Title
Opiate receptor-effect relationships in intact rats and cultured cell lines

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OPIATE RECEPTOR - EFFECT RELATIONSHIPS IN INTACT RATS AND CULTURED CELL LINES

by

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DISSERTATION

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This thesis and the work chronicled within are dedicated to my parents,

Geraldine D. Richards and Paul W. Richards

ABSTRACT

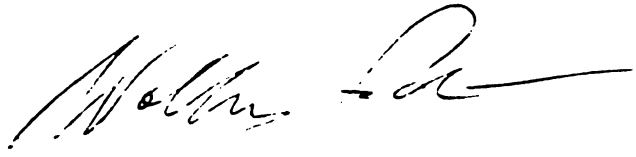
The receptor occupancy and pharmacological effects of opioids were compared in both intact rats and cultured neuronal cell lines in order to establish opiate site-effect relationships. A novel ex vivo labeling method was established for characterizing in vivo opiate receptor occupancy in rat brain. This method was applied to oripavine and benzomorphan opioids and found to compare well with previously developed in vivo labeling methods. However unlike the latter approach, the ex vivo labeling technique was able to differentiate occupancy of delta and kappa sites. Further, the contrast of relative binding affinities determined in vivo versus in vitro demonstrates the importance of the in vivo measures when making quantitative comparisons with pharmacological effects.

The in vivo occupancy of oripavine and benzomorphan opioid antagonists were compared with their blocking action on bremazocine-induced urine flow. The in vivo fractional occupancies of the antagonists were ~0.5 at the kappa site, while variable and ≠ 0.5 at the mu and delta sites. This supports the alleged role for kappa sites in mediating opioid-stimulated diuresis. Accordingly, buprenorphine caused a similar inhibition of uresis in both naive and morphine (mu) tolerant, bremazocine-treated rats, suggesting that buprenorphine acts as an antagonist at the kappa site.

The discovery in our laboratory of a human neuroblastoma cell line that expresses mu and delta opiate binding sites prompted an investigation into their potential functional significance. SK-N-SH cells were found to produce cyclic AMP, and the PGE_1-stimulated production was inhibited to ~ 80% control by etorphine, DADL and morphine. Further, the opioid effects were blocked by naloxone. However, the inhibitory signal was insufficient to make quantitative comparisons for determining the receptor site(s) responsible for the observed effect.

Opioids did not affect catecholamine uptake in human neuroblastoma cell lines, SK-N-SH and IMR-32, found to contain both opiate receptors and noradrenergic

uptake$_1$ sites. Based on comparisons with brain particulates, the uptake$_1$ system on the 2 human neuroblastoma cell lines appears to be an excellent model system for catecholamine reuptake in noradrenergic pathways of rodent and human brain.



Acknowledgements

The content of this thesis directly results from an association with my research advisor, mentor and friend, Wolfgang Sadee. He is responsible for integrating the tabulated numbers into the advancement of science.

Also I recognize my wife and friend, Mary. During the past 4-plus years she made the bad days tolerable and the good days bliss.

But it has been my Father who, in spite of his reluctance to take credit for my meandering through 15 years of College, provided the impetus to complete my education. His inspiration, support and friendship have laid the foundation for all the positive things that I accomplish professionally.

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INTRODUCTION

A. Overall Objective

The objectives of this dissertation are to: establish the in vivo binding character of oripavine and benzomorphan opioids using a novel ex vivo labeling approach; compare in vivo binding parameters of oripavine and benzomorphan opioids with their effects on urine output in the intact animal; and determine the effect of prototypical mu, delta and kappa opioids upon second messengers and neurotransmitters in human neuroblastoma cells.

B. BACKGROUND

1. Opiate receptor system

Diversity of opiate receptors

Since the first description of the multiple opiate receptor system by Martin (Martin et al., 1976; Gilbert and Martin, 1976), considerable pharmacological and biochemical evidence has been submitted in support of opiate receptor heterogeneity (Chang and Cuatrecasas, 1979; Schultz et al., 1981; Wood et al., 1981a; Magnan et al., 1982). In rodent central nervous system (CNS), the mu, delta and kappa opiate binding sites have been well characterized in vitro using radioligand binding techniques (Magnan et al., 1982; Pfeiffer and Herz, 1982; Gillan and Kosterlitz, 1982). Opioid alkaloids, such as morphine, bind selectively to the mu site, whereas delta sites are preferred by opioid peptides (eg., enkephalins) (Chang and Cuatrecasas, 1979). Benzomorphans (eg., ethylketocyclazocine and ketazocine) and the dynorphins are the prototypical ligands for the kappa site

(Chang et al., 1981b; Huidobro-Toro et al., 1982). Additional sites have been identified to which opioids bind. These include sigma (Zukin and Zukin, 1981) and lambda sites (Grevel and Sadee, 1983) in rat brain, and epsilon sites (Miller, 1982) in rat vas deferens. However, the number of opioid drugs that bind to these sites are limited and, thus, likely do not serve an important role in the pharmacological actions of most opioids.

An additional source of evidence supporting the concept of opiate receptor heterogeneity comes from pharmacological studies utilizing isolated peripheral organs. The large differences in potencies of opioids to elicit an effect (inhibition of electrically stimulated contractions) in guinea pig ileum, mouse vas deferens and rat vas deferens suggests that different receptors control their responses. Guinea pig ileum is very sensitive to mu selective opioids such as morphine (Wuster et al., 1981). Conversely, opioid peptides that are selective for delta receptors are more potent in the mouse vas deferens preparation than guinea pig ileum (Wuster et al., 1981). Rat vas deferens appears to be responsive only to β-endorphin (Wuster et al., 1981).

The results of in vivo opioid binding studies in rat brain recently have provided further support for multiple receptors (Rosenbaum et al., 1984a; 1985). The in vivo labeling method, while apparently selective for the mu site, was unable to differentiate delta and kappa sites. This limitation likely reflects the lack of tracer specificity at delta and kappa sites and the paucity of kappa receptors in rat brain (Gillan and Kosterlitz, 1982).

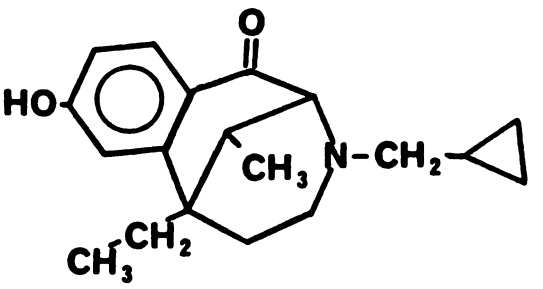
Pharmacology of opioid drugs

The dominant pharmacological effects associated with opioid drugs are localized in the CNS and bowel: analgesia, tolerance, physical dependence, respiratory depression and other effects associated with CNS depression, and slowing of gastro-intestinal motility (Jaffe and Martin, 1980). Effects that are less commonly associated with opioids include alterations of urine output (Huidobro,

Selected Structural Classes of Opioid Drugs

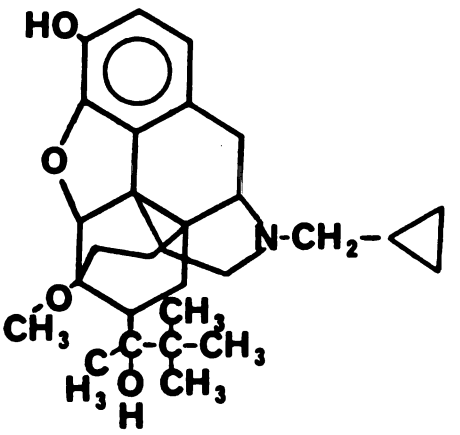
Benzomorphans

ethylketocyclazocine



Oripavines

buprenorphine

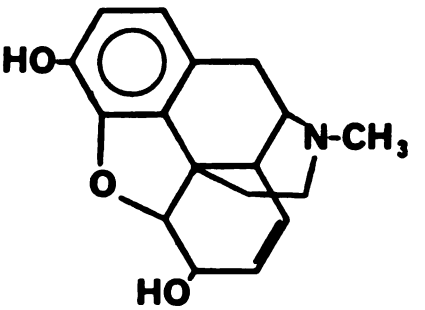


Enkephalins

D-Ala, D-Leu enkephalin L-Tyr-D-Ala-Gly-L-Phe-D-Leu-OH

Alkaloids

morphine



1978; Leander, 1983a) and psychotomimetic/dysphoric reactions (Zukin and Zukin, 1981). The pharmacological effects associated with each opioid drug depends on whether it is an agonist, antagonist or mixed agonist/antagonist. Moreover, it's receptor binding selectivity will determine it's action. Knowledge of the profile of each opioid drug with respect to these characteristics is necessary to establish receptor site-effect relationships.

Opiate receptor site-effect relationships

Martin's initial description of the pharmacological profiles for opioid drugs was based on a comparison of drug class (hence, receptor class) versus it's pharmacological effects (Martin et al., 1976; Gilbert and Martin, 1976): alkaloid opioids (eg., morphine; mu receptors) caused supraspinal analgesia, respiratory depression, euphoria and physical dependence; and benzomorphans (eg., ketocyclazocine; kappa receptors) caused spinal analgesia, miosis and sedation. For the most part, establishment of site-effect relationships have since been limited to attempts at linking in vitro binding character with in vivo pharmacological potencies. Although such comparisons have resulted in defining mu (Gacel et al., 1981; Kosterlitz et al., 1980), delta (Fredrikson et al., 1981) and kappa (Wood et al., 1981b; Tyres, 1980) sites as responsible for mediating analgesia, the mu receptor is generally regarded as playing a dominant role in antinociceptive responses. Similar approaches have implicated kappa sites in increasing urine output (Slizgi and Ludens, 1982; Leander, 1983a), whereas morphine and other 'mu agonists' decrease urination in water loaded rats (Huidobro, 1978; Huidobro and Huidobro-Toro, 1979). Less is known about the consequences of agonist binding to delta sites beyond the putative analgesic effects (Fredrikson et al., 1981) and the inhibition of contractile responses in mouse vas deferens (Wuster et al., 1981). In vitro binding studies have indicated possible allosteric interactions between mu and delta sites with their respective ligands (Rothman and Westfall, 1982).

Considerable difficulties exist with regard to linking in vitro binding affinities

with pharmacological effects in the intact animal. In part, this stems from the observation that the in vitro homogenization and washing procedures change the receptor binding properties of opioid agonists and antagonists (Sadee et al., 1982a; Kurowski et al., 1982). Moreover, the recent demonstration of discrepancies between the relative in vitro versus in vivo receptor occupancy of several opioid ligands (Rosenbaum et al., 1984a; 1985) suggests that binding affinities in vitro do not accurately reflect the relative affinities in vivo. Further, to identify the receptor types responsible for mediating a physiological response, it is insufficient to determine receptor occupancy by agonists, since fractional receptor occupancy would not be expected to be proportional to the response in most cases. Rather, the fractional in vivo receptor occupancy of antagonist drugs should be compared with their ability to inhibit the effects of agonists. If antagonism is competitive and not accompanied by agonist effects, this fractional occupancy should be directly proportional to the inhibitory effect. As an illustration, the in vivo receptor occupancy of several opioids were measured at the mu, delta and kappa sites using an in vivo labeling technique (Rosenbaum et al., 1984a). Consistent occupancy-effect relationships for mu antagonism versus sufentanil and etorphine analgesia demonstrates the suitability of this approach to delineate functions of the individual opioid receptor types.

2. Pharmacology of opioids in clonal cell lines

Opiate receptors in clonal cell lines

Because of the heterogeneity of opiate receptors in the CNS, it is desirable to find clonal cell lines that express homogeneous opiate receptor populations so that their functions can be more easily characterized at the cellular level. However until recently, only delta type of opiate binding sites have been identified in cultured cells. These cell lines are subclones and hybrids of murine neuroblastoma cells

(West and Miller, 1983). Although these cell systems have been invaluable in providing insight into the delta site modulation of second messenger systems (Sharma et al., 1975; Brandt et al., 1976; Law et al., 1982), the role of delta receptors in mediation of opioid effects in the intact animal is unknown. The recent discovery of a human neuroblastoma cell line (SK-N-SH) that expresses mu and delta opiate binding sites provides a potential tool for understanding the function of mu sites at the cellular level (Yu et al., 1986).

Modulation of cyclic nucleotides

Opioid drugs have been shown to depress the basal and hormonally-stimulated levels of cyclic AMP in rat brain homogenates (Law et al., 1981; Collier and Roy, 1974) and cultured cells (Law et al., 1982; Sharma et al., 1975; Brandt et al., 1976). Because the effect is observed in the presence of phosphodiesterase inhibitors, it is likely that inhibition of adenylate cyclase is responsible. Further, it appears that this effect on adenylate cyclase is mediated by the inhibitory subunit (N_i) of the adenylate cyclase receptor system located in the cell membrane (Blume, 1980).

Opioids stimulate increases of cyclic GMP in neuroblastoma clones/hybrids (Gwynn and Costa, 1982). The mechanism of this change is less well understood although a rapid Ca^{++} influx has been implicated (West and Miller, 1983).

The receptor system(s) mediating opioid effects on cyclic nucleotides has not been fully characterized. However, because the cell lines studied contain only delta sites, it is reasonable to assume that the opioid activity is mediated, at the least, through delta receptors. Whether other opioid receptors are involved in modulating the adenylate cyclase system is yet to be determined.

Effects on catecholamines

Interactions between opioids and various neurotransmitter systems have been described in particulates from rat brain. Although some dispute exists regarding

their mechanism, it is generally believed that opioids promote an increase of dopamine turnover (Gauchy et al., 1973; Yonehara and Clouet; 1984; Mulder et al., 1984). This has been variously attributed to an effect on DA release and/or metabolism (Feigenbaum and Yanai, 1984; Wood, 1983b). Conflicting reports also have indicated morphine-induced changes in NE turnover and inhibition of NE release following stimulation of noradrenergic neurons (Schoffelmeer and Mulder, 1984; Attila and Ahtee, 1984). Additionally, there appear to be interactions of opioids and α-adrenergic agonists in the locus coeruleus (Korf et al., 1974).

However, because of the variable experimental conditions, different tissue sources and the complexities of heterocellular systems, there remains much debate regarding the effects of endogenous and exogenous opioids on catecholaminergic cells. Use of cloned neuronal cell preparations would provide an opportunity to measure changes in catecholamine uptake/release at the cellular level under well controlled conditions.

C. Specific Aims

1. To determine the in vivo binding character of the oripavine opioids, buprenorphine, etorphine and diprenorphine, to the mu, delta and kappa sites by a novel ex vivo labeling method, and compare these results with in vivo labeling methods and relative affinities determined in vitro.

2. To compare the in vivo binding affinities of benzomorphan and oripavine antagonist opioids at the mu, delta and kappa sites with their ability to inhibit bremazocine-induced diuresis in the rat.

3. To characterize buprenorphine's activity at the kappa site by assessing it's effect upon bremazocine-induced diuresis in naive and morphine- tolerant rats.

4. To compare the effect of mu and delta agonists on the prostaglandin E_1 stimulated cyclic AMP production in a human neuroblastoma cell line (SK-N-SH) that expresses mu and delta sites.

5. To characterize the catechol uptake system(s) identified in human neuroblastoma cell lines, and to demonstrate it's usefulness as a model system for determining uptake propensity in human neuronal cells.

Chapter 1

In vivo opiate receptor binding of oripavines to mu, delta and kappa sites in rat brain as determined by an *ex vivo* labeling method.

