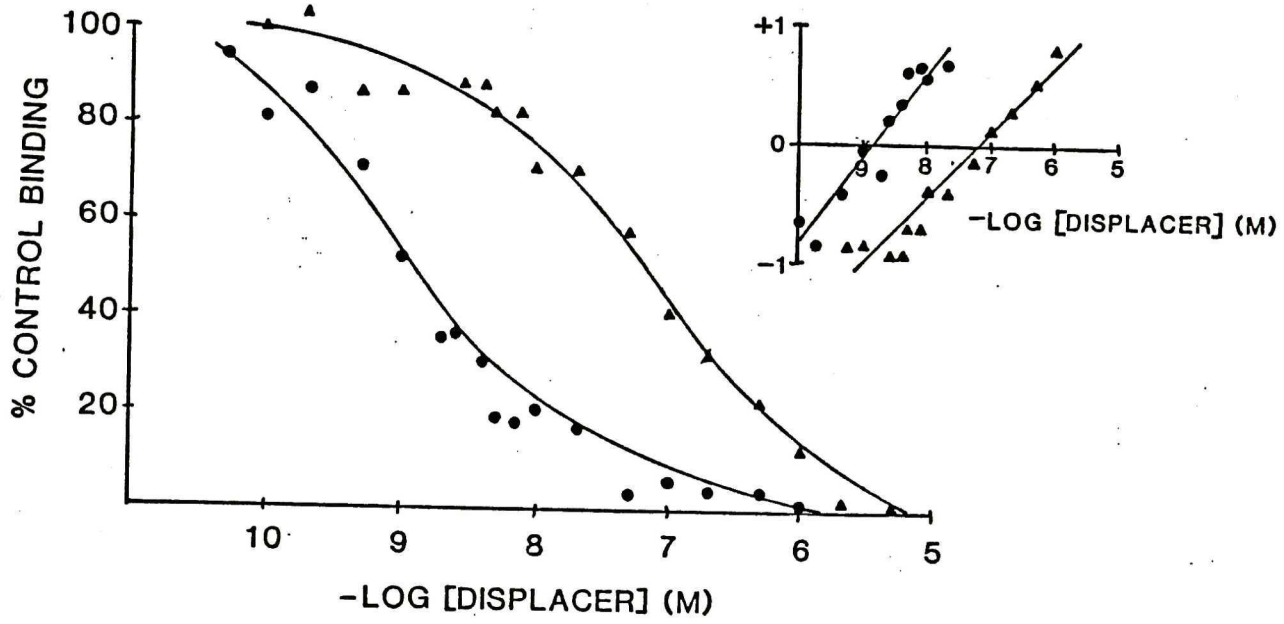


FIGURE 11. SPECIFIC BINDING OF 2 nM [^{3}H]EKC IN PRESENCE OF INCREASING CONCENTRATIONS DSTLE WITH AND WITHOUT 1 µM DAGO BLOCK. Representative results from one of two experiments are presented. Data are shown for displacement by DSTLE without 1µM DAGO block(▲), and displacement by DSTLE with 1µM DAGO block (●).

FIGURE 12. SPECIFIC BINDING of 2 nM [^{3}H]EKC IN PRESENCE OF EKC WITH AND WITHOUT 1 µM DAGO BLOCK (A), OR U50488H WITH OR WITHOUT 1µM DAGO BLOCK (B). Data are shown for displacement by EKC (A) without 1 µM DAGO block (▲), and with 1µM DAGO block (●). Data are also shown for displacement with U50488H (B) without1µM DAGO block (▲), and with 1µM DAGO block (●). Hill plot (inset) Y axis= Log (P/1-P) and P= % displacement of control binding. One of two represenative experiments shown, except where indicated by *, N = 1.

A


100
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% CONTROL BINDING
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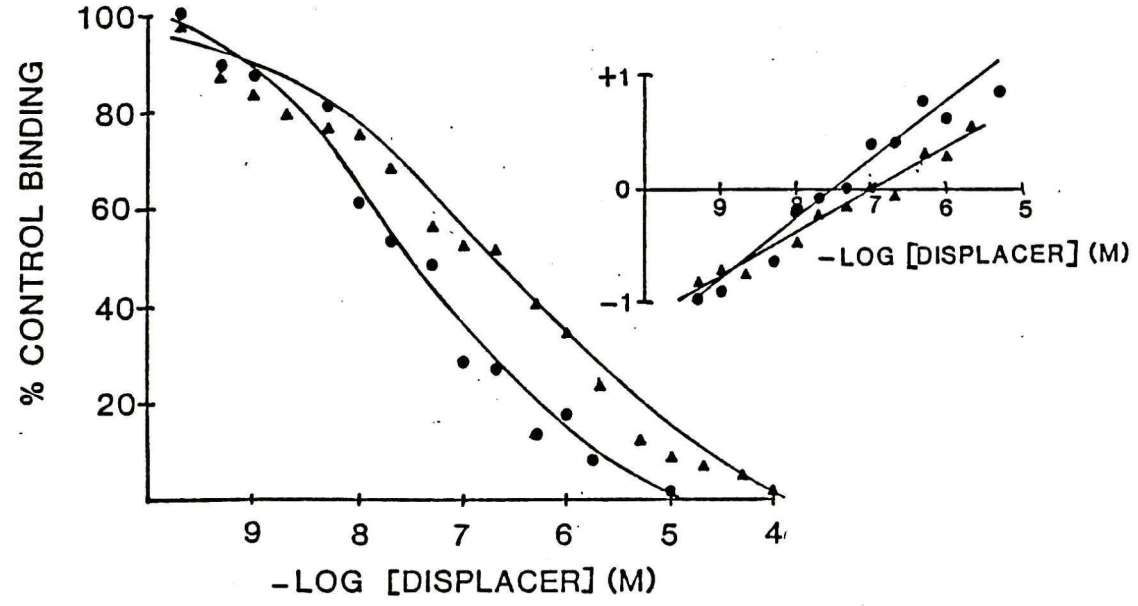
5
-LOG [DISPLACER] (M)
+1
0
-1
9
8
7
6
5
-LOG [DISPLACER] (M)