1. SUMMARY

The relative in vivo receptor affinities of three oripavine drugs given subcutaneously were determined at the mu, delta and kappa type of opiate binding sites in rat brain. The oripavines include the agonist etorphine, the antagonist diprenorphine and the mixed agonist- antagonist buprenorphine. With the use of mu, delta and kappa specific labeling conditions in brain homogenates immediately after sacrifice (ex vivo labeling), the method relies on the assay of those receptor sites that remain unbound in vivo. Because of the slow receptor binding kinetics of the oripavines, little or no dissociation of the in vivo ligand occurs during the ex vivo labeling period. All three drugs displayed lower affinity in vivo at the delta sites relative to mu sites, whereas the kappa affinities were highly variable. Etorphine displayed considerable mu selectivity, while buprenorphine's affinity at the mu and kappa sites was similar. The apparent in vivo binding affinities obtained from the ex vivo labeling approach are compatible with previous results where tracers were applied in vivo. The dramatic differences of the in vivo and in vitro opiate receptor binding properties of the oripavines suggest the need for in vivo receptor binding parameters in the analysis of the function of individual receptor types.

2. INTRODUCTION

Pharmacological and biochemical evidence supports the existence of multiple opiate receptors (Martin et al., 1976; Gilbert and Martin, 1976; Chang and Cuatrecasas, 1979; Schultz et al., 1981; Wood et al., 1981a; Magnan et al., 1982). In rodent central nervous system (CNS), the mu, delta and kappa sites have been well characterized in vitro (Magnan et al., 1982; Pfeiffer and Herz, 1982; Gillan and Kosterlitz, 1982). The presence of additional sites (e.g., sigma and lambda) also

has been suggested (Pasternak et al., 1981; Grevel and Sadee, 1983), although the number of opioid drugs that bind to these sites is limited. Recent in vivo binding studies have provided further support for opiate receptor multiplicity (Rosenbaum et al., 1984a; 1985); however, differences in relative receptor affinities and binding site populations were observed when compared with in vitro binding results. Therefore, determination of opiate receptor binding in the intact animal might be crucial to the understanding of the functions of each opiate receptor site.

Previous in vivo labeling methods (Rosenbaum et al., 1984a; 1985) while apparently selective for the mu site, failed to differentiate the delta and kappa sites because of the lack of tracer specificity at these sites and the paucity of kappa receptors in rat brain (Gillan and Kosterlitz, 1982). To assess more selectively opioid binding affinity to the mu, delta and kappa sites in the rat CNS, an alternative method has been developed that utilizes an ex vivo tracer labeling approach. Upon subcutaneous administration of opioids with slow receptor binding kinetics, it is possible to estimate their in vivo receptor occupancy immediately following sacrifice of the rat. This is accomplished with specific labeling conditions in fresh membrane homogenates to quantitatively assess the remaining mu, delta and kappa sites which are not bound by the drug administered in vivo. Relative binding affinities of buprenorphine, etorphine and diprenorphine determined by this method are compared both with results obtained from studies employing in vivo tracer administration and with published in vitro binding studies using brain membrane homogenates.

3. MATERIALS AND METHODS

3.1 Ex vivo labeling technique

Male Sprague-Dawley rats weighing 120-140 g were subcutaneously administered 400 ul of normal saline with or without varying doses of either buprenorphine, etorphine or diprenorphine. Time of sacrifice was chosen to

coincide with either peak brain concentrations or maximum effect in order to approximate a pseudo-equilibrium state with respect to the in vivo ligand and it's binding site(s), i.e., when net changes in binding are at a minimum. This has been estimated to occur at approximately 20 min for etorphine (Perry et al., 1982) and diprenorphine (Perry et al., 1980) and at 60 min for buprenorphine (Dum and Herz, 1981). After decapitation, the brain (minus cerebellum) is quickly removed, weighed and homogenized (Brinkman Polytron, setting 6) for 20 s in a 1:100

Table I-1. In vitro labeling conditions. Incubation conditions of ligands for determining site selective tracer binding to mu, delta and kappa sites in fresh membrane homogenates which are not occupied bt the oripavine administered in vivo. Specifically bound radioactivity is determined for each site as the difference in radioactivity of filtered aliquots from incubates A and B. Radioligand concentrations were approximately 3 nM [^{3}H]naloxone, 2 nM [^{3}H]DADL and 1 nM [^{3}H]diprenorphine.

site	A	B
mu	[^{3}H]naloxone	[^{3}H]naloxone + 5×10^{-6} M morphiceptin
delta	[^{3}H]DADL + 5×10^{-6} M morphiceptin	[^{3}H]DADL + 3×10^{-7} M diprenorphine
kappa	[^{3}H]diprenorphine + 5×10^{-6} M morphiceptin 5×10^{-7} M DADL	[^{3}H]diprenorphine + 3×10^{-7} M diprenorphine

dilution (brain wet weight : volume) of 50 mM pH 7.4 Tris buffer at 20°C. Aliquots of this homogenate were immediately transferred to incubation flasks containing appropriate tracers and blockers according to the conditions described in table I-1. Bound radioactivity was determined by removing several samples of 1 ml (mu and delta) or 3 ml (kappa) from the incubation flasks, filtering through Whatman GF/B filters and washing with three portions of 5 ml ice-cold Tris buffer and determining the radioactivity associated with the filter via liquid scintillation counting. The scintillant was Aquasol (New England Nuclear) and counting efficiency ranged from 41-44%.

Specifically bound tracer at the mu, delta and kappa sites is calculated from the difference in radioactivity bound to filter from incubates A and B (table I-1). For example, although ^{3}H-naloxone preferentially labels mu sites in vitro, it binds to delta, kappa, lambda and non-specific sites as well. Thus the binding of ^{3}H-naloxone to sites other than mu is determined in a separate incubate containing 5 X 10^{-6} M morphiceptin. This concentration of morphiceptin has been found to selectively block tracer binding to mu sites in fresh membrane homogenates under the described conditions (see Results). Similarly, 5 X 10^{-7} M D-Ala, D-Leu-enkephalin (DADL) was found to block mu and delta sites while not affecting tracer binding to kappa sites. Moreover, [^{3}H]DADL in the presence of 5 X 10^{-6} M morphiceptin labels delta and nonspecific sites, while nonspecific binding can be determined using a saturating concentration of diprenorphine in the presence of DADL tracer. Specific labeling of kappa sites is accomplished using diprenorphine tracer in conjunction with mu and delta blockers (incubate A), and separately determining nonspecific binding in the presence of unlabeled diprenorphine (3 X 10^{-7} M) to saturate mu, delta and kappa sites (incubate B).

To minimize the error associated with the redistributing in vivo ligand, in vitro tracer binding was determined according to the scheme illustrated in fig. I-1. A four min sampling time is chosen to minimize redistribution at 20°C of the in vivo ligand while achieving sufficient tracer binding for quantitative analysis. Several ($\geq$ 4)

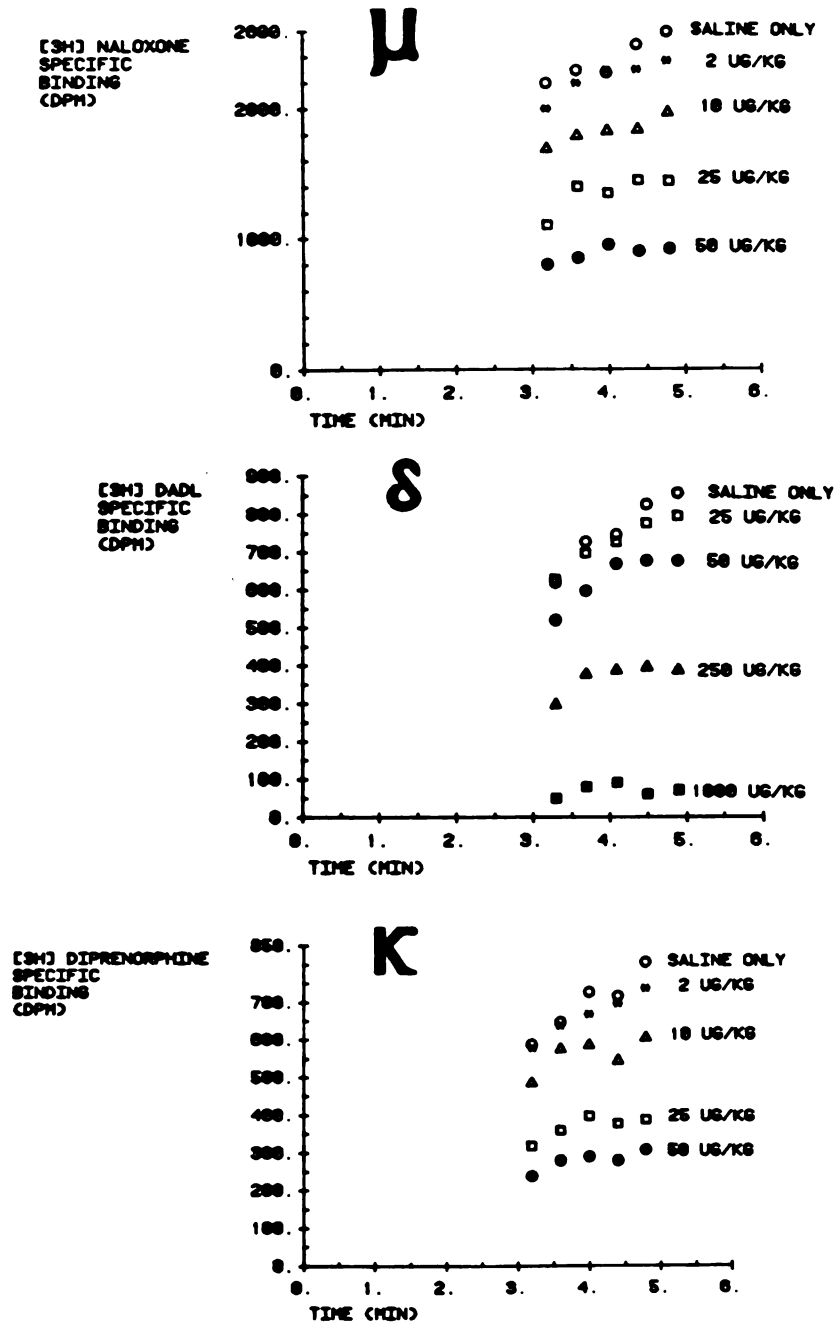


Figure I-1. Determination of in vivo oripavine binding by ex vivo labeling. Buprenorphine was given s.c. in the doses indicated, and the rats were sacrificed after 60 min. Between 3 and 5 min after addition of fresh membrane homogenates to tracer incubates A and B (see table 1), several (>4) aliquots of this homogenate mixture are quickly removed and filtered in a sequential fashion. The amount of specifically bound tracer at four min is determined by linear regression for homogenates obtained from each rat receiving saline or varying doses of buprenorphine. The average standard deviation of the tracer binding estimate at 4 min was 8.7% for the three binding sites (n=5 independent rat experiments).

samples were obtained between 3.5 and 5 min following homogenization, and specifically bound tracer was determined at four min using linear regression of the results over the sampling period. Deviations from linearity of the association curves did not appear to be significant during this brief observation period. Exemplary equilibrium binding curves are illustrated for each tracer using membranes obtained from untreated rats (fig. I-2).

At any time point, the amount of [^{3}H]naloxone, [^{3}H]DADL and [^{3}H]diprenorphine specifically bound in vitro to the mu, delta and kappa sites, respectively, should be directly proportional to the number of sites remaining unoccupied by the in vivo drug. The reduction in radioactivity specifically bound to membranes obtained from drug treated rats is expressed as a percentage of control binding, i.e., bound radioactivity to mu, delta and kappa sites in membranes from saline treated rats.

3.2 Determination of in vivo occupancy by ex vivo labeling

The K (in vivo) parameters for buprenorphine, etorphine and diprenorphine to each the mu, delta and kappa sites in rat brain were determined using a model derived from the law of mass action:

$$B = Tmax - \frac{Tmax}{1 + (K/D)^N}$$

where B is the amount of tracer specifically bound in vitro relative to the control condition (Tmax = maximal tracer binding). K represents the drug dose (ug/kg) that displaces 50 percent of the specific binding of the ex vivo applied tracers at the mu, delta or kappa receptors 20 min (etorphine and diprenorphine) or 60 min (buprenorphine) after the dose. The slope parameter is indicated by the exponent N. The unbound concentration in the brain is assumed to be proportional to the

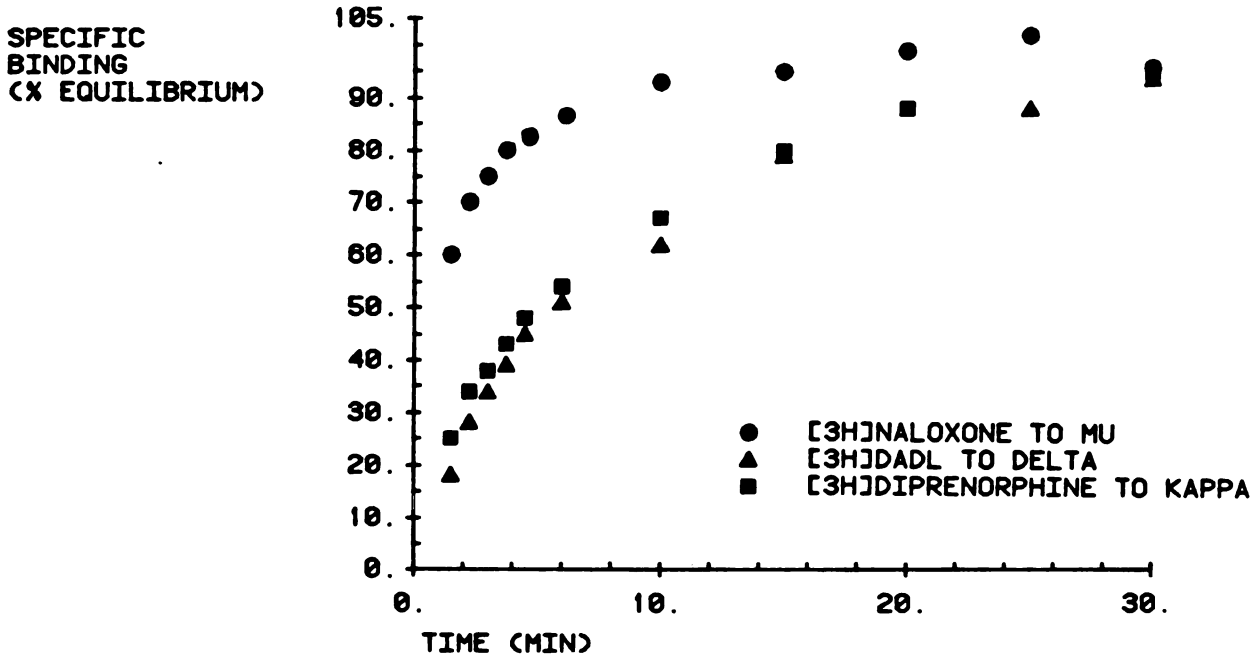


Figure I-2. Tracer binding curves at the mu, delta and kappa sites as a function of time. Each curve represents specifically bound radioactivity to fresh rat brain homogenates. ●, [^{3}H]naloxone; ▲, [^{3}HDADL] + 5 X 10^{-6} M morphiceptin; ■ ,[^{3}H]diprenorphine + 5 X 10^{-6} M morphiceptin + 5 X 10^{-7} M DADL. The data represents mean values from 2 or 3 experiments.

subcutaneous dose (D). This assumption has been experimentally verified for etorphine and diprenorphine (Rosenbaum et al., 1984a; Perry et al., 1982). Moreover, greater than 90 to 95 percent of bound etorphine and diprenorphine in the brain can be accounted for as unchanged drug (Perry et al., 1982; Sadee et al., 1982a). Distribution and metabolism experiments were not performed for buprenorphine. Data fitting and graphical analyses were accomplished using the MKMODEL program of the PROPHET computer system (Holford, 1982).

3.3 Materials

In vitro and in vivo applied ligands were obtained from the following sources: [^{3}H]naloxone (56-60 Ci/mmol), [^{3}H]ethylketocyclazocine (EKC) (16.4 Ci/mmol) and [^{3}H]diprenorphine (11-34 Ci/mmol) from Amersham Corp. (Arlington Hts., IL); [^{3}H]DADL (43.6 Ci/mmol) from New England Nuclear (Boston, MA). Tracer purities were determined by HPLC to exceed 90 percent. Buprenorphine HCl was a gift from Reckitt and Colman, Ltd. (Hull, England); unlabeled morphiceptin and DADL were purchased from Peninsula Laboratories (San Carlos, CA); etorphine HCl and diprenorphine HCl were obtained from the NIDA (Rockville, MD). Sprague-Dawley rats were purchased from Bantin and Kingman, Inc. (Fremont, CA).

4. RESULTS

4.1 Selection of mu, delta and kappa labeling conditions

Receptor labeling and blocking conditions (table I-1) were defined using 1:100 dilutions of fresh rat brain homogenates. Blocking conditions for the mu site were determined by obtaining a displacement curve for [^{3}H]DADL with the mu selective peptide, morphiceptin (Chang et al., 1981a). A displacement shoulder, implying saturation of mu sites, appeared in the concentration range of 5×10^{-7} to 10^{-5} M morphiceptin. Because [^{3}H]DADL selectively labels delta sites and to a lesser extent mu sites in vitro (Wood, 1983), its specific binding in the presence of 5×10^{-6} M morphiceptin therefore is assumed to occur selectively at the delta site. A

concentration of 5 X 10^{-7} M DADL completely suppressed [^{3}H]DADL specific binding at all time points and was within the range of concentrations which gave rise to a shoulder of [^{3}H]EKC binding. This concentration of DADL also displaced [^{3}H]diprenorphine, a ligand with similar affinity to mu, delta and kappa sites in vitro (Kosterlitz et al., 1981; Pfeiffer and Herz, 1982), to approximately 20 percent of total specific [^{3}H]diprenorphine binding. The addition of 5 X 10^{-6} M morphiceptin did not further reduce [^{3}H]diprenorphine binding in the presence of 5 X 10^{-7} M DADL, which suggests that only kappa sites were labeled at equilibrium in vitro. However, at the time of the ex vivo labeling analysis prior to equilibrium, 5 X 10^{-7} M DADL was insufficient to completely suppress mu binding of [^{3}H]diprenorphine. This is presumably caused by the different binding kinetics of tracer and blocker. Therefore, it was necessary to add both 5 X 10^{-7} M DADL and 5 X 10^{-6} M morphiceptin in order to permit selective labeling of kappa sites with [^{3}H]diprenorphine. Similarly, [^{3}H]DADL binding to delta sites in fresh membrane homogenates was compared when added either simultaneously or 2 min after addition of the blocker (5 X 10^{-6} M morphiceptin). The superimposability of the binding curves again indicates that only delta sites are labeled throughout the period prior to equilibrium.

4.2 Ligand redistribution

The in vitro redistribution of in vivo bound and free ligands was determined by including a centrifugation step (40,000 X g, 5 min) at 0°C immediately following brain homogenization. Rats were given either no drug or an oripavine at doses that nearly saturate each receptor type. The resulting brain membrane pellets from the drug treated rats were mixed with supernatants from naive rat brain homogenates and incubated with tracers for mu, delta or kappa labeling at 20°C. Fast off-rates from either mu, delta or kappa sites thus can be detected as an increase in bound tracer radioactivity during a 25 min labeling period. This control experiment with membranes that were presaturated with an oripavine in vivo was performed at each the mu, delta and kappa sites using a dose expected to result in receptor

saturation: diprenorphine, 50 ug/kg (mu, delta, kappa); buprenorphine, 250 ug/kg (mu, kappa) and 10 mg/kg (delta); etorphine, 100 ug/kg (mu) and 1.5 mg/kg (delta, kappa). Tracer binding remained suppressed over the test period in all cases but one, suggesting that receptor dissociation of the oripavines did not occur to a significant degree. The exception was etorphine's binding at the kappa sites; the 1.5 mg/kg s.c. dose failed to suppress tracer binding in the centrifuged membrane preparations. This result suggests either a fast etorphine off-rate from kappa sites following homogenization or a very low etorphine in vivo affinity to kappa sites, or both. All three drugs are known to dissociate extremely slowly from the mu site in Tris buffer brain homogenates (Perry et al., 1982; Sadee et al., 1982a; 1982b), particularly at 20°C as used here. Hence, with the possible exception of etorphine binding to kappa sites, post homogenization dissociation of the in vivo administered oripavines does not interfere with the ex vivo labeling assay.

Potential for post-homogenization association of the unbound in vivo ligand was similarly assessed by immediate centrifugation of brain homogenates after sacrifice. Tracer binding was measured in naive membrane pellets reconstituted with supernatants from brain homogenates of saline and drug treated rats. Where accumulation of unbound oripavine in the brain was sufficient to decrease the 4 min binding level of the tracer applied to the reconstituted homogenate, the displacement caused by the in vitro reassociation was quantitated for the dosage range under study. Administration of oripavines in doses at or above the in vivo K values caused a depression of tracer binding for each of the following: buprenorphine, delta site (250 ug/kg, 92% control); etorphine, delta site (500 ug/kg, 51% of control) and kappa site (500 ug/kg, 57% of control). For all other labeling conditions, doses 2 to 9 fold above the in vivo K of the oripavines caused little or no reassociation in the reconstituted brain membrane homogenate.

4.3 Relative in vivo binding potencies at the mu, delta and kappa sites

Estimates of the in vivo binding constants (K) of buprenorphine, etorphine and diprenorphine to the mu, delta and kappa sites are listed in table I-2. Graphs

containing the actual data points along with the fitted lines are included in fig. I-3 a-c. Slope factors (N) of the fitted lines are listed in table I-3. In vivo affinities to delta sites are approximately 4 to 20 fold lower than to mu sites. Moreover, substantial differences in relative binding potencies of the 3 ligands to kappa sites are evident. Buprenorphine displays approximately equal potency at mu and kappa sites, whereas etorphine appears to exhibit much lower kappa than mu affinity. Because of the possible redistribution of etorphine at the kappa site in vitro, however, a quantitative estimate of etorphine's kappa binding was not feasible. Diprenorphine's potency at kappa sites is intermediate to that for mu and delta and exceeding that of buprenorphine and etorphine for all three sites. In most cases, fitted lines and K approximations for each drug were determined using data from rats receiving doses ranging from well below to approximately 2 to 9 fold greater than the in vivo K at each site, without the need to account for ligand reassociation in vitro. However, for buprenorphine and etorphine, actual tracer binding was corrected for the measured degree of ligand reassociation at doses exceeding their K at the delta sites during the in vitro incubation.

Table I-2. In vivo binding constants with standard errors (S.E.) as determined by the ex vivo labeling method. The K values (K = dose to occupy 50% of the receptor sites) were derived from computer fits of the data illustrated in fig. I-3. K values indicated with an asterisk must be considered as rough estimates because of interference from unbound in vivo drug with the binding of the tracer applied ex vivo.

	K (S.E.) (ug/kg)		
	mu	delta	kappa
buprenorphine[a]	23 (2.3)	253* (45)	28 (2.4)
etorphine[b]	19 (3.5)	374* (90)	---[c]
diprenorphine[b]	7.0 (0.1)	28 (11)	11 (2.1)

[a] In vivo labeling period of 60 min.

[b] In vivo labeling period of 20 min.

[c] cannot evaluate because of ligand redistribution in vitro

Table I-3. Slope factors of the fitted lines illustrated in fig. I-3.

	N factors		
	mu	delta	kappa
buprenorphine	1.6 (.25)	0.88 (.15)	1.4 (.17)
etorphine	0.91 (.16)	0.60 (.12)	
diprenorphine	1.2 (.02)	0.59 (.17)	0.78 (.09)

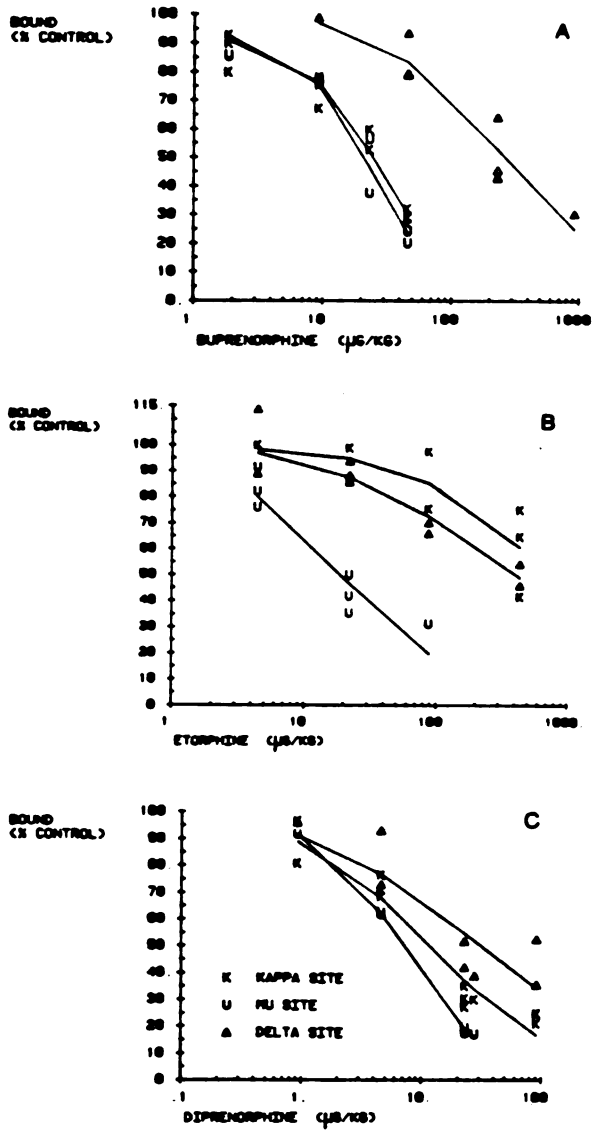


Figure I-3. In vivo displacement curves. The binding displacement of in vitro tracers to mu, delta and kappa sites as a function of the in vivo dose of buprenorphine, etorphine and diprenorphine are illustrated in panels A, B and C, respectively. The actual data points (8 to 14 per curve) each representing a single rat, are displayed with their fitted lines. In most cases, membranes from a single rat were used to determine binding to two or more sites. Etorphine displayed lowest potency in suppressing kappa site labeling ex vivo; however, the dissociation rate of in vivo bound etorphine from kappa sites following preparation of homogenates could not be measured. Hence, the kappa site binding affinity of etorphine appears to be too low for the ex vivo labeling approach, and therefore, the K value is not listed in table I-2.

5. DISCUSSION

This chapter describes the development of a novel ex vivo labeling technique for assessing in vivo binding affinity to mu, delta and kappa opiate binding sites. The method is based on clearly different principles when compared to previous methods that utilize in vivo tracer application. Therefore, it can serve to validate previous estimates of in vivo opiate receptor binding affinities. Moreover, in contrast to in vivo labeling methods, the ease of establishing specific ex vivo labeling conditions allows discrimination of binding at each the mu, delta and kappa sites.

Application of ex vivo labeling methods is limited by redistribution of the in vivo drug between the bound and free form following sacrifice and brain homogenization. Although dissociation half-lives of buprenorphine, etorphine and diprenorphine bound in vivo generally exceed 20 to 30 min following brain homogenization in Tris buffer at 37°C (Perry et al., 1982; Sadee et al., 1982a; 1982b) and are much longer at 20°C (unpublished), these represent hybrid dissociation rates from all sites to which the tracers specifically bind; dissociation rates from the individual sites are unknown. Therefore, control experiments were performed with brain membrane fractions containing fully blocked mu delta and kappa sites by in vivo drug administration. These membranes were incubated with drug free supernatant and tracers to detect any oripavine receptor dissociation. The absence of significant tracer binding over a 25 min incubation period rules out any substantial dissociation of drug bound in vivo to the receptor sites in all cases with the possible exception of etorphine at the kappa site. Moreover, rapid assessment of tracer binding immediately after sacrifice of the animal minimizes the potential for redistribution. Tracer binding can be evaluated prior to equilibrium since specifically bound radioactivity at any time point should be directly proportional to the number of unbound sites available, if the tracer (present at a virtually constant concentration) binds to a homogeneous group of receptors according to the law of mass action under the in vitro conditions. Fig. I-2 demonstrates that the three

tracers that were chosen to label mu, delta and kappa sites equilibrate at different rates. Because in vivo pre-administration of oripavines reduced tracer binding in the same proportion at different time points of the association curves, any time point of the association curve appeared to be suitable for the construction of displacement curves. At the selected 4 min time point, tracer binding was sufficiently high for quantitative measurements.

Because of a 100-fold dilution of the brain homogenate, one would not expect reassociation of unbound drug in the brain to the receptor sites in vitro to be significant. However, it is possible that, upon tissue homogenization, the drug's affinity to a receptor type immediately increases so that reassociation occurs despite the homogenate dilution. This in vivo - in vitro change is best illustrated with buprenorphine which displays equal affinity at each the mu, delta and kappa sites in vitro (Sadee et al., 1982b), while it's in vivo binding affinity at the delta site is more than an order of magnitude lower than at the mu and kappa sites (table I-2). The reassociation noted for buprenorphine (delta site) and etorphine (delta and kappa sites), despite the 100-fold dilution of the homogenate supernatants, suggests a selective increase of affinity in vitro for these two drugs at the indicated sites. The documented decrease of agonist binding affinity by the endogenous regulators, Na^+ and GTP (Blume et al., 1979) could account for the lower relative in vivo affinity when compared to the in vitro affinity in Tris buffer. However buprenorphine's in vitro binding affinity is not affected by Na^+ (Sadee et al., 1982b) and therefore other in vivo - in vitro changes might contribute to the observed affinity increase. For example, we have recently postulated that the opiate receptor partially loses its sensitivity towards guanyl nucleotides upon homogenization in Tris buffer (Rosenbaum and Sadee, 1983). Therefore, caution must be exercised with the interpretation of K values for in vivo binding where reassociation of unbound in vivo ligand has been documented (table I-2, marked with asterisk). While it is possible to correct for in vitro reassociation of unbound in vivo ligand in the labeling assay, some uncertainty remains because reassociation cannot be tested during the time period (<1 min) after sacrifice and before homogenization.

Death of the animal results in an immediate drop of cellular GTP levels (Maybaum et al., 1980), a putative regulator of the opiate receptor system. This could cause a rapid change of binding affinity in the brain tissue during the brief time (<1 min) before homogenization and, thereby, result in an overestimation of the drug's in vivo affinity. Thus, even lower potencies than indicated in table I-2 might apply for buprenorphine or etorphine at the delta site.

In order to approach in vivo binding equilibrium most closely, animals were sacrificed when peak brain levels or effects are observed. Peak brain levels occur at approximately 20 min for etorphine after subcutaneous dosing (Perry et al., 1982). Because of diprenorphine's in vivo binding kinetics and a discrepancy in the time of peak serum and brain (bound) concentrations, we have recently proposed systematic deviations from the law of mass action for diprenorphine's in vivo binding (receptor microcompartment hypothesis) (Perry et al., 1980). Hence, a time of 20 min was selected arbitrarily. Buprenorphine's binding character is determined at 60 min to coincide with its peak analgesic effect (Dum and Herz, 1981).