B


100
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% CONTROL BINDING
9
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4
-LOG [DISPLACER] (M)
+1
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-1
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8
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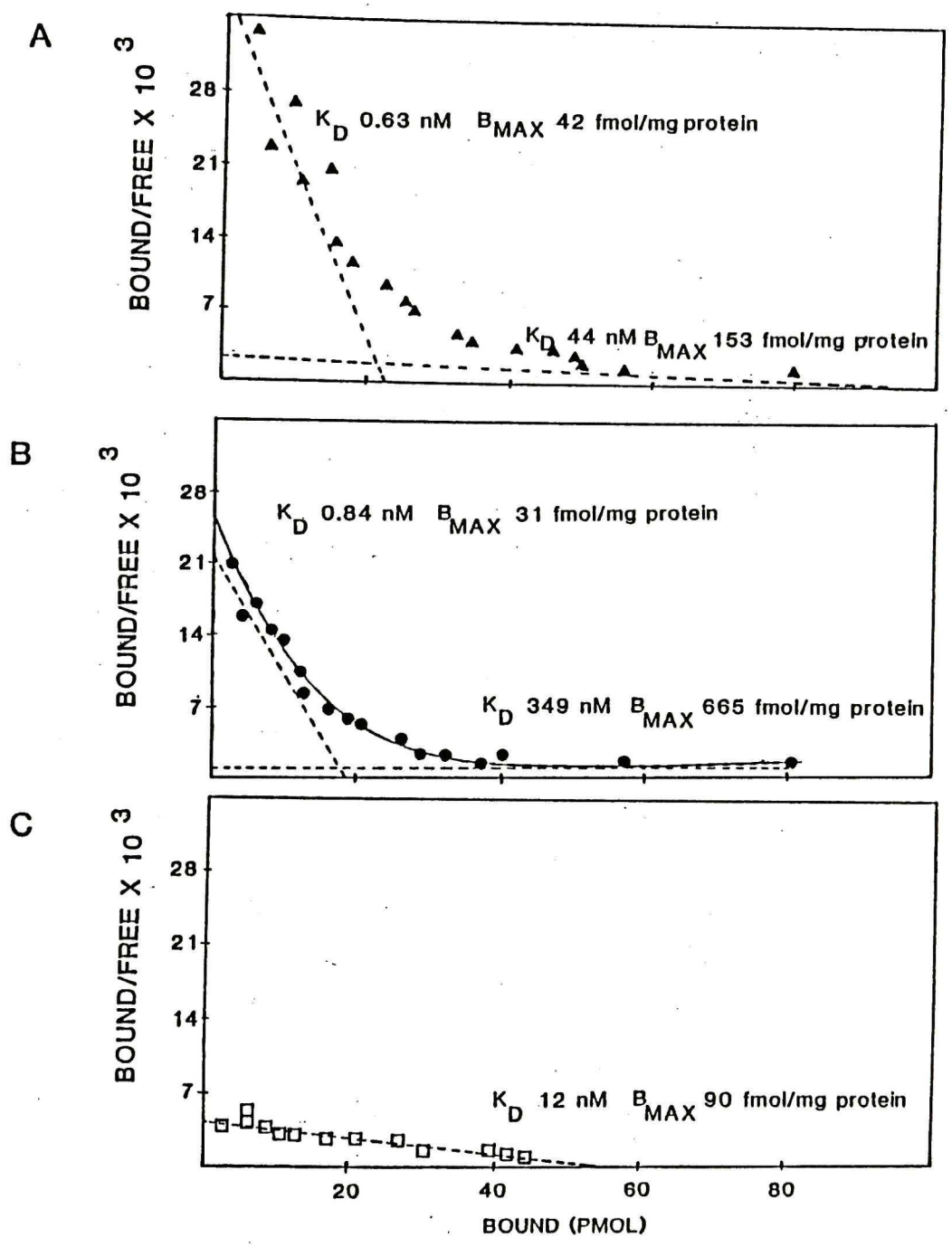
6
5
-LOG [DISPLACER] (M)

Scatchard transformation of the saturation curve for [^{3}H]EKC showed a curvilinear plot (Fig. 13a.). Computer analysis prefered a model that assumed two independent binding sites over a one-site model (F=33 P=0.001). Assuming the existence of two independent binding sites a high affinity site (k site) with a K_D of 0.63 nM and a B_{MAX} of 42 fmol/mg protein and a low affinity site (μ site) with a K_D of 44 nM and a B_{MAX} of 153 fmol/mg protein were identified. When 1μM DAGO block was used in the [^{3}H]EKC saturation assay, Scatchard transformation of the data still produced a curvilinear plot (Fig. 13b.). When this data was subjected to computer analysis, a model assuming two independent binding sites fit the data better than a one-site model (F=93 P=.001), a high affinity site with a K_D of 0.84 nM and a B_{MAX} of 31 fmol/mg protein, and a low affinity site with a K_D of 349 nM and a B_{MAX} of 665 fmol/mg protein were found. Finally, when a 1μM U50488H block was used in the [^{3}H]EKC saturation assay, Scatchard transformation yielded a linear plot and computer modeling was only able to fit a one-site model with a K_D of 12 nM and a B_{MAX} of 90 fmol/mg protein (Fig. 13c.). The high B_{MAX} for the low affinity site in the saturation curves for [^{3}H] EKC with and without a DAGO or U50488H block is too high to be consistent with the B_{MAX} measured for the μ site by [^{3}H]DAGO saturation or displacement curves. This high B_{MAX} is probably an artifact of saturable non-specific binding or the higher concentrations of [^{3}H]EKC in the saturation experiments displacing the blocking ligand from μ sites resulting in a false estimate of the density of the μ site.

Additional displacement studies were performed to analyze ligand binding selectivity at an isolated k site labelled with [^{3}H]EKC in guinea pig spinal cord membranes. WIN 44,441-3, a putative selective k antagonist and its positive isomer WIN 44,441-2, as well as two clinically useful analgesics, nalbuphine HCl and morphine sulfate, were used to displace 2 nM [^{3}H]EKC. WIN 44,441-3 had a K_I of 0.69 nM and a B_{MAX} of 35 fmol/mg protein while

FIGURE 13. SPECIFIC BINDING OF [^{3}H]EKC TO GUINEA PIG SPINAL CORD MEMBRANES AT INCREASING RADIOLIGAND CONCENTRATIONS IN A PARALLEL EXPERIMENT. Membranes were incubated with 0.1-50 nM [^{3}H]EKC as described in Methods section. Increasing quantities of [^{3}H]EKC were added up to 25 nM and higher concentrations were achieved by adding non-radioactive EKC. Data presented is from one experiment without any blocking drugs (A), with 1 µM DAGO block (B), and with 1 µM U50488H block (C). N = 1 for each curve shown.