It must be noted that the assumptions of the law of mass action are probably not valid for in vivo binding studies, and that the binding model is the simplest model that is suitable to quantitatively describe the data. Deviations from the law of mass action could result from a lack of equilibrium in vivo, binding of the tracers to multiple sites, coopertivity, receptor occupancy by endogenous ligands (which would shift drug binding curves to the right) or any combination of these factors. The exponential constant N provides an estimate of the steepness of the dose - binding curve. No conclusions as to the nature of the ligand - receptor interaction can be drawn on the basis of the goodness of fit (fig. I-3). However, despite potential systematic errors incurred in the curve fitting procedure, it is likely that binding measurements and K estimates reflect in vivo receptor occupancy of the oripavine drugs at the time of sacrifice. Therefore, the binding model is used here to provide a quantitative description of the observed results rather than testing the validity of specific receptor binding models. As such, the in vivo binding results can be used for comparison to previously reported in vivo binding parameters and to

known pharmacological effects.

Expression of the apparent in vivo potency (K) in terms of the drug dosages requires a linear relationship between drug dosage and unbound concentrations in the brain. This has been verified for diprenorphine and etorphine (Rosenbaum et al., 1984a) but not tested for buprenorphine. The fraction of the dose present in the brain at 20 min was 0.21% for diprenorphine (Rosenbaum et al., 1984a) and 0.22% for etorphine (Perry et al., 1982), whereas it only reached 0.04% for buprenorphine at 60 min (Sadee et al., 1982b). Since the in vivo K's are based on the dose rather than brain concentrations, these values must be considered with the interpretation of the in vivo dissociation constants provided in table I-2. The lower fraction of buprenorphine reaching the brain might simply result from the longer in vivo binding period (1 hr versus 20 min) that was chosen because of the extremely slow receptor equilibration and a resulting delay of maximum pharmacological effects of buprenorphine.

Apparent in vivo K values provided by the ex vivo labeling procedures (table I-2) are compared to previous results from in vivo labeling techniques (Rosenbaum et al., 1984a; 1985) (table I-4) and to published in vitro binding affinities at each site (table I-5). Three sites were recently differentiated by in vivo labeling with multiple tracers and displacers (Rosenbaum et al., 1984a). The third site represents a novel type (lambda) that rapidly disappears in vitro at 20°C and does not bind the oripavine drugs (Grevel and Sadee, 1983). Therefore, it is not considered here. Site 1 was proposed to be identical to the mu site. Indeed the results for in vivo binding to the mu sites obtained by ex vivo labeling (table I-2) are consistent with those calculated for site 1 on the basis of the in vivo labeling method (table I-4). Buprenorphine's affinity to site 1 was calculated with the procedure given by Rosenbaum et al. (1984a) on the basis of the data provided by Sadee et al. (1982b). Considering the largely different methods, the close agreement between the results in tables 2 and 4 for the mu site (site 1) strongly suggest that the two labeling techniques provide an accurate measure of the actual in vivo binding of the oripavines. Comparison of the data given for site 2 is hampered by the fact that

Table I-4. In vivo binding selectivity of oripavines determined by methods which employ tracers applied in vivo (Rosenbaum et al., 1984a; 1985). Site 1 is equivalent to the mu site, while site 2 is thought to represent a hybrid of the delta and kappa sites. (K = dose to occupy 50% of the receptor sites)

	K (ug/kg)	
	site 1	site 2
buprenorphine[a]	26	1050
etorphine[b]	28	650
diprenorphine[b]	6.8	25

[a] in vivo labeling period of 60 min.

[b] in vivo labeling period of 20 min.

Table I-5. In vitro dissociation constants of the oripavines at the opiate receptor. Indicated parameters are representative of literature reports for the three oripavines. All values were determined in rat brain homogenates unless noted otherwise.

	Dissociation constants (nM)			
	mu	delta	kappa	Ref.
buprenorphine	0.86[a]	0.86[a]		(Villiger and Taylor, 1981)
	~0.1[a]	~0.1[a]	~0.1[a]	(Sadee et al., 1982b)
etorphine	0.11[b]	0.9[c]	4.8[d]	(Wood et al., 1981a)
	0.8[e]	0.5[f]		(Chang et al., 1978)
		0.8[g]		
diprenorphine	0.2[h]	0.18[i]	0.47[j]	(Chang et al., 1981a)
	0.26[k]	0.26[k]	0.26[k]	(Pfeiffer and Herz, 1982)

[a] IC_{50} value for total buprenorphine binding against [^{3}H]diprenorphine.
[b] Kd against [^{3}H]dihydromorphine (0.5 nM).
[c] Kd against [^{3}H]DADL (1 nM).
[d] Kd against [^{3}H]EKC (2 nM).
[e] IC_{50} against [^{3}H]naloxone (1.5 nM).
[f] IC_{50} against [I^{125}]DADL (1 nM).
[g] IC_{50} against [I^{125}]DADL (1nM) in N4TG1 cells.
[h] Kd against [I^{125}](D-Ala,N^{O}-MePhe^4,Met(O)ol^5)enkephalin (0.05 nM).
[i] Kd against [I^{125}]DADL (0.05 nM).
[j] Kd against [^{3}H]diprenorphine (0.5 nM) (+ 10 uM morphiceptin + 0.1 uM DADL).
[k] computerized multiple curve-fitting technique, Ki.

site 2 probably represents a hybrid of the delta and kappa sites. However, given the greater abundance of delta over kappa sites in rat brain (Gillan and Kosterlitz, 1982) one might expect that site 2 affinities are dominated by the delta site affinities. Apparently this is the case when comparing the data for site 2 (table I-4) with that for the delta site (table I-2).

Comparison of the oripavine binding affinities to the mu, delta and kappa sites in vivo (table I-2) to those in vitro (table I-5) immediately reveals dramatic differences. While the usefulness of comparing absolute binding affinities and their rank order for the three drugs is limited because of the differences in pharmacokinetics (e.g., access to the brain), the relative affinities of each individual drug at the mu delta and kappa sites in vivo and in vitro should be directly comparable. Although both diprenorphine and buprenorphine were shown to have equal mu, delta and kappa affinities in vitro (table I-5), significant differences were observed in vivo (table I-2). Recent in vitro studies for etorphine suggest that this agonist in fact is somewhat mu selective (Chang et al., 1978; Chang and Cuatrecasas, 1979; Wood et al., 1981a), while etorphine's mu preference is pronounced in vivo. These relative in vitro - in vivo binding differences do not appear to be related to the use of fresh membrane homogenates in the ex vivo labeling procedures since similar mu, delta and kappa affinities were found in fresh membrane homogenates relative to washed membrane homogenates (Pfeiffer et al., 1982a).

Because of the large differences observed between in vitro and in vivo binding affinities of the oripavines to the mu, delta and kappa sites (tables I-2 and I-5), some reinterpretation is suggested with regard to the sites which mediate the known effects of these opioids. Etorphine can be characterized as mu-selective; a concept which is consistent with it's known pharmacological effects (Rosenbaum et al., 1984b). Because of the sizable preference with which etorphine binds to the mu sites (table I-2), it is unlikely that the analgesic and catatonic effects of etrorphine can be attributed to delta and kappa receptors. Diprenorphine's binding affinity to the mu sites is in agreement with it's antagonistic ID_{50} against morphine analgesia

(Blane and Dugdall, 1968; Levine et al., 1980). Likewise, the in vivo K of diprenorphine at the kappa site is similar to it's ID_{50} against bremazocine-induced increases in urine output (Richards and Sadee, 1985b), a putative kappa-mediated effect. One would expect a lower potency of diprenorphine at the delta sites; however, the delta effect of diprenorphine remains unclear. The in vivo binding affinity of buprenorphine permits a possible explanation of it's bell-shaped dose response curve (Sadee et al., 1982b) on the basis of differential receptor occupancy. The analgesic effect of buprenorphine can be roughly linked to it's binding to mu and/or kappa sites. However the down-regulation of analgesia occurs with doses above those that saturate mu and kappa sites, but in the range of doses which result in delta occupancy. It remains to be investigated whether buprenorphine acts as an agonist at the kappa sites. Our results on the effects of buprenorphine on bremazocine-induced increases of urinary output suggest that the drug acts as an antagonist at dosages that are consistent with it's in vivo kappa binding affinity (Richards and Sadee, 1985c). In conclusion, the differences in relative affinities of oripavines in vitro and in vivo and the correlation of in vivo binding affinity with known pharmacological effects demonstrate the need for in vivo binding characterizations in order to understand opiate receptor functions.

Chapter 2

In vivo binding of benzomorphans to mu, delta and kappa opiate receptors: comparison with urine output in the rat

1. SUMMARY

In vivo binding affinities of three benzomorphans, Win 44,441-3, bremazocine and MR 2266, were determined at the mu, delta and kappa types of opiate binding sites in rat brain, using an ex vivo labeling technique. The receptor occupancy of the benzomorphans and of previously tested diprenorphine were compared with their activities in increasing urine output (agonist ED_{50}: bremazocine) or inhibiting bremazocine-induced diuresis (antagonist ID_{50}: Win 44,441-3, MR 2266 and diprenorphine). The agonist, bremazocine, bound (in order of decreasing affinity) to the kappa = mu > delta binding sites, and it's pharmacological effects appeared in the dosage range of kappa and mu binding. In order to positively identify which receptor type is responsible, the potency of the three antagonists to block the effects of bremazocine were compared to their ability to occupy the individual sites in vivo. A fractional occupancy of 0.5 would be expected at the ID_{50} if one assumes a linear relationship between receptor occupancy by the antagonist and the antagonistic effect. Such a linear relationship was observed for the three antagonists only at the kappa site, while variable occupancies were observed at the mu and delta sites. These results support the previously proposed hypothesis that kappa receptors mediate the effects of benzomorphan opioid drugs on urine flow.

2. INTRODUCTION

Various opioid drugs are capable of altering urine output in animals. Morphine and other mu agonists decrease urination in water loaded rats (Huidobro, 1978; Huidobro and Huidobro-Toro, 1979), while chronic administration of morphine

(Marchand and Davis, 1968) or single doses of benzomorphans, e.g., bremazocine, ethylketocyclazocine (EKC) and ketazocine (Leander, 1983a; 1983b), have been found to increase urine output in normally hydrated animals.

The events which ultimately lead to these changes in urine flow probably originate in the central nervous system (CNS) (Leander, 1983b; Huidobro and Huidobro-Toro, 1979). It has been suggested that increases of urine output result from opioid mediated inhibition of vasopressin release from the hypothalamus (Clarke et al., 1979; Iversen et al., 1980). Because many of the agents capable of stimulating urine flow also are kappa agonists, the diuretic effect was proposed to be mediated by kappa receptors (Slizgi and Ludens, 1982; Leander, 1983a). However, since many drugs that are highly active in stimulating urine flow bind nondiscriminantly to mu, delta and kappa sites in vitro (Wood, 1983a), some ambiguity remains as to where these opioid drugs act.

Hence, the experiments described here were designed to test the hypothesis that kappa sites in rat brain are responsible for mediating the enhanced urine output associated with putative kappa agonists. The ability of three opioid antagonists, diprenorphine, MR 2266 and Win 44,441-3, to inhibit bremazocine induced diuresis in the rat was compared with their in vivo receptor binding character in rat brain. These antagonists were chosen because of their general opioid antagonist character (VonVoigtlander and Lewis, 1982; Yoshimura et al., 1982; Ward et al., 1983) and high binding affinties in vitro (Pfeiffer and Herz, 1982; Wood, 1983a). The latter feature offers a potential advantage for determining in vivo binding affinities by ex vivo labeling methods (Richards and Sadee, 1985a; Sadee et al., 1983). This approach permits one to determine the relationship between drug dosage and in vivo receptor binding separately at each of the mu, delta and kappa types of binding sites. The parallel between the inhibition of bremazocine's diuretic effect and in vivo fractional receptor occupancy at the kappa site by the three antagonists demonstrates that kappa receptors mediate the increased urine flow.

3. MATERIALS AND METHODS

3.1 In vivo binding experiments

The in vivo binding affinities of bremazocine, Win 44,441-3 and MR 2266 at the mu, delta and kappa types of binding sites were determined using an ex vivo labeling approach. This method previously has been applied to characterize the in vivo binding of diprenorphine, as well as buprenorphine and etorphine (Richards and Sadee, 1985a). Briefly, the method entails subcutaneous administration of 400 ul normal saline or drug (in normal saline) to male Sprague-Dawley albino rats, weighing 120 to 140 grams. Rats were sacrificed 20 min after injection and the brain (minus cerebellum) immediately removed and homogenized in 1:100 dilution of 50 mM Tris buffer at 20°C. With the use of mu, delta and kappa specific labeling conditions in fresh rat brain homogenates, the fraction of sites remaining unbound in vivo is determined. This is accomplished by immediately transferring aliquots of brain homogenates to separate incubation flasks containing appropriate tracers and blockers that focus binding radioactivity to each the mu, delta and kappa sites (table I-1, chapter 1). Bound radioactivity is determined by filtering aliquots of the homogenate mixture through Whatman GF/B glass fiber filters. Assays were performed after sacrifice at 20 min or occasionally, for comparison, 60 min after drug administration. To limit the post-homogenization redistribution of the in vivo drug, tracer binding to each the mu, delta and kappa sites in vitro was determined at four minutes after tracer-homogenate addition, i.e., prior to the achievement of tracer binding equilibrium. Binding at each point of the association curve is assumed to be proportional to the number of available binding sites. Prior in vivo drug administration reduces the number of binding sites available for ex vivo labeling in a dose dependent fashion.

3.2 Redistribution of in vivo drug following sacrifice

The possible redistribution of free and bound drug after sacrifice and before binding analysis over a period of approximately 5 to 6 min could introduce artifacts into the ex vivo binding analysis. Therefore, control experiments were performed by immediately separating the brain homogenates of drug treated rats into membrane pellets and supernatants (40,000 x g for 5 min at 0°C). Membrane pellets obtained from rats treated with saturating doses of drug are then reconstituted with supernatants obtained from naive rat brain homogenates. Short dissociation half-lives of a ligand from a binding site will appear as a large increase in tracer binding to mu, delta or kappa sites during a 25 min ex vivo labeling period (20°C). However, because this was not evident for Win 44,441-3, MR 2266 or bremazocine, dissociation of in vivo ligand would not be expected to interfere with ex vivo labeling.

Similarly, post-homogenization association of the unbound in vivo ligand can be estimated by reconstituting naive membrane pellets with supernatants from brain homogenates of drug treated rats. Reconstituted naive pellets and naive supernatants served as the control. If unbound drug accumulated to a sufficient extent in vivo so as to decrease the 4 min tracer binding level in the reconstituted homogenate, then reassociation of the in vivo drug will cause a systematic error. However, it should be noted that brain tissues are diluted 100-fold before analysis, so that reassociation is expected to occur only at very high dosages. A depression of tracer binding was quantitated for each of the following: bremazocine, mu site (100 ug/kg, 66% control), kappa site (100 ug/kg, 74% control) and delta site (100 ug/kg, 61% control); MR 2266, mu site (100 ug/kg, 66% control) and delta site (400 ug/kg, 72% control). In vitro tracer binding at these doses was corrected accordingly in order to account for in vitro reassociation of the drug present in the brain in vivo, and higher dosages were not employed in these cases. Significant error arising from reassociation was not evident for: MR 2266, kappa site ($\leq$400

ug/kg); Win 44,441-3, mu site (≤100 ug/kg), delta site (≤1500 ug/kg) and kappa site (≤400 ug/kg).

3.3 Estimation of in vivo binding affinities

The in vivo binding affinities (K's) for bremazocine, Win 44,441-3, MR 2266 and diprenorphine at each the mu, delta and kappa sites in rat brain were determined using the following model based on the law of mass action:

$$B = Tmax - \frac{Tmax}{1 + (K/D)^N} \qquad (1)$$

where B represents specifically bound tracer to mu, delta or kappa sites in homogenates obtained from drug treated relative to saline treated animals (Tmax = maximal tracer binding) animals. The binding parameter, K, of the ligand administered in vivo is the dose (D) that occupies 50% of either the mu, delta or kappa sites 20 min after subcutaneous injection. The slope parameter (N) is equivalent to the Hill coefficient. All binding and pharmacodynamic data fits and graphics were performed using the MKMODEL program of the PROPHET computer system (Holford, 1982).

3.4 Measurement of urine output

A total of 38 male Sprague-Dawley albino rats, weighing 300 to 450 g, were used for this series of experiments. Generally rats were used once each week, although occasionally up to twice a week. Except during urine collection periods, the rats were housed in a colony room maintained at 22° to 23°C and illuminated for 12 hours daily. Free access to water and rodent chow was allowed except

during urine collections, when food and water were withheld. Urine collections were initiated immediately following subcutaneous injections and placement in metabolism cages. All drugs were administered in normal saline (400 ul) and dosages expressed as the free base. When two drugs were to be given simultaneously to the same animal, they were co-administered in a single injection. Urine output was recorded at 2 and 5 hr after drug injections.

3.5 Estimation of pharmacodynamic parameters

The parameters describing bremazocine's activity in promoting urine output were obtained by fitting the data to the following pharmacodynamic model derived from the law of mass action:

$$E = \frac{(Emax - Emin)\, A^N}{A^N + ED_{50}{}^N} \qquad (2)$$

The influence of varying doses of the opioid antagonists diprenorphine, Win 44,441-3 and MR 2266 upon the bremazocine dose-response curve was analyzed in a simultaneous fashion using the ED_{50} value for bremazocine determined in equation 2. This model (eq. 3) has been previously employed in this laboratory (Rosenbaum et al., 1984b), and it is based on the same principles used in eq. 2 with an additional factor that allows for competitive antagonism:

$$E = \frac{(Emax - Emin)\, A^N}{A^N + [ED_{50}\,(1 + B/ID_{50})]^N} \qquad (3)$$

where E represents the gm of urine produced at a given dose of bremazocine

(A) (± antagonist) and Emax is the maximum output of urine expected at high doses of bremazocine. Emin is the minimum quantity of urine expected for the collection period, i.e., in the absence of bremazocine. ED_{50} is the dose of bremazocine that will produce a level of urine output equivalent to [0.5 (Emax - Emin) + Emin]. The dose of antagonist (B) is held constant for each bremazocine dose-response curve. The ID_{50} is the dose of antagonist which necessitates a doubling of the agonist dose to achieve the same level of effect at it's ED_{50}. The slope parameter (N) permits deviations from simple law of mass action predictions. It was allowed to differ for bremazocine dose-response curves in the absence and presence of each of the 3 antagonists.

3.6 Materials

Sprague-Dawley rats were purchased from Bantin and Kingman, Inc. (Freemont, CA). Tracers and unlabeled ligands used for binding experiments were obtained from the following sources: [^{3}H]D-Ala,D-Leu-enkephalin ([^{3}H]DADL) (43.6 Ci/mmol) from New England Nuclear (Boston, MA); [^{3}H]naloxone (60 Ci/mmol) and [^{3}H]diprenorphine (34 Ci/mmol) from Amersham Corp. (Arlington Hts., IL); morphiceptin and unlabeled DADL from Peninsula Laboratories (San Carlos, CA). Diprenorphine HCl was obtained from the NIDA (Rockville, MD). The following drugs were donated by Pharmaceutical companies which are indicated parenthetically: MR 2266 (Boehringer Ingelheim Ltd., Ridgefield, CT), Win 44,441-3 (Sterling-Winthrop, Rennselaer, NY), and bremazocine HCl (Sandoz Ltd., Basel, Switzerland).

4. RESULTS

4.1 In vivo binding affinities

In vivo displacement curves of bremazocine, Win 44,441-3 and MR 2266 at the mu, delta and kappa sites are illustrated in figs. II-1 a-c. Computer assisted estimates of the in vivo binding parameter, K, for the benzomorphans are indicated in table II-1, as is the previously reported estimate for diprenorphine using the ex vivo labeling method (Richards and Sadee, 1985a). Because of constraints imposed by the in vitro reassociation of unbound in vivo ligand, the useful dosing range for affinity estimates was limited to the following : MR 2266, 400 ug/kg (mu, delta and kappa); Win 44,441-3, 100 ug/kg (mu), 400 ug/kg (kappa) and 1500 ug/kg (delta); and bremazocine, 100 ug/kg (mu and kappa). All ligands have variable but consistently lower affinity in vivo to delta relative to mu or kappa sites, whereas the affinity ratio at the mu and kappa sites are different for each drug.

Because K estimates at the delta site contained rather large errors with the ex vivo labeling methods, delta site affinities of the benzomorphans were evaluated independently using an in vivo labeling technique (table II-2) with [^{3}H]diprenorphine in the presence of 250 ug/kg buprenorphine to block mu and kappa sites (Richards and Sadee, 1985a). The results for bremazocine and Win 44,441-3 were compatible, whereas a discrepancy between the in vivo and ex vivo labeling methods was noted for MR 2266. A comparison of bremazocine's K at the mu site (table II-1, ex vivo method) with it's K versus [^{3}H]etorphine administered in vivo (table II-2), a mu-selective ligand (Rosenbaum et al., 1984a), also reveals a higher estimate for the in vivo labeling procedure (43 versus 25 ug/kg).

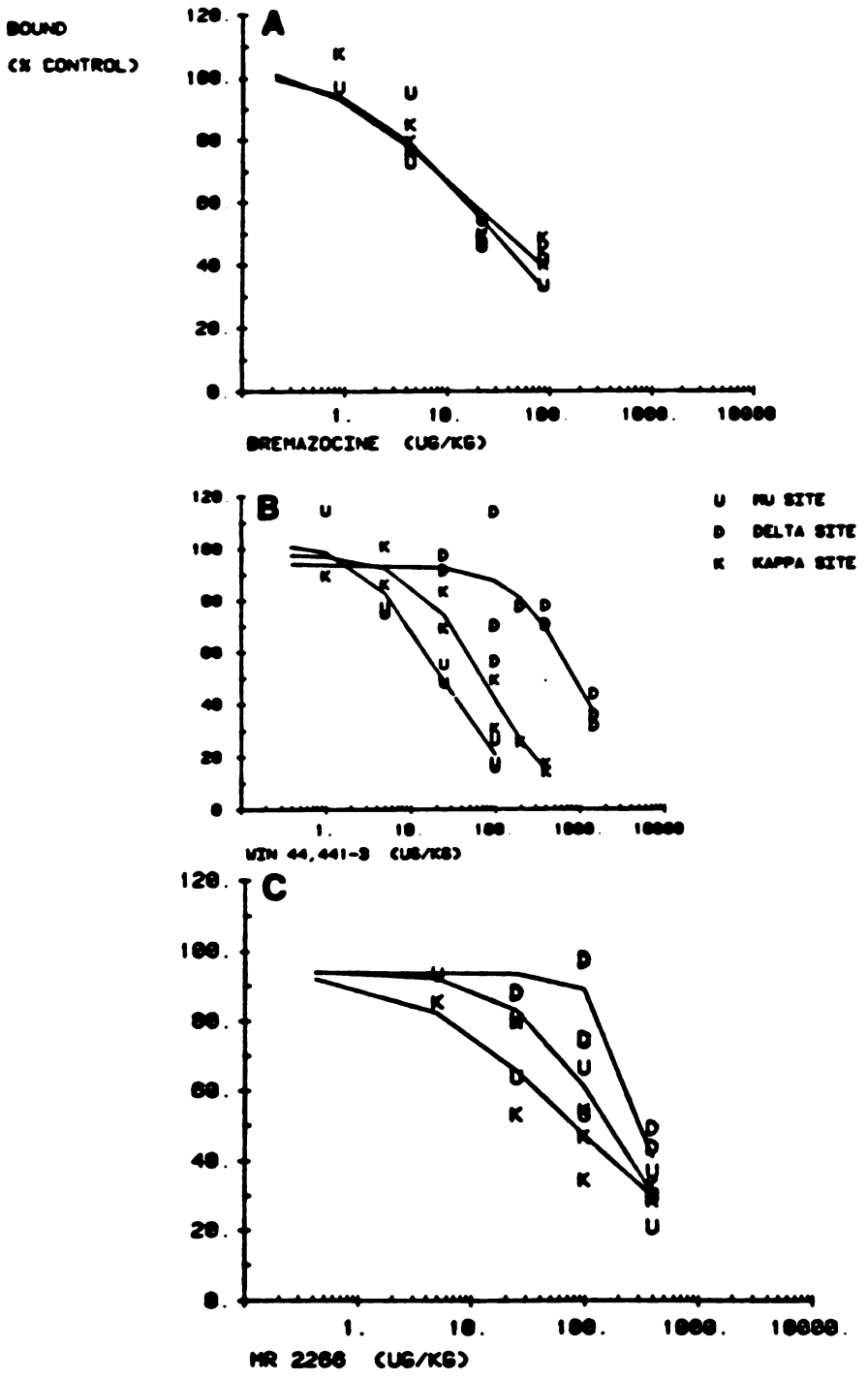


Figure II-1. In vivo displacement curves. The displacement of tracer binding to mu, delta and kappa sites in vitro as a function of bremazocine (A), Win 44,441-3 (B) and MR 2266 (C) dose is illustrated above. The observed values, each representing determinations in a single rat (7 to 11 per curve), are displayed with their fitted lines. The binding data for bremazocine at the delta sites are not included because of extensive in vitro reassociation of the drug at that site.

Table II-1. In vivo binding parameters. K values (dose required to occupy 50% of receptor) were derived from computer fits of the data illustrated in fig. II-1. K values are listed with standard errors (S.E.) as determined by the ex vivo labeling method 20 min after drug administration.

	K (S.E.) ug/kg		
	mu	delta	kappa
bremazocine	25 (7.7)[b]	>100[a]	23 (12)[b]
Win 44,441-3	22 (4.7)	1017 (237)	76 (12)
MR 2266	185 (47)[b]	358 (42)[b]	83 (39)
diprenorphine[c]	7 (0.1)	28 (11)	11 (2.1)

[a]Substantial redistribution of bremazocine occurred at doses below the in vivo K at this site, preventing a reasonably accurate estimate of binding affinity.

[b]Interference from unbound in vivo drug with the binding of the tracer applied ex vivo made necessary the adjustment of these values.

[c]Data from Richards and Sadee, 1985a.

4.2 Urine output activity of bremazocine

Bremazocine-stimulated increases of urine flow in the rat (27 observations) over a 5 hr period resulted in computer generated predictions for Emax of 18.1 gm (S.E. = 0.95), Emin of 1.2 gm and ED_{50} of 8.8 ug/kg (table II-3). The change in urine output as a function of bremazocine dose is illustrated with it's fitted line in fig. II-2.

4.3 Effects of opioid antagonists on bremazocine-augmented diuresis

Simultaneous computer analysis of bremazocine dose-response curves in the presence of 10 different antagonist doses (97 observations) resulted in Emax and Emin estimates of 21.4 (S.E. = 0.4) and 1.0 gm of urine, respectively, during a five hour urine collection period. Graphs showing bremazocine dose-response curves in the presence of antagonists are displayed in fig. II-3. Estimates of the ID_{50}'s of Win 44,441-3, MR 2266 and diprenorphine against bremazocine-induced increases in urine flow are listed in table II-3. Urine production at 2 hr generally was 10 to 20 percent less than the 5 hr accumulation, indicating similar relative potency at 2 and 5 hr for all drugs tested. Moreover, variability was greater for the 2 hr levels. Hence, parameter estimates and dose-response curves are shown for the 5 hr collections only.

Table II-2. Displacement of tracers applied in vivo. ID_{50}s of bremazocine, Win 44,441-3 and MR 2266 against the in vivo binding of [^{3}H]etorphine (preferential mu labeling) or [^{3}H]diprenorphine plus 250 ug/kg buprenorphine (preferential delta labeling). Values (ID_{50}) represent the doses of the 3 ligands that displace 50% of the specific binding of the tracers in vivo.

	ID_{50} ug/kg	
displacer	[^{3}H]etorphine[a]	[^{3}H]diprenorphine[b] (+ buprenorphine, 250 ug/kg)
bremazocine	43	175
Win 44,441-3		2000
MR 2266		2200

[a]tracer dose = 0.85 ug/kg

[b]tracer dose = 2.0 ug/kg

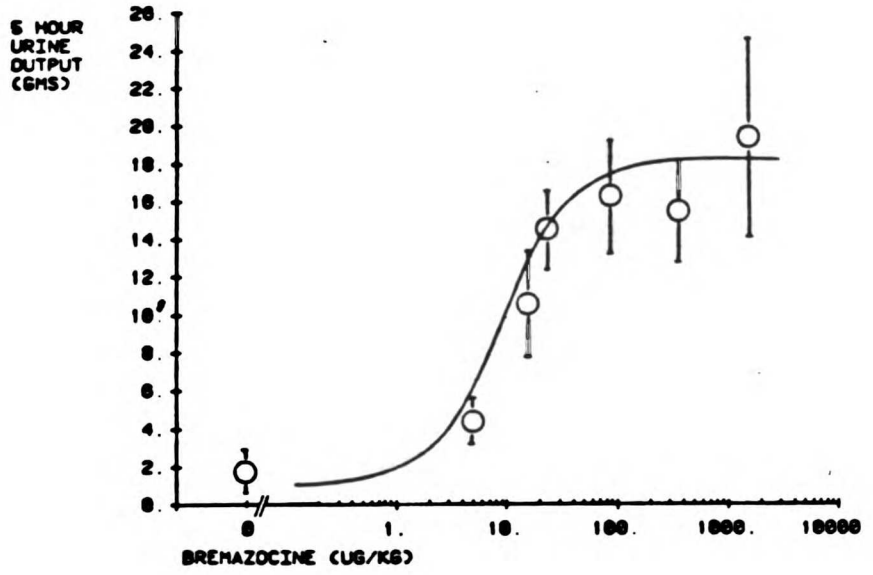


Figure II-2. Bremazocine dose-response curve. Each point (± S.E.) represents the mean of at least 3 determinations.

Table II-3. Comparison of in vivo receptor occupancy with effect. Receptor occupancy at the D_{50} (ED_{50} or ID_{50}) was calculated on the basis of the in vivo K estimates provided in table II-1. Range of occupancy extends from 0 (absence of bound drug) to 1 (saturation of receptor site).

drug	ED_{50} (S.E.) ug/kg	ID_{50} ug/kg	occupancy at D_{50}[a] mu	delta	kappa
bremazocine	8.8 (0.1)		.254	<.1[b]	.277
Win 44,441-3		81 (25)	.786	.074	.516
MR 2266		94 (21)	.337	.208	.531
diprenorphine		18 (6)	.720	.391	.621

[a] $\text{Occupancy} = \dfrac{D_{50}}{K + D_{50}}$;

this is thought to be a reasonable approximation of receptor occupancy, since the slope factor N did not deviate greatly from 1 for the in vivo binding curves (see table II-4).

[b] See table II-1, footnote a.