A
B
C
BOUND/FREE X 10 3
28
21
14
7
K_D 0.63 nM B_{MAX} 42 fmol/mg protein
K_D 44 nM B_{MAX} 153 fmol/mg protein
K_D 0.84 nM B_{MAX} 31 fmol/mg protein
K_D 349 nM B_{MAX} 665 fmol/mg protein
K_D 12 nM B_{MAX} 90 fmol/mg protein
20
40
60
80
BOUND (PMOL)

the positive isomer WIN 44,441-2 had a K_I of 1.9 µM and a B_{MAX} of 48 fmol/mg protein. Morphine sulfate had a low affinity K_I of 5 µM and a B_{MAX} of 39 fmol/mg protein at the isolated k site labelled with [^{3}H]EKC. In the absence of a 1 µM DAGO block, nalbuphine HCl displaced 2 nM [^{3}H]EKC with an IC_{50} of 100 nM and gave a Hill Slope of 0.84 (Fig. 14). In the presence of 1 µM DAGO block the IC_{50} shifted to 400 nM and the Hill Slope was 0.91. The K_I for nalbuphine HCl at isolated k receptors labelled with [^{3}H]EKC was 118 nM and had a B_{MAX} of 30 fmol/mg protein. The results of [^{3}H]EKC binding at the k site are summarized in table 2.

Characterization of Specific Binding of [^{3}H]DADLE

Specific binding was found to be saturable when membrane suspension was incubated with increasing concentrations of [^{3}H]DADLE 0.3-30 nM (Fig. 15a.). Increasing concentrations of [^{3}H]DADLE were added up to 15 nM. Higher concentrations were achieved by adding non-radioactive DADLE. At higher concentrations of [^{3}H]DADLE non-specific binding was 70-80% of total binding. A linear Scatchard plot was obtained from transformation of saturation curves (Fig. 15b.). Computer analysis of [^{3}H]DADLE saturation curves preferred a one site model, with a K_D of 8.0 nM and a B_{MAX} of 28 fmol/mg protein. Previous work has shown that [^{3}H]DADLE discriminates poorly between µ and ∂ opioid receptors (Gillan and Kosterlitz, 1982; James and Goldstein, 1984), and labels ∂ receptors with only a five fold preference over µ receptors. Displacement of 5 nM [^{3}H]DADLE with DSTLE, a putative ∂ selective ligand, was attempted in order to identify [^{3}H]DADLE binding to µ and ∂ subtypes (Fig. 16). DSTLE displaced specific [^{3}H]DADLE binding with an IC_{50} of 15 nM, and displacement of [^{3}H]DADLE was complete within two orders of magnitude. Hill plots of DSTLE displacement of [^{3}H]DADLE had a slope of 0.70 and a R^2 of 0.93 (inset Fig. 16). DSTLE's

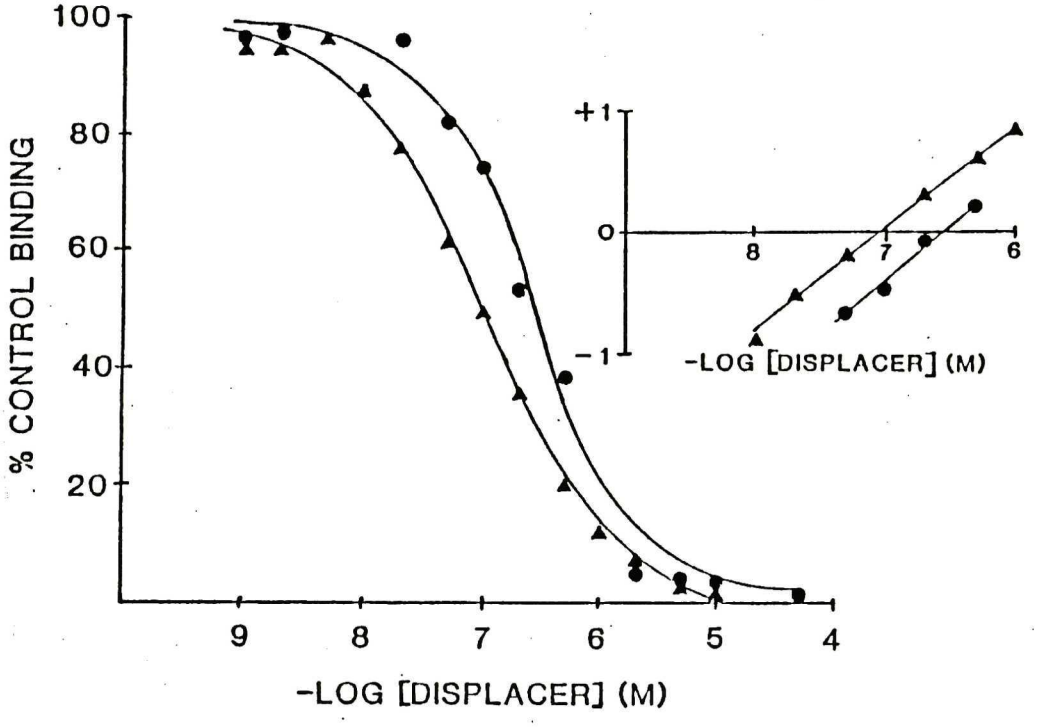


FIGURE 14. SPECIFIC BINDING OF 2 nM [^{3}H]EKC IN PRESENCE OF NALBUPHINE HCL WITH AND WITHOUT 1 µM DAGO BLOCK. Data are shown for displacement without 1 µM DAGO block (▲), and with 1 µM DAGO block (●). Hill plot (inset) Y axis= Log (P/1-P) and P= % displacement of control binding. N = 2-3 for each curve shown.

Table 2

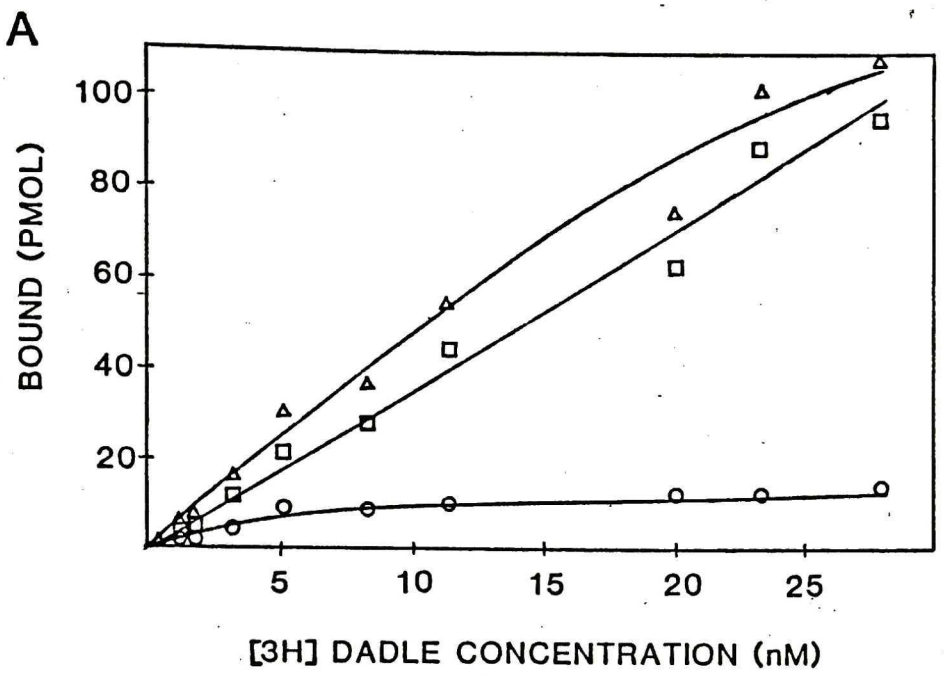
Parameters for k site binding of [^{3}H]EKC in guinea pig spinal cord measured by saturation and displacement studies.

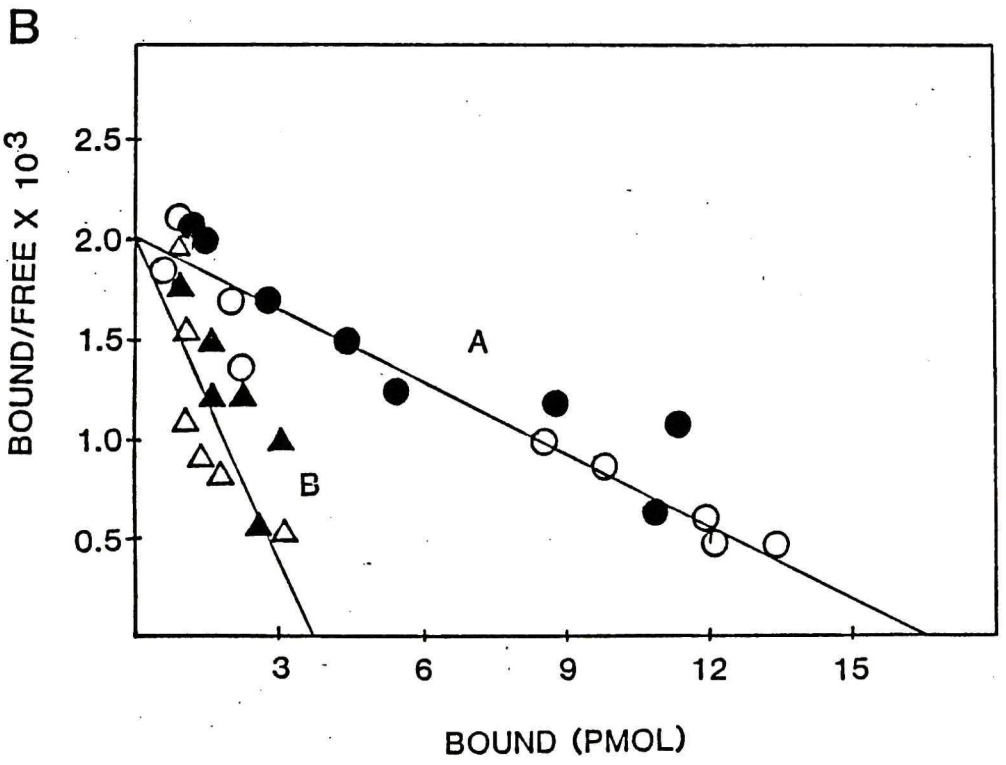
Parameters were derived by the LIGAND computer program for analysis of saturation or displacement data. For displacement experiments increasing concentrations of unlabelled ligand were used to compete against the binding of 2 nM [^{3}H]EKC. Saturation experiments were performed as described in methods. K_D and K_I values are expressed as nM, and B_{MAX} as fmol/mg protein. N = 2-3 for each value listed, except where indicated by *, in which case N = 1.

	K_D	K_I	B_{MAX}
Experimental Condition			
[^{3}H] EKC Scatchard	1.0	---	40
[^{3}H] EKC Scatchard Site 1, 1 µM DAGO Block	0.84 *	---	31 *
[^{3}H] EKC Displaced With EKC, 1 µM DAGO Block	---	0.5 *	30 *
[^{3}H] EKC Displaced With U50488H, 1 µM DAGO Block	---	14.0	29
[^{3}H] EKC Displaced With Nalbuphine HCl, 1 µM DAGO Block	---	118.0	30
[^{3}H] EKC Displaced With WIN 44,441-3, 1 µM DAGO Block	---	0.70	35
[^{3}H] EKC Displaced With WIN 44,441-2, 1µM DAGO Block	---	1900.0	48

FIGURE 15a. SPECIFIC BINDING OF [^{3}H]DADLE TO GUINEA PIG SPINAL CORD MEMBRANES AT INCREASING RADIOLIGAND CONCENTRATIONS. Membranes were incubated with 0.3-30 nM [^{3}H]DADLE as described in Materials and Methods. Increasing concentrations of [^{3}H]DADLE were added up to 15 nM. Higher concentrations were achieved by adding non-radioactive DADLE. Plot of saturation curve data show: total binding (Δ); non-specific binding (□); specific binding (o). Data from one of two typical experiments shown

FIGURE 15B. SPECIFIC BINDING OF [^{3}H]DADLE TO GUINEA PIG SPINAL CORD MEMBRANES AT INCREASING RADIOLIGAND CONCENTRATIONS IN (●o) ABSENCE AND IN (▲Δ) PRESENCE OF 10 nM DAGO BLOCK. Data for (●o) are experimental results described in Fig. 11b. Data for (▲Δ) are from 2 independent experiments where membranes were incubated with 0.5-25 nM [^{3}H]DADLE. Solid lines are computer-generated fits for experiments with similar experimental conditions modelled together.