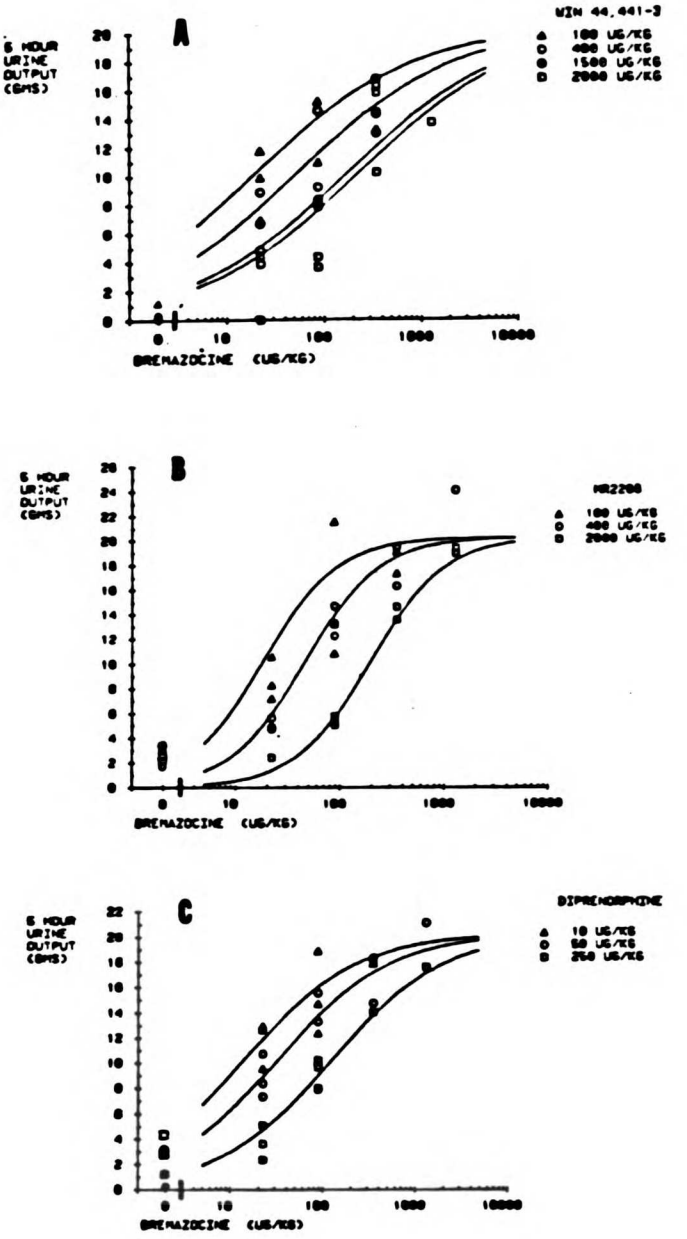


Figure II-3. Dose-response curves for bremazocine are displayed as a function of increasing doses of Win 44,441-3, MR 2266 or diprenorphine in panels A, B and C, respectively. The curves represent computer predictions for equation 3 using an ED_{50} of bremazocine = 8.8 ug/kg. All bremazocine dose-response curves in the presence of antagonist were simultaneously analyzed. Each data point represents a single observation.

Table II-4. Slope factors. Computer estimates of the slopes of the fitted curves are illustrated for the binding (fig. II-1) and effect (figs. II-2 and II-3) models.

	N factors (S.E.)			
	binding			effect
	mu	delta	kappa	
bremazocine	0.63 (.14)		0.46 (.11)	1.47
Win 44,441-3	0.91 (.13)	1.12 (.35)	1.22 (.21)	0.54
MR 2266	0.97 (.21)	2.3 (1.29)	0.62 (.10)	1.2
diprenorphine	1.2 (.02)	0.59 (.17)	0.78 (.09)	0.71

5. DISCUSSION

This study provides first estimates of the in vivo receptor occupancy of 3 benzomorphan drugs at the mu, delta and kappa sites, in comparison to their effects on urine flow. The ID_{50}s of the benzomorphan antagonists Win 44,441-3 and MR 2266 and the oripavine antagonist diprenorphine for inhibition of bremazocine-associated diuresis correspond to a fractional kappa receptor occupancy of approximately 0.5. Conversely, occupancy of mu and delta sites at each antagonist ID_{50} are more variable and divergent from 0.5. If principles of classical receptor theory are observed, a linear relationship should exist between the occupancy of a receptor site by a competitive antagonist drug and the inhibition of an agonist's effect (see Hollenberg, 1978, for review). That is, the ID_{50} of an antagonist should equal the binding K for that drug at the site in question, resulting in a fractional occupancy of 0.5. Hence, the parallel changes between urine flow inhibition and kappa site binding in vivo for the antagonists supports the proposed kappa receptor involvement in mediating increases of urine output.

5.1 Measurements of benzomorphan receptor binding in vivo

The ex vivo labeling approach for characterizing in vivo binding affinities previously has been applied to three oripavine opioids, diprenorphine, buprenorphine and etorphine (Richards and Sadee,1985a). The slow binding kinetics of the oripavines were demonstrated by in vitro dissociation rate studies (Sadee et al., 1982b; Perry et al.,1982). Moreover, a linear relationship between dose and unbound brain concentration was established (Sadee et al., 1982a; Rosenbaum et al., 1984a), and the bound ligand was accounted for as unchanged drug (Sadee et al., 1982a; Perry et al., 1982). Finally, the apparent in vivo affinity of diprenorphine, buprenorphine and etorphine, determined by the ex vivo labeling methods, were consistent with the findings of the in vivo labeling procedures

(Rosenbaum et al., 1984a; 1985). These results support the accuracy of the ex vivo labeling approach for determining in vivo receptor occupancies of the 3 oripavines.

The techniques have been applied here to the benzomorphans without specifically testing either for the contribution of metabolites or the relationship between dosage and brain concentrations. However, it is unlikely that these factors disrupt the measurement of unbound receptor sites and thus, the ex vivo labeling assay should reflect drug receptor occupancy at the time of sacrifice. No attempt was made to validate the assumptions of the in vivo binding model (e.g., attainment of equilibrium, etc.); rather, the numerical data analysis simply serves to describe the results in a quantitative fashion, with potential systematic deviation accounted for by the slope factor N (table II-4). The major potential shortcoming of the occupancy measurments by this technique relates to redistribution of unbound and bound in vivo drug during the five min period between brain homogenization and binding characterization. In order to test for redistribution, supernatants and pellets of brain homogenates taken from saline and drug treated rats were separated and reconstituted to distinguish the influence of the bound and free ligand upon the ex vivo applied tracer. The results indicate that little drug dissociation occurs after homogenization even during a 25 min labeling period. However, association of unbound drug present in vivo was observed under some of the assay conditions. Partial reassociation was corrected for in these cases when estimating the receptor occupancy (table II-1), and in vivo doses were limited to a dosage range with less than 40% reassociation in vitro.

In order to further test the validity of the ex vivo labeling approach for the benzomorphans and to obtain another estimate of their delta binding, we have again employed the in vivo labeling technique (Rosenbaum et al., 1984a) for comparison. The K value for bremazocine (43 ug/kg) against [^{3}H]etorphine co-injected with the bremazocine was fairly consistent with the ex vivo result at the mu site (25 ug/kg), if one considers that [^{3}H]etorphine also labels delta sites to a small extent (Rosenbaum et al., 1984a) and that bremazocine has rather low

affinity to the delta sites in vivo. Hence, the two labeling methods yield compatible results in this case, with the ex vivo labeling method likely providing a more accurate analysis because of the more specific labeling procedure.

Because of the low affinity of benzomorphans to delta sites in vivo and the large errors inherent with ex vivo labeling of low affinity in vivo sites, an independent estimate was obtained by labeling delta sites in vivo with [^{3}H]diprenorphine, while blocking tracer binding to mu and kappa sites using 250 ug/kg buprenorphine (table II-2). This permits fairly selective labeling of delta sites because of the 10 fold greater affinity of buprenorphine to mu and kappa sites in vivo (Richards and Sadee, 1985a), although a fraction of the delta sites also would be blocked by buprenorphine. The in vivo binding affinities of either Win 44,441-3 or bremazocine at the delta site are roughly comparable when measured by ex vivo and in vivo labeling techniques, if one allows for the rather large error of both methods for the delta site. However, K estimates at delta for MR 2266 by ex vivo labeling (358 ug/kg) and in vivo labeling (2200 ug/kg) are largely discrepant and point to a systematic limitation of the ex vivo labeling technique with ligands of low in vivo affinity to the delta site. Specific labeling of kappa receptors in vivo is not currently possible because of the relatively small number of kappa sites in rat brain (Gillan and Kosterlitz, 1982) and the lack of ligands that are capable of selectively labeling kappa receptors in vivo.

When comparing the rank order of in vivo and in vitro binding affinities, large discrepancies become apparent. The similar affinities of the oripavines to mu, delta and kappa sites in vitro (Pfeiffer and Herz, 1982; Sadee et al., 1982b; Chang et al., 1978) contrasts with their variable K estimates in vivo (Rosenbaum et al., 1984a; Richards and Sadee, 1985a). Differences also are noted here for the benzomorphans. In vitro binding studies are not always in agreement, but indications are that MR 2266 binds to mu, delta and kappa sites with similar affinity (Kosterlitz et al., 1981; Lahti et al., 1982; Wood, 1983a). On the other hand, bremazocine and Win 44,441-3 each have comparable affinity to mu and delta

sites and approximately 4 to 20 fold lower preference for kappa sites (Lahti et al., 1982; Gillan and Kosterlitz, 1982; Wood, 1983a; Wood et al., 1984). One report, however, indicated that bremazocine binds to all 3 sites in vitro with similar affinity (Kosterlitz et al., 1981). The lower affinities of the benzomorphans to delta relative to mu and kappa sites in vivo (table II-1) is consistent with our previous results with the oripavines and, again, in contrast to the approximately equal affinities of these opioids to all sites in vitro. In part, the in vivo binding results are corroborated in that the activity of Win 44,441-3 against mu agonists in the guinea pig ileum is greater than ten fold higher that against DADL in the mouse vas deferens (delta site preparation) (Ward et al., 1983). This is consistent with the low in vivo delta affinity of Win 44,441-3 observed here. Further, MR 2266 has been found to antagonize both mu and kappa effects in vivo; but, in line with it's binding pattern (table II-1), is more potent against the latter (VonVoigtlander and Lewis, 1982). The large differences which distinguish relative in vitro and in vivo binding affinities of the benzomorphan and oripavine opioids demonstrate the need for in vivo binding characterization when making site-effect comparisons.

5.2 Pharmacodynamic measurements of urine flow

Simultaneous fitting of pharmacodynamic data with a non-linear regression program allows utilization of the 97 observations from all bremazocine (+ antagonist) dose-response curves to estimate Emax and Emin. Moreover, at least 30 observations are used for determining each antagonist ID_{50}. Hence, although the data for each bremazocine dose-response curve might be insufficient for characterizing that curve if analyzed alone, the combined data from the 3 or 4 dose-response curves in the presence of each antagonist are adequate for estimating their ID_{50}s against the diuresis caused by bremazocine. The data and fitted lines presented in fig. II-3, thus, should be evaluated with consideration of the simultaneous fitting procedure that permits one to minimize the sample size, but

introduce rather large data scatter for the individual dose-response curves. The fitting program readily converged on final parameter estimates suggesting that the competitive antagonism model is compatible with the results.

Because of quantitative differences in urine output, depending on whether antagonists were present or not, the results describing bremazocine's agonist activity in promoting diuresis were independently fitted in the absence of antagonists. Therefore, a large sample number was employed for the dose-response curve of bremazocine alone (fig. II-2). The Emax estimate obtained from dose-response curves for rats treated with bremazocine only is more than 3 gm less than for rats receiving both the agonist and antagonist. As it has been shown that mu agonists inhibit urine flow (Huidobro, 1978; Huidobro and Huidobro-Toro, 1979), the lower Emax estimate for the rats treated with bremazocine alone might be explained by a weak mu agonist effect of bremazocine (Wolozin et al., 1982; Schmauss and Yaksh, 1984). Inhibition of this mu action by the antagonists, Win 44,441-3, MR 2266 and diprenorphine, would then promote increases of Emax. This might be expected particularly for Win 44,441-3 and diprenorphine which have higher affinity to mu than kappa sites in vivo (table II-1). The ED_{50} estimate for bremazocine was then used in the mathematical model for simultaneous fitting of bremazocine dose-response curves in the presence of varying doses of antagonist to determine the parameters in eq. 3.

The bremazocine dose-response curves in the absence and presence of the antagonists, diprenorphine and MR 2266 are comparable to previous findings (Leander, 1983a; 1983b). However, Leander (1983b) has previously concluded that Win 44,441-3 did not inhibit urine output by blocking the same sites that mediate bremazocine's agonist effect. This conclusion was based on the shallow dose-response curve for Win 44,441-3 against 80 ug/kg bremazocine and the fact that substantial residual effect was evident after doses of up to 20 mg/kg Win 44,441-3 (Leander, 1983b). These discrepancies in the results obtained for Win

44,441-3 are not easily reconcilable, although the continuity of four bremazocine dose-response curves in the presence of varying doses of Win 44,441-3 (fig. II-3a) would appear to be more reliable than the single Win 44,441-3 dose-response curve reported by Leander (1983b). Moreover, use of large subcutaneous doses of Win 44,441-3 in the latter case might result in a non-linear dose-response relationship because of problems such as precipitation at the injection site, as we previously have observed for ethylketocyclazocine (Rosenbaum et al., 1984b).

Slope parameters (N) determined from the fitted lines of the binding and effect curves were in most cases close to unity (table II-4). In contrast, large slope values were obtained for the quantal analgesia dose-response curves of etorphine and sufentanil (3.55 and 16.25, respectively) (Rosenbaum et al., 1984b). At the least, the N values obtained here suggest that effects on urine flow are not of a quantal nature. The Hill coefficients estimated for the benzomorphan receptor binding curves were in the same range noted for the oripavines (Richards and Sadee, 1985a).

5.3 Comparison of receptor binding in vivo with the effect

Extrapolation of receptor occupancy determined 20 min after injection to an effect measured over 5 hours presents a possible systematic error in the interpretation of the binding-effect relationship. All ligands tested have high affinities to opiate binding sites in vitro, a necessary feature for accurate measurement of in vivo binding by ex vivo labeling. The slow receptor binding kinetics also could reflect rather slow changes of the in vivo occupancies over the pharmacological assay period. Comparative binding experiments at 20 and 60 min have shown that the binding of Win 44,441-3, MR 2266 and bremazocine remains rather stable from 20 to 60 min after injection. In addition, Maurer (1984) demonstrated through in vivo autoradiography that the binding of 0.1 - 5 ug/kg bremazocine did not differ when tested at 5 to 60 min following IV injection.

Moreover, the bremazocine induced increases of urine output occur largely over the initial 2 hr collection period, and the 3 antagonists inhibit bremazocine's effect to a similar degree over 2 versus 5 hr. These findings suggest that the receptor occupancy assay at 20 min can be considered relevant to the entire pharmacological observation period, although the optimal time for the receptor assay might differ somewhat for each agent. More importantly, however, one can compare the relative occupancies for each agent at the mu, delta and kappa sites to identify that site with consistent fractional occupancy at the ID_{50}.

The discrepancy between bremazocine's K at the kappa site (25 ug/kg) and it's ED_{50} for increasing urine output (8.8 ug/kg) is not unexpected. Several examples are cited in the literature where a linear relation between fractional receptor occupancy and degree of effect do not hold for agonists (Rodbell, 1980; Terasaki and Brooker, 1978; Rosenbaum et al., 1984b). Such apparent discrepancies have been explained by evoking concepts such as low fractional occupancy at 50 percent activation of receptors (Furchgott, 1978). However, the differences between the time frame for binding and effect measurements will influence occupancy estimates. Further, there is no knowledge as to what degree of activation is required to maximally stimulate urine flow. Hence, agonist binding data alone are not useful to link receptor occupancy with pharmacological effect.