A
BOUND (PMOL)
100
80
60
40
20
5
10
15
20
25
[3H] DADLE CONCENTRATION (nM)


B
BOUND/FREE X 10^3
2.5
2.0
1.5
1.0
0.5
A
B
3
6
9
12
15
BOUND (PMOL)

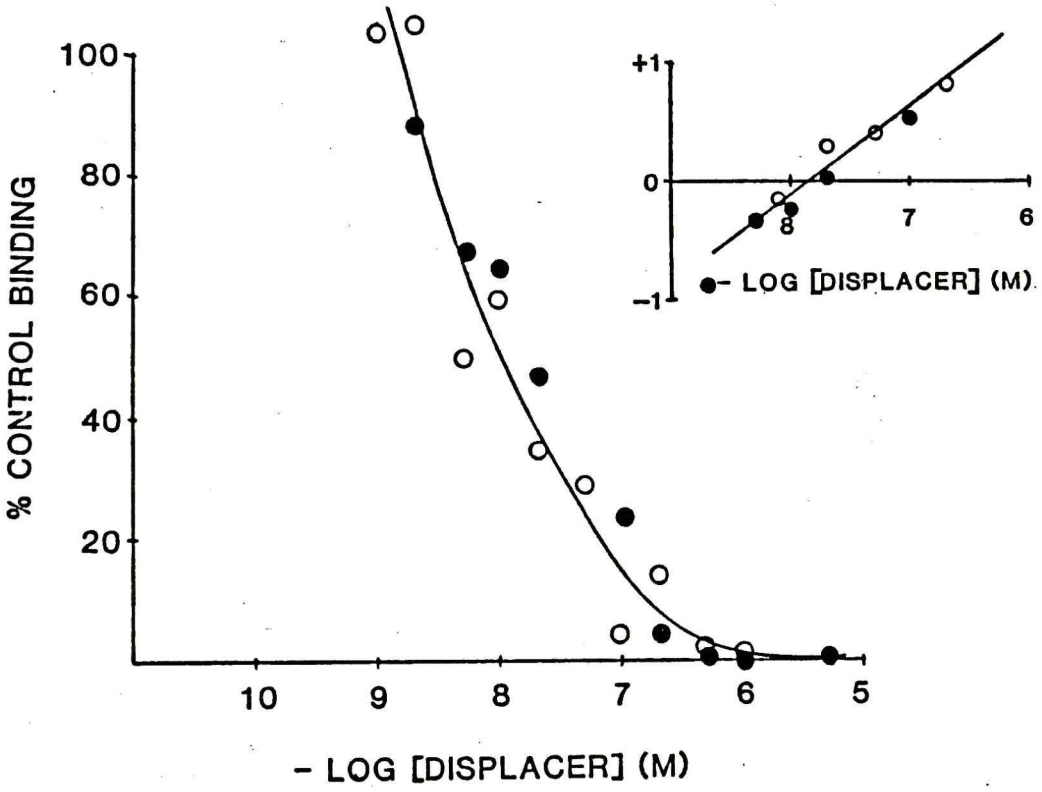


FIGURE 16. SPECIFIC BINDING OF 5 nM [^{3}H]DADLE IN PRESENCE OF VARYING CONCENTRATIONS OF DSTLE. (●) and (o) are data points from independent experiments. Hill plot of displacement experiments (inset) Y axis= Log (P/1-P) with P= % displacement of control binding.

inability to discriminate two binding sites for [^{3}H]DADLE at μ and ∂, resulted in our using DAGO, a μ selective ligand for displacement of [^{3}H]DADLE to achieve this goal. Displacement of 5 nM [^{3}H]DADLE with DAGO showed a high affinity displacement with an IC_{50} of 3 nM and 80 % of control binding complete at 10 nM DAGO (Fig. 17). DAGO was unable to displace the remaining 20 % of control binding of [^{3}H]DADLE even at 50 μM concentrations of DAGO. Based on these experiments, a concentration of 10 nM DAGO was selected to block [^{3}H] DADLE binding to μ sites and allow binding studies of isolated ∂ sites. Saturation analysis of 0.5-25 nM a[^{3}H]DADLE binding was repeated in the presence of 10 nM DAGO (Fig. 15b). Scatchard analysis of the data showed a linear plot. Computer analysis of this data fit a model that assumed the existence of a single type of opioid receptors, while a two site model was not compatible with the observed values. Assuming the existence of a single site, the computer fit a model with a K_D of 1.9 nM and a B_{MAX} of 6.0 fmol/mg protein. Blocking μ sites with 10 nM DAGO resulted in an approximate four fold increase in [^{3}H]DADLE's affinity for the remaining sites and a 80 % reduction in the number of receptor sites. Displacement studies of 5 nM [^{3}H]DADLE binding in the presence of 10 nM DAGO block was not possible due to too few remaining specifically bound dpm (< 75 dpm). Increasing the concentration of [^{3}H]DADLE to increase the amount of specific binding was not possible for two reasons. First the concentration of [^{3}H]DADLE would be far above its K_D at ∂ sites and second increasing the concentration of [^{3}H]DADLE above 5 nM results in large increases of nonspecific binding. A solution to this problem may be to increase the amount of tissue (and assay volume) in order to increase specifically bound dpm. Displacement of 5 nM [^{3}H]DADLE without DAGO block was carried out using nalbuphine HCl. Nalbuphine HCl had a IC_{50} of 31 nM and displacement of specific binding was complete at 10 μM nalbuphine HCl. This IC_{50} is higher than its IC_{50} against [^{3}H]DAGO binding to μ sites, again suggesting that a component of [^{3}H]DADLE binding has low affinity for nalbuphine. It is unlikely that nalbuphine has high affinity at the ∂ site.

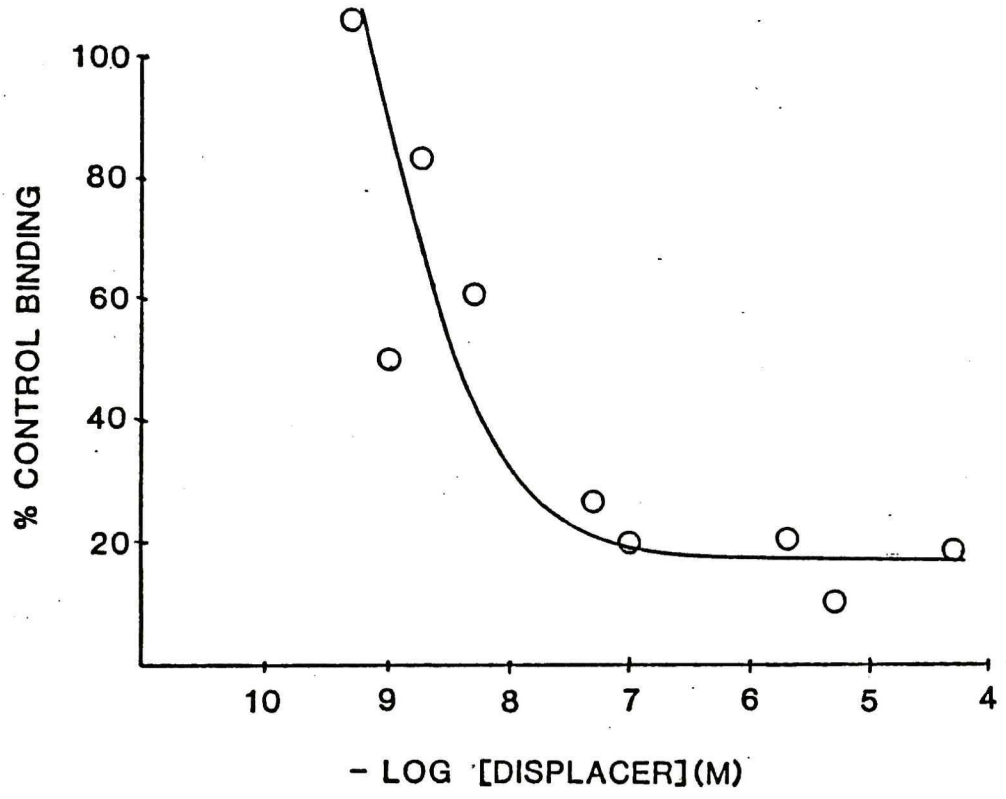


FIGURE 17. SPECIFIC BINDING OF 5 nM [^{3}H]DADLE IN PRESENCE OF VARYING CONCENTRATIONS OF DAGO. Results shown are from one of two similar experiments.

Discussion

Heterogeneity of Spinal Opioid Receptors

It is clear from these data that the opiate receptors in the spinal cord are heterogeneous like those in brain (Kosterlitz et al., 1981; James and Goldstein, 1984; Werling et al., 1984c). It is also clear that guinea pig spinal cord has a lower density of receptors than guinea pig cortex Table 3. The K_D's of [^{3}H]DAGO at μ sites, [^{3}H]DADLE at ∂ sites and [^{3}H]EKC at k sites are similar for all neural tissues studied in this laboratory under our conditions. The relative proportions of μ, ∂ and k receptors are similar for guinea pig cortex and spinal cord. In rat whole brain there are fewer k receptors and more μ and ∂ receptors. Thus, species differences influence the relative content of μ, ∂ and k receptors in a given tissue but appear to have a minimal effect on the affinity of a radioligand at an isolated opioid receptor type. In our work with whole guinea pig spinal cord we noted a total opioid receptor density of 50 fmol/mg protein. The relative content of μ, ∂ and k receptors in guinea pig spinal cord are in good agreement with the relative content of opioid receptor types in postmortem whole human spinal cord and guinea pig lumbo-sacral cord described by Czlonkowski and co-workers (1983). However the density of opioid receptor types in whole human spinal cord minus cervical segments (Czlonkowski et al., 1983) was 22 fmol/mg protein. The addition of sodium to our assay system would be anticipated to produce a reduction in the density of only ∂ type opioid receptors (Werling et al., 1984b), which are only 10 % of the total opioid receptors in guinea pig spinal cord. Therefore the addition of sodium to our assay system would only be expected to produce a small decrease in the total density of opioid receptors in whole spinal cord tissue. It is possible that the 1.5 - 5.5 hour delay from time of death to removal of human postmortem spinal cord tissue resulted in a slow degradation of tissue, and in a proportional decrease of all opioid receptor types. Rat whole spinal cord had a three fold greater density of opiate receptors (Mack et al., 1984) than we found in these studies in whole guinea pig spinal cord. The relative content of μ, ∂ and k opioid

Table 3.

Parameters for binding of radioligands in guinea pig and rat neural tissue

Parameters were derived by using the LIGAND computer program to analyze saturation data. [^{3}H]DAGO was used to characterize μ binding, [^{3}H]DADLE in the presence of 10nM DAGO for ∂ binding, and [^{3}H]EKC in the presence of 1μM DAGO for K binding. K_D values are expressed in nM, and B_{max} values as fmol/mg protein. Percentages in parentheses following B_{max} values are proportions of total receptor number represented by that receptor type. For guinea pig cortex, n = 2-6 for each K_D and B_{max} listed; for other preparations n = 2-3.

	μ		∂		K	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
Guinea Pig Cortex *	6.7	36 (23%)	2.5	18 (12%)	1.4	10 (68%)
Guinea Pig Spinal Cord	6.0	18 (30%)	1.9	6.1 (10%)	1.4	35 (59%)
Guinea Pig Striatum *	7.2	32 (20%)	4.7	41 (26%)	1.0	87 (54%)
Rat Whole Brain *	7.2	70 (47%)	3.5	51 (34%)	1.6	29 (19%)

* Work From:
Werling, L. L., Zarr, G., and Cox, B.M. : Opioid binding characteristics of rat and guinea pig brain membranes in the presence of physiological cations at 37°C. (Manuscript in preparation)

receptors in rat spinal cord reported by Mack and co-workers is in good agreement with the relative proportion of opioid receptors in rat brain reported by Werling and co-workers (1984c). It is important to remember that the content of k receptors in rat spinal cord is 13% while the relative proportions of ∂ receptors is 33% of total opioid receptors (Mack et al., 1984). Previous intrathecal opiate analgesia studies in rats using drugs thought to have k agonist properties (Schamauss et al.,1984) may have underestimated the potency of these compounds due to the low number of k receptors in rat spinal cord for k agonists to bind to and produce a pharmacologic effect. Use of an animal model with a relative concentration of opioid receptors closer to that of man may yield potency estimates for k agonists that would have greater clinical relevance.