However comparison of the binding of Win 44,441-3, MR 2266 and diprenorphine with their activity against bremazocine augmented urine flow supports the conclusion that the kappa receptor mediates the diuretic effect. The fractional occupancy of antagonists is usually assumed to be directly proportional to their inhibitory effects, which is observed only at the kappa site. In contrast, variable occupancies at the mu and delta site after ID_{50} doses argue against these sites as being responsible for the bremazocine enhanced urine flow. Hence, these results corroborate previous findings that diuresis is stimulated by full or partial kappa agonists (Slizgi and Ludens, 1982; Leander, 1983a; 1983b), an effect that is

thought to occur through inhibition of vasopressin release from the neurohypophysis (Slizgi and Ludens, 1982; Clarke et al., 1979; Iversen et al., 1980). Because of the opposite effects of kappa and mu agonists in the modulation of urine output, the activities at both sites must be considered. By avoiding use of strong mu agonists, the present study demonstrates the role that kappa receptors play in the mediation of urine output.

It is likely that the antidiuretic effect of kappa agonists is mediated by a very small subset of the overall kappa receptor population in the brain. One cannot know a priori, whether this subset of kappa sites is biochemically different or is sequestered in a brain area with poor drug access. The observation here that the in vivo binding parameters, K, (obtained for the entire kappa population) of the 3 antagonists are similar to their ID_{50} values suggests that the functional kappa receptor subset is similar to the total kappa population. However, this important question requires further study.

Chapter 3

Buprenorphine is an antagonist at the kappa opiate receptor

1. SUMMARY

The effect of buprenorphine on bremazocine-induced diuresis was tested in the rat to determine the nature of buprenorphine's action at the kappa opiate receptor. Both morphine tolerant and naive rats were used to account for possible antidiuretic effects of buprenorphine at the mu site. Separate experiments established that the morphine pretreatment caused profound tolerance with respect to the antidiuretic action of mu agonists. Buprenorphine acted as a potent antagonist (ID_{50} = 11 ug/kg) of the diuretic action of the kappa agonist bremazocine (ED_{50} = 10 ug/kg). The similar potency of buprenorphine as an antagonist of bremazocine in naive and morphine tolerant rats further supports the hypothesis that buprenorphine exerts it's antidiuresis via an antagonistic effect at kappa sites, rather than as an agonist at the mu sites. The high affinity displayed by buprenorphine at the kappa opiate receptor in vivo is consistent with this conclusion. Hence, buprenorphine can now be classified as a partial agonist at the mu site and as an antagonist at the kappa site against bremazocine induced urine flow, while it's action at the delta site to which it has much lower affinity in vivo remains unknown.

2. INTRODUCTION

Buprenorphine is an opioid agonist/antagonist that gives rise to a bell-shaped dose-response curve in analgesia tests with rats (Dum and Herz, 1981; Rance et al., 1980). The rising portion of buprenorphine's dose-response curve is associated with low doses (< 500 ug/kg) while higher doses result in decreasing the observed

analgesic effect. Recently, it was shown that buprenorphine saturates mu and kappa sites in vivo at doses that also cause peak analgesia (500 ug/kg), while the downslope of the bell shaped curve occurs at doses that give rise to delta site occupancy (Richards and Sadee, 1985a). However, the relative involvement of mu and kappa sites in buprenorphine analgesia remains uncertain. Martin et al. (1976) characterized buprenorphine as a partial mu agonist. Alternatively, others have suggested on the basis of heat versus pressure stimulus antinociceptive assays that kappa sites play a dominant role in mediating buprenorphine analgesia (Tyers, 1980; Skingle and Tyers, 1980).

Hence to clarify the nature of buprenorphine's action at the kappa site, it's influence on the diuretic response to bremazocine was tested in rats. The increased urine output in rats caused by bremazocine recently has been shown to be mediated by kappa receptors in the brain. However, because strong mu agonists are known to decrease urine output (Huidobro, 1978; Huidobro and Huidobro-Toro, 1979; Dray and Metsch, 1984a; 1984b), a decrease in the diuretic response to bremazocine could be caused by an antagonist or a partial agonist/antagonist effect of buprenorphine at the kappa receptor, or by an agonist effect at the mu receptor. Thus, buprenorphine's action at the mu and kappa sites were differentiated by comparing it's effect on bremazocine stimulated urine flow in naive and morphine tolerant rats. The latter group received a regimen of morphine previously demonstrated to cause tolerance to morphine's analgesic effect. Thus, this group might be expected to exhibit a significantly diminished response to buprenorphine if it were acting via the mu receptor. Buprenorphine's similar antagonistic potency against bremazocine-associated diuresis in both morphine (mu) tolerant and naive rats indicates that buprenorphine is an antagonist at the kappa receptor in this pharmacological test.

3. MATERIALS AND METHODS

3.1 Urine collections

Urine samples were collected as previously described (Richards and Sadee, 1985b) from 28 male Sprague-Dawley albino rats, weighing 350 to 450 g. Except during urine collections, the animals were housed at 22° to 23°C in a colony room with a 12 hr dark-light cycle and allowed free access to food and water. Bremazocine (± buprenorphine) was administered s.c. in 400 ul normal saline. Immediately after injection of drugs and placement of the rats in metabolism cages, urine collections were initiated and the output recorded 2 and 5 hr later. Each rat was used no more than twice weekly. Upon completion of studies with normal rats, many of the same animals were then used for the morphine tolerance studies.

3.2 Induction of morphine tolerance

Two groups of 12 rats each were administered either 10 mg/kg morphine twice daily or 20 mg/kg morphine three times a day in 600 ul normal saline. Four rats died as a result of the initial 20 mg/kg dose of morphine. Urine flow response to bremazocine (± buprenorphine) was tested at both 3 and 5 days after beginning morphine injections and 12 to 13 hr after the previous dose of morphine. Hence, there were four animal groups with somewhat different morphine treatment schedules; rats receiving either 20 or 60 mg/kg morphine daily for 3 and 5 days. Rats in all morphine treatment groups demonstrated a profound depression of activity after initial injections. In all cases, this was replaced by normal or excessive levels of activity following later morphine injections, indicating the development of tolerance.

The effectiveness of morphine administration for inducing tolerance to the

inhibitory action of mu agonists on urine flow was tested in a separate experiment. Urine output response to concurrent administration of bremazocine (25 ug/kg) and etorphine (25 ug/kg), a selective mu agonist (Richards and Sadee, 1985a; Rosenbaum et al., 1984a), was measured both in naive rats (N = 10) and in rats which had received 10 mg/kg morphine twice daily for 3 days (N = 10).

3.3 Parameter estimation

The pharmacodynamic parameters describing the activity of bremazocine and buprenorphine on modulating the output of urine were obtained by simultaneously analyzing bremazocine's dose-response curves in the absence and presence of varying doses of buprenorphine. This was performed separately for naive and morphine tolerant rats. Buprenorphine's ability to decrease bremazocine stimulated diuresis in morphine tolerant rats did not differ when fitting the urine output data obtained from rats receiving morphine doses (over 3 and 5 days) of either 60 mg/kg daily (buprenorphine ID_{50} = 7.1 ug/kg, S.E. = 5.9) or 20 mg/kg daily (buprenorphine ID_{50} = 11.2 ug/kg, S.E. = 5.7). Hence the data obtained from all morphine tolerant rats were pooled. The pharmacodynamic model, derived from the law of mass action, has been described previously (Richards and Sadee, 1985b):

$$E = \frac{(Emax - Emin)\, A^N}{A^N + [ED_{50}\,(1 + B/ID_{50})]^N} \qquad (1)$$

The estimate of urine output (E) caused by a s.c. dose of bremazocine (A) in the absence or presence of a fixed dose of buprenorphine (B) ranges from the expected values for minimum (Emin) and maximum (Emax) urine output. ED_{50}

represents the dose of bremazocine that will cause an output of urine equal to [0.5 (Emax - Emin) + Emin]. The N parameter describes the slope of the dose-response curves as determined from the simultaneous fits. Simultaneous data fitting with a non-linear regression program allows use of all observations from the naive and morphine tolerant rat groups to estimate the pharmacological parameters in each group. Hence, although each bremazocine dose-response curve (± buprenorphine) might be inadequately described when considered individually, the combined data from the 3 or 4 dose-response curves of either the naive or morphine tolerant animals are sufficient for providing reasonably accurate parameter estimates, while permitting one to minimize sample size. Moreover, the fitting program readily converged on final parameter estimates suggesting that the competitive antagonist model and the parameter estimates are compatible with the results.

4. RESULTS

The simultaneous fits of the bremazocine (± buprenorphine) dose-response curves in naive rats (fig. III-1A, table III-1) show that buprenorphine antagonizes bremazocine associated diuresis in a dose dependent manner. Similarly, buprenorphine antagonized urine output in morphine tolerant animals resulting in parameter estimates close to the values observed for naive rats (fig. III-1B, table III-1).

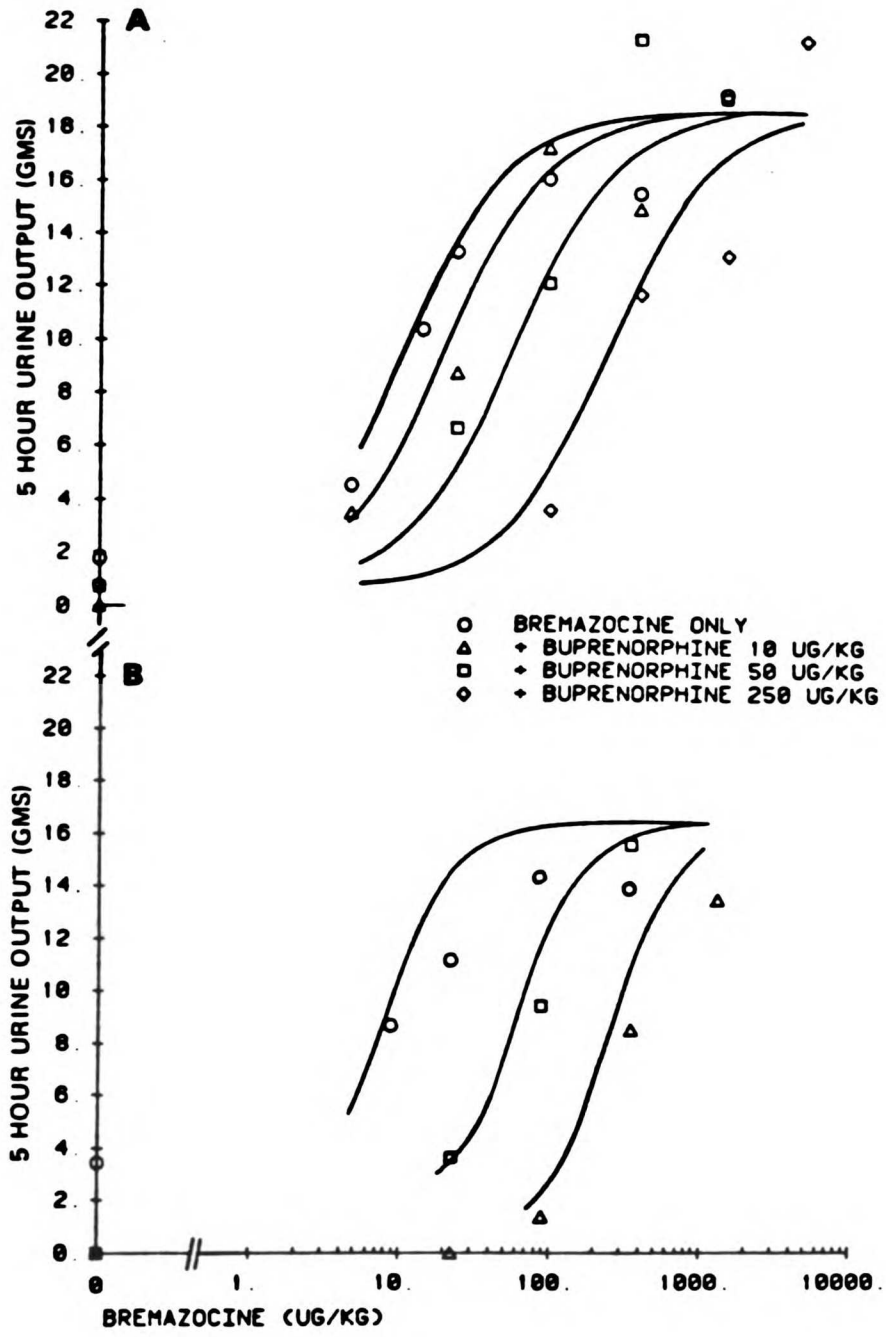


Figure III-1. Bremazocine dose-response curves in the absence and presence of buprenorphine as measured in (A) naive and (B) morphine tolerant rats. Each data point represents the mean of two or more observations. The fitted lines are based on the parameter estimates of table III-1 as generated from the raw data.

Table III-1. Computer generated parameter estimates derived from simultaneous fitting of urine output data obtained from naive or morphine tolerant rats (see eq. 1). Doses are expressed as the free base.

	naive	morphine tolerant
observations	72	38
Emax (± S.E.)	18.7 (± 1.4) g	17 (± 0.6) g
Emin	0.8 g	3 g
N (± S.E.)	1.3 (± 0.1)	1.8 (± 0.2)
ED_{50} (bremazocine) (± S.E.)	10.3 (± 3.5) ug/kg	7.3 (± 2.7) ug/kg
ID_{50} (buprenorphine) (± S.E.)	11.2 (± 3.2) ug/kg	6.8 (± 3.2) ug/kg

5. DISCUSSION

It has been suggested that kappa receptors mediate the increased urine output caused by certain opioids, i.e., bremazocine, ethylketocyclazocine and ketazocine (Martin et al., 1976; Leander,1983b). This was supported more recently by comparisons of in vivo fractional occupancy of mu, delta and kappa sites by general opioid antagonists with their ability to inhibit bremazocine-induced diuresis (Richards and Sadee, 1985b). The ID_{50} values (as defined in this chapter for buprenorphine) for diprenorphine, Win 44,441-3 and MR 2266 coincided with the doses of these antagonists required to block 50% of the kappa receptors in vivo, while receptor occupancy at the ID_{50} doses was variable for the mu and delta sites. This result clearly implicates the kappa site as the primary receptor for mediating bremazocine's effects on urine flow.

Hence, the shift of bremazocine dose-response curves to the right by increasing doses of buprenorphine (fig. III-1A) suggests that buprenorphine is acting as a kappa antagonist. Alternatively, because strong mu agonists have been found to decrease urine output in water loaded rats (Huidobro, 1978; Huidobro and Huidobro-Toro, 1979), the inhibition of bremazocine diuresis by buprenorphine could be a result of agonist activity at mu receptors. However, Leander (1983b) demonstrated that 20 mg/kg morphine does not alter the diuretic response to 80 ug/kg bremazocine. Because 10 mg/kg morphine and 500 ug/kg buprenorphine s.c. produce an equivalent level of analgesia in the electrically evoked vocalization test (Dum et al., 1981), it can be implied that, in the doses employed, buprenorphine's efficacy at the mu site is insufficient to antagonize the bremazocine effect on urine flow. Nonetheless, in relation to Leander's (1983b) findings, it should be pointed out that 80 ug/kg bremazocine occupies a substantial proportion of mu sites in vivo, based on in vivo binding affinities (Richards and Sadee, 1985b), which could have prevented morphine's mu activity.