Lumbo-sacral Spinal Cord Opioid Receptors

Our work in guinea pig lumbo-sacral spinal cord has demonstrated the existence of specific binding of [^{3}H]DAGO, a μ opioid receptor agonist. The binding parameters for [^{3}H]DAGO in lumbo-sacral spinal cord are similar to those seen in whole guinea pig lumbo-sacral spinal cord. Displacement of [^{3}H]DAGO in lumbo-sacral spinal cord with morphine and U50488H also indicate that the binding of [^{3}H]DAGO in guinea pig lumbo-sacral spinal cord is to a site with μ opioid receptor characteristics. The demonstration of μ binding in guinea pig lumbo-sacral spinal cord is in agreement with work by Czlonkowski and co-workers who found μ binding in guinea pig, rat and human lumbo-sacral spinal cord. These findings are not in agreement with previous reports of absence of μ binding in rat and guinea pig lumbo-sacral spinal cord (Attali et al., 1982; Gouarderes et al., 1982). The absence of specific binding for [^{3}H]DHM or [^{3}H]DADLE in guinea pig lumbo-sacral spinal cord lead Attali and co-workers to assume the absence of μ or ∂ opioid receptor binding in this tissue. The reasons for this group's inability to identify specific binding for [^{3}H]DHM or [^{3}H]DADLE remains uncertain, but may be a result of a high level of non-specific relative to specific binding in this tissue. Attali and co-workers

proposed the existence of two receptor sites that could be isolated by the differential effects of a 5 μM DADLE block on [^{3}H]EKC binding. These two sites were termed DADLE sensitive site (DAL_s) which was a portion of the [^{3}H]EKC binding that was lost when a 5 μM DADLE block was utilized. The use of a 5 μM DADLE block resulted in the loss of [^{3}H]etorphine binding as well. The DADLE insensitive site (DAL_i) was defined by the binding of [^{3}H]EKC that remained when a 5 μM DADLE block was used. The DAL_i site had a K_D of 0.36 nM and a B_{MAX} of 30 fmol/mg protein. The DAL_i site was proposed to have characteristics similar to a k site identified in guinea pig brain by Kosterlitz and co-workers (1981). Our work in whole guinea pig spinal cord revealed a single k site with a K_D of 0.84 nM and a B_{MAX} of 31 fmol/mg protein. We believe that this isolated k site is the same site that Attali and co-workers (1982) identified in guinea pig lumbo-sacral spinal cord because the K_D's and the B_{MAX}'s are quite similar. The 2.3 ..fold reduction in the affinity of [^{3}H]EKC at this site is due to the addition of salts to our assay system. Assuming that our results showing that μ opioid receptor binding is present in lumbo-sacral spinal cord is correct, a portion or all of the DAL_s site would be due to DADLE blocking [^{3}H]EKC to μ binding sites and not DADLE blocking [^{3}H]EKC binding to benzomorphan sites as proposed (Attali et al., 1982). Scatchard transformation of [^{3}H]EKC saturation curves in the presence of 5 μM DADLE block remained curvilinear with a low affinity site that had a K_D of 5.6 nM and a B_{MAX} of 108 fmol/mg protein. Attali and co-workers did not address the issue of what receptor site could be associated with this low affinity site. In our Scatchard transformations and computer modelling of [^{3}H]EKC saturation curves we noted a low affinity site for [^{3}H]EKC binding in whole guinea pig spinal cord both in the presence and the absence of 1μM DAGO block. In our unblocked [^{3}H]EKC saturation curves the density of these sites is too high to be only μ receptor sites. The possibility exists that a portion of this low affinity site is a

benzomorphan site. However, when a 1 μM DAGO block is used in [^{3}H]EKC saturation curves, the density of the low affinity site increases 5 fold. This type of finding is most consistant with high concentrations of [^{3}H]EKC displacing the DAGO block from μ sites and the labelling of a component of saturable low affinity non-specific binding resulting in the artifactual appearance of an additional binding site. In view of our findings and those and Czlonkowski and co-workers (1983), interpretation of binding experiments in lumbo-sacral spinal cord under the assumption of absence of μ or ∂ binding may lead to inaccurate conclusions.

Mu Receptor Subtypes

The existence of μ_1 and μ_2 opioid receptor subtypes (Pasternak et al., 1980) in whole or lumbo-sacral guinea pig spinal cord has not been established at this time. The broad displacement of [^{3}H]DAGO by DAGO and DSTLE in whole spinal cord (Fig. 7b) and morphine in lumbo-sacral spinal cord (Fig. 8b) may be an indication that [^{3}H]DAGO is not binding to a homogeneous population of opioid receptors in this tissue. In addition, Scatchard transformations of [^{3}H]DAGO saturation curves in lumbo-sacral and cervical-thoracic spinal cord have a slightly curvilinear appearance. However, computer analysis is unable to fit this data to a two site model. Additional [^{3}H]DAGO saturation curves with more low concentration points as well as additional displacement studies with DSTLE and DAGO may be able to establish firm evidence for μ subtypes in guinea pig spinal cord. Should further studies establish evidence for μ subtypes in this tissue, naloxazone or naloxonazine blocking could be added to these studies. Past work with naloxazone (Pasternak et al., 1980) and naloxonazine (Houghton et al., 1984) has shown that these long lasting opioid antagonists have a preference for μ_1 sites. Addition of naloxazone or naloxonazine to saturation or displacement assaies would be expected to produce a loss of the very high affinity, low capacity binding that would be associated with μ_1

binding and have no effect on the lower affinity μ_2 binding site. The work of Pasternak and co-workers (1980) showed that in mice, μ_1 sites seemed to have a selective role in producing morphine analgesia and that loss of these sites by *in vivo* naloxazone injections, resulted in loss of analgesia from morphine administration. Should further research establish firm evidence of μ_1 opioid receptor subtypes, and a selective ligand for these sites be developed, systemic or intrathecal opiate analgesia without significent respiratory depression might be possible.