To address further the question of whether buprenorphine's inhibitory effects on bremazocine induced urine flow are mediated by it's antagonistic action at the kappa site or it's agonistic action at the mu site, we have induced selective mu tolerance by repeated morphine administration. In the presence of tolerance to the antidiuretic action of mu agonists, one would expect no diminution of buprenorphine's anti-bremazocine potency, if it primarily acts as an antagonist at the kappa site. Tolerance has been reported to develop to the antidiuretic effect of morphine (Huidobro, 1978). Moreover, twice daily injections of 10 mg/kg morphine were shown to cause tolerance to the analgesic effects of buprenorphine at the mu site (Dum et al.,1981). Therefore, this dosage schedule as well as higher doses of morphine were chosen to induce tolerance over 3 to 5 days.

To avoid saturation of the mu receptors with high doses of bremazocine (e.g., 80 ug/kg in a previous study; Leander, 1983b), we have chosen a rather low dose (25 ug/kg) that elicits ~ 70% of maximal response (Richards and Sadee, 1985b). In contrast to the findings of Leander (1983b) with 80 ug/kg bremazocine, morphine (20 mg/kg) was capable of preventing the diuresis induced by the low bremazocine dose. Further, this effect was attenuated in morphine pretreated animals (data not shown). Because etorphine is considered to be a potent and rather selective mu agonist with a chemical structure similar to that of buprenorphine, it was chosen to document the degree of mu tolerance attained after 3 days of twice daily injections with 10 mg/kg morphine, i.e., the minimum pretreatment schedule for the induction of mu tolerance. At 25 ug/kg etorphine, which is at least 30-fold greater than its ED_{50} in the rat tail flick analgesia assay (Rosenbaum et al., 1984b), etorphine virtually eliminated the diuretic action of bremazocine (25 ug/kg) over at least 2 hr, reducing urine flow from 10.0 ± 3.2 g (± S.D.) and 12.3 ± 3.1 g to 1.8 ± 1.9 g and 8.6 ± 3.2 g over 2 and 5 hr, respectively. In contrast, etorphine (25 ug/kg) was ineffective in reducing bremazocine stimulated urine flow in morphine pretreated animals (8.2 ± 2.9 g over 2 hr and 12.3 ± 1.9 g over 5 hr). The differences between

naive and morphine pretreated animals were significant to the <0.0005 and <0.01 levels for 2 and 5 hr, respectively (paired t test). The apparent diminution of etorphine's antidiuretic effect at 5 hr relative to 2 hr might be explained by a longer residence time of bremazocine in the brain relative to that of etorphine (Maurer, 1984). No significant differences were observed between naive and tolerant animals treated with bremazocine alone (see also fig. III-1 and table III-1) and tolerant animals treated with bremazocine plus etorphine.

These results clearly establish the development of tolerance to the antidiuretic effects of mu agonists by morphine pretreatment. Since etorphine at the selected dosage (25 ug/kg) was shown to selectively interact with the mu sites in vivo, with no measurable binding to delta and kappa sites (Richards and Sadee, 1985a), one can conclude that the morphine induced tolerance is specific to the mu site. This finding allows one to interpret any effects of buprenorphine on bremazocine induced diuresis in morphine tolerant animals, which are shown in fig. III-1B and table III-1. The lack of any significant change of buprenorphine's ability to antagonize bremazocine in the mu tolerant animals strongly argues against the hypothesis that its effect on urine flow is mediated via agonistic actions at the mu site. Rather, the hypothesis that buprenorphine acts as a potent antagonist at the kappa site remains unchallenged.

The conclusion that buprenorphine represents a kappa antagonist contrasts with that of Tyers (1980) who classified buprenorphine as a kappa agonist on the basis of comparing it's ED_{50} values against various nociceptive stimuli. It is possible that an agonist of low efficacy could behave like an antagonist in one test system (urine flow), while causing an agonistic response in another system (antinociception) because of differences in the required receptor activation. However, the complexity introduced by the bell shaped dose-analgesia curve of buprenorphine makes comparison of ED_{50}s subject to erroneous conclusions.

The view that buprenorphine does not act as a kappa agonist is supported by

behavioral studies. Discriminative stimulus experiments by Shearman and Herz (1982) revealed that bremazocine generalized to the effects of MR 2033, a putative kappa agonist, but did not generalize to the effects of buprenorphine or etorphine, the latter being a preferential mu agonist (Dum et al., 1981).

We have recently measured the relative affinity of buprenorphine at the mu, delta and kappa sites in vivo (Richards and Sadee, 1985a). The dose (s.c.) required to occupy 50 % of each receptor population at 60 min was approximately 20, >200 and 20 ug/kg, respectively. This result appears to rule out any delta site involvement with buprenorphine's action against bremazocine. Although both mu and kappa affinities are in the range that is consistent with the involvement of either of these receptor types, the combined pharmacological evidence presented here and elsewhere strongly supports the notion that buprenorphine, while acting as a partial agonist at the mu sites in analgesia tests, represents a potent kappa antagonist in the bremazocine-stimulated urine flow test. However, it cannot be ruled out at present that buprenorphine displays weak agonistic kappa effects in other selected pharmacological test systems. Buprenorphine's effect at the delta site remains unknown. Nonetheless, because it now appears that buprenorphine's analgesic effect might predominantly be elicited through mu receptors while exerting little or no agonistic effect at the kappa site, it is possible that buprenorphine's action at the delta sites mitigates it's action at the mu site, thereby creating a bell-shaped dose-analgesia curve.

Chapter 4

Effect of opioids on cyclic nucleotide accumulation in SK-N-SH human neuroblastoma cells

1. SUMMARY

The availability of cultured cell lines that express delta opioid receptors has provided much information on the interaction of these receptors with the cyclic nucleotides. However the functional significance of this is unknown as the role of delta receptors in the intact animal has yet to be identified. The recent discovery of a human neuroblastoma cell line (SK-N-SH) with mu and delta opiate binding sites provides a potential opportunity to test for a possible link between the mu receptor and the modulation of second messengers. However although etorphine, DADL and morphine inhibited PGE_1-stimulated cyclic AMP accumulation in SK-N-SH cells, the data variability and small signal inhibition prohibited delineation of the receptor(s) responsible for the cyclic AMP inhibition. Opioids did not affect cyclic GMP concentrations in the SK-N-SH human neuroblastoma cells.

2. INTRODUCTION

In view of the multitude of opiate binding sites in heterogeneous tissue preparations, it is important to identify transformed neuronal cell lines in which the distinct opiate binding sites can be individually studied. Among cell lines that have been examined for the presence of opiate receptors, only the delta type of binding sites was found in murine neuroblastoma cells (West and Miller, 1983). Nevertheless, these cell lines have proven excellent model systems for studying the molecular functions of the opiate receptor (Klee and Nierenberg, 1974; Sharma et al., 1975; Gilbert and Richelson, 1983).

We have recently identified a human cell line, SK-N-SH, that displays readily measurable specific binding for both the mu, delta and kappa ligand [^{3}H]diprenorphine and the mu-selective ligand [^{3}H]naloxone (Yu et al., 1985). This

human neuroblastoma cell line was derived from a metastatic tumor in the bone marrow of a 4-year old girl (Biedler et al., 1973). The neuroblastoma nature of the cells was previously established by the presence of neuritic processes that contain dense core granules and neurotubules. Moreover, the isoenzyme pattern and dopamine secretion of these cells are consistent with a human neuronal origin (Biedler et al., 1973).

Binding studies with multiple tracers and ligands revealed that SK-N-SH cells express both mu and delta opiate receptor sites (Yu et al., 1985). The mu sites displayed high or selective affinity for naloxone, morphine, D-Ala^2, MePhe^4, Gly-ol^5 enkephalin (DAGO) and morphiceptin, while delta sites preferentially bound DADL and leu- and met-enkephalin. The spectrum of binding affinities for the studied opioid ligands establishes the presence of mu and delta sites that closely resemble mu and delta sites observed in human (Maurer, 1982; Pfeiffer et al., 1981; Pfeiffer et al., 1982b; Bonnet et al., 1981) and rodent (Wood, 1982; Chang et al., 1981b; Gillan and Kosterlitz, 1982; Lord et al., 1977) brain, and the delta sites in the neuroblastoma x glioma hybrid cell line, NG 108-15 (Gilbert and Richelson, 1983).

The total number of opiate binding sites were estimated at 70,000 sites per cell on the basis of saturation curves with diprenorphine. The Bmax value obtained here compares well with the delta site population on the NG 108-15 cells (300,000 sites per cell) (Klee and Nierenberg, 1974), if one considers the smaller size of the SK-N-SH cells.

The differential effects of sodium ions and GppNHp on opioid agonist and antagonist binding observed in SK-N-SH cells were similar to previous results in the mouse neuroblastoma x rat glioma hybrid, NG 108-15 (Blume, 1978a) and rodent brain (Blume, 1978b; Childers and Snyder, 1980). This finding suggests that the human opiate receptor of SK-N-SH cells has a similar molecular organization as the rodent receptor.

The intent of this study was to determine the effect of opioid drugs on the production of cyclic nucleotides in SK-N-SH cells. In cultured cell lines containing delta receptors, opioids partially inhibit prostaglandin E_1 (PGE_1) stimulated cyclic AMP production through inhibition of adenylate cyclase (Klee and Nierenberg, 1974; Sharma et al., 1975; Robson et al., 1983). The discovery of SK-N-SH cells provides an opportunity to test whether mu sites are involved with these second messenger systems (Yu et al., 1986).

3. MATERIALS AND METHODS

3.1 Cyclic nucleotide assays

SK-N-SH cells were grown at 37°C in monolayer in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were transferred to 17 mm culture wells (Falcon Primaria) and allowed to grow at confluence for 24 hr prior to assay. Medium was removed and 0.3 ml of fresh medium was added to each well containing 0.5 mM of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), followed by addition of opioid at 5 min. For cyclic AMP measurements, PGE_1 was added at 10 min, allowed to incubate at 37°C for another 10 min and terminated by removal of the medium and addition of 100 ul 6% trichloroacetic acid (TCA) (0°C). Cyclic GMP was measured in the absence of PGE_1 and usually at 30 s after addition of opioid. The cyclic nucleotides were measured at various times following stimulation of nucleotide accumulation, with similar results (cyclic AMP, 5-55 min; cyclic GMP, 0.5-10 min). After addition of TCA, the cells were scraped from the dish and transferred to 1.5 ml polypropylene microcentrifuge tubes, sonicated (30 s) and centrifuged (600 g, 15 min, 0°C). Supernatants were washed

twice with 1 volume fresh freon: tri-N-octylamine (78:22), and aliquots of the aqueous layer were tested for cyclic nucleotides using a competitive protein binding assay kit (Amersham) for cyclic AMP and a radioimmunoassay kit (Amersham) for cyclic GMP. The presence of cyclic AMP in SK-N-SH cells was quantitated through the ability of cell extracts to displace [^{3}H]adenosine 3',5'-cyclic phosphate from a specific binding protein isolated from bovine muscle. A similar method was used for measuring guanosine 3',5'-cyclic phosphate except that a specific antibody is used in place of the bovine muscle binding protein. Pellets were assayed for protein concentration using the method of Lowry et al. (1951).

3.2 Materials

SK-N-SH cell line was provided by Dr. J. Biedler. Cyclic AMP and cyclic GMP assay kits were purchased from Amersham Corp. DADL was purchased from Peninsula labs. Morphine sulfate was obtained from Mallinckrodt, Inc. and naloxone from Endo Labs. Etorphine HCl and bremazocine HCl were obtained from the National Institute on Drug Abuse. PGE_1 and IBMX were puchased from Sigma.

4. RESULTS

The levels of cyclic GMP produced by SK-N-SH cells were barely detectable (< 1 pmol/mg protein) and not measurably affected by the addition of 1 uM etorphine, morphine, DADL or bremazocine. However, basal and PGE_1-stimulated accumulations of cyclic AMP were approximately 5 and 150 pmol/mg protein, respectively. Significant decreases of PGE_1 stimulated cyclic AMP accumulation were observed when cells were pretreated with 1 uM etorphine (81 ± 10.8%, $p < .0005$), DADL (87.8 ± 5.6%, $p < .005$) and morphine (82.8 ± 11.7%, $p < .025$).

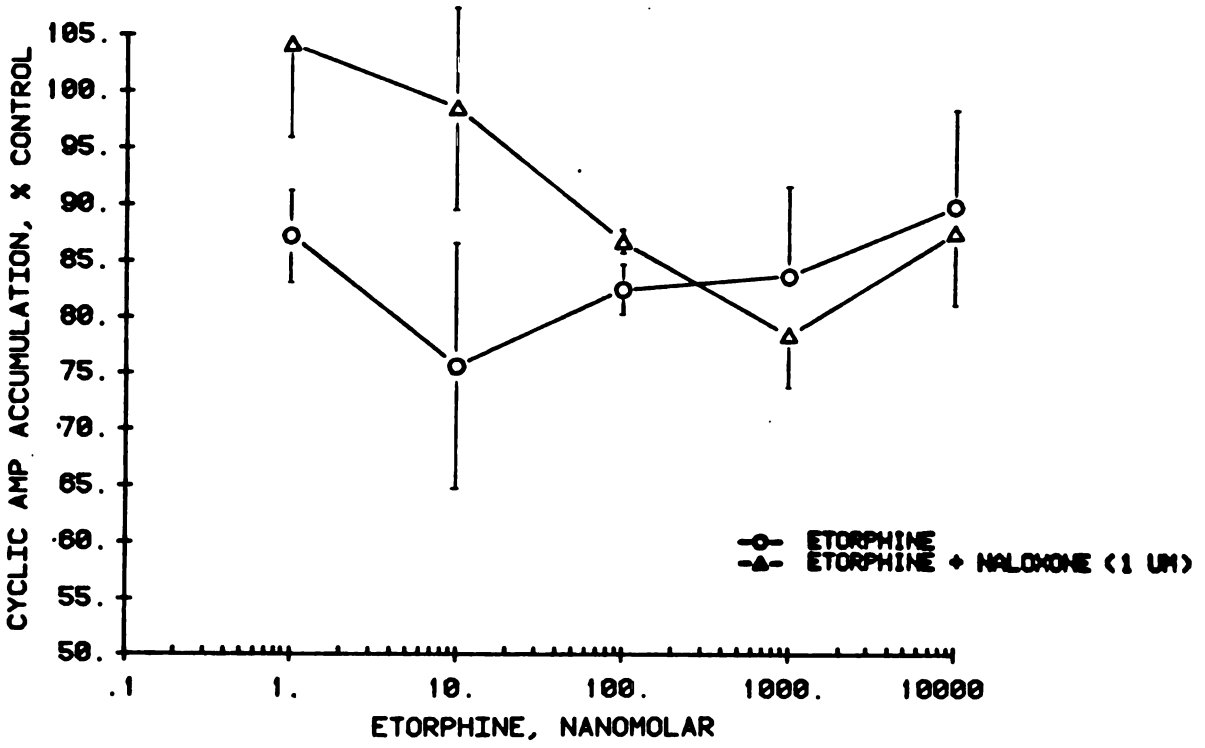


Figure IV-1. The influence of etorphine upon the PGE_1-stimulated accumulation of cyclic AMP in SK-N-SH cells and the reversal by naloxone. Data points represent the mean of at least 6 etorphine (-◯-) or 3 etorphine + 1 uM naloxone (-△-) observations.

Values are expressed as % control ± S.D. and statistically tested by Students T test. No difference was noted for 1 uM bremazocine (99.3%) or naloxone (98.3%). The inhibitory effect noted for etorphine (fig. IV-1) and DADL (data not shown) was reversed by naloxone (1 uM).

5. DISCUSSION

In rodent neuroblastoma