Delta Opioid Receptors

These data clearly establish the existence of ∂ receptors in guinea pig spinal cord. The low density of ∂ receptors in this tissue and the high non-specific binding of [^{3}H]DADLE in this system present a technical difficulty when trying to characterize various ligand affinities at isolated ∂ sites labelled with [^{3}H]DADLE in the presence of a 10 nM DAGO block. The amount of specific binding that remains under these conditions is less than 100 dpm. This amount of specific binding is not sufficent to construct accurate displacement curves under our assay conditions. Increasing the concentration of [^{3}H]DADLE to increase the bound dpm is not possible because the concentration of radioligand needs to be near the K_D of ∂ sites. The introduction of a highly selective ∂ radioligand with lower non-specific binding than [^{3}H]DADLE may allow for further radioligand binding characterization of ∂ sites in this system.

Nalbuphine HCl Affinities at Isolated k and μ Opioid Receptor Sites

The data for nalbuphine HCl show a ten fold selectivity for μ binding in guinea pig whole spinal cord. These *in vitro* binding results do not indicate the agonistic or antagonistic activity of nalbuphine HCl on isolated opioid receptor sites. Further displacement studies with nalbuphine HCl at isolated μ and k receptor sites in the presence and absence of manganese may

provide biochemical evidence of nalbuphine's agonistic or antagonistic activity at isolated opioid receptor sites. It has been shown that manganese in the presence of sodium increases the binding of agonist opiates while having little effect on the binding of antagonists (Pasternak et al., 1975). The results of co-administration of nalbuphine HCl and morphine intrathecally in rats (Schamauss et al., 1983) would suggest that nalbuphine HCl is an agonist at k sites and an antagonist at μ sites. Bioassay techniques in electrically stimulated GPI that has been pretreated by site directed alkalation to produce an enriched population of desired opioid receptors (James et al., 1982) would provide information as to nalbuphine's agonistic and antagonistic properties at isolated receptor sites.

Comparison of *In Vitro* K_D and K_I Values With *In Vivo* EC_{50} Estimates

There is good agreement between published EC_{50} values for *in vivo* opiate and opioid analgesia and estimates of binding affinities derived for isolated opioid receptor sites from *in vitro* radioligand binding studies presented in these data. Yaksh (1981) has published intrathecal EC_{50} values for a wide variety of opiate and opioid drugs. Morphine has an EC_{50} of 12.7 nM, while in our morphine displacement assays of μ sites labelled with [^{3}H]DAGO, morphine had a K_I of 15 nM. DADLE had an EC_{50} of 11.4 nM. In our unblocked [^{3}H]DADLE saturation curves where [^{3}H]DADLE is binding to both μ and ∂ sites a K_D of 8 nM was noted. When comparing EC_{50} and K_D values for DADLE, it is reasonable to use K_D values derived from unblocked saturation curves, since in the in vivo intrathecal opioid model DADLE is most likely producing its analgesia via both μ and ∂ receptors (Yaksh, 1984). The EC_{50} for EKC in rat spinal cord was 52.9 nM. This is a value that is close to our measured K_D of 40 nM for [^{3}H]EKC at μ sites in guinea pig spinal cord. The analgesic activity of EKC in rat spinal cord is most

likely due to binding to μ receptors since the density of k receptors is low in rat spinal cord (Mack et al., 1984).

These comparisons suggest a remarkable similarity between estimates of potency and affinity at the receptors apparently mediating the spinal analgesia. However, the similarity should be treated with caution. The opioid potency estimates are based on assumptions regarding the volume of distribution of the intrathecally administered opioid which may not be correct. Secondly, in other opioid systems which have been studied in more detail, there is evidence for the existence of "spare" receptors. If there were spare receptors on neurons mediating opioid analgesia in spinal cord, EC_{50} values would be expected to be lower than estimates of receptor affinity.It remains to be determined if k-agonists have a higher analgesic potency in species with a higher density of k type receptors. It would also be of interest to compare potencies of k agonists in the relief of chemically mediated pain in species with relatively high and low concentrations of k receptors, in view of the suggestion by Schmauss and co-workers (1983) that this kind of painful stimulus is particularly susceptible to k agonist induced analgesia.

Conclusions

These data clearly establish the heterogeneity of opioid receptors in guinea pig spinal cord under more physiologic assay conditions than have been used in past studies. The relative proportion of opioid receptor types in the spinal cord is in close agreement with the relative proportion of receptor types in guinea pig cortex. In addition, these data clearly show that μ binding exists in guinea pig lumbo-sacral spinal cord. These data also show the binding affinities of two clinically useful spinal analgesics, nalbuphine HCl and morphine sulfate. It is apparent from these data that morphine, a μ selective ligand, and DSTLE, a ∂ selective ligand, have very low affinity at isolated k sites. Morphine sulfate in displacement studies of 2 nM [^{3}H]EKC, in presence of 1 μM DAGO block, had an IC_{50} greater than 5 μM. While DSTLE had no measurable affinity at isolated k sites labelled by 2 nM [^{3}H]EKC in the presence of 1 μM DAGO block. Displacement of [^{3}H]DAGO binding with morphine, DSTLE, and DAGO all show possible evidence of binding to μ_1 and to μ_2 sites. In this system DAGO remains a μ site selective ligand but may be able to discriminate subtypes of μ receptors. The affinities of morphine and EKC at μ sites, and DADLE at μ and ∂ sites, correspond closely with their reported EC_{50} in inducing spinal analgesia in the rat.

Radioligand receptor binding studies in tissue homogenates can give quantitative information about the density and relative proportions of opioid receptor types and binding affinities of these receptors for various ligands. However, radioligand receptor binding studies can not provide information about the anatomic localization of opioid receptors. Future studies in selected levels of guinea pig spinal cord, utilizing opiate and opioid site selective radioligand binding studies in slices of spinal cord tissue and autoradiographic techniques combined with computer analysis of autoradiograms, could provide quantitative as well as qualitative information about opioid receptors in this tissue. In addition these types of studies could provide information as to the location of opioid receptor types in spinal cord and the potential functions

of each receptor type in the regulation of different aspects of spinal cord function. Several lines of evidence suggest that spinal cord opioids have functions in systems unrelated to processing of pain sensation. Comparison of receptor selective opioid potencies in some of these systems with estimates of their affinities at isolated opioid binding sites under physiological conditions may assist in the identification of the receptor types mediating these affects.

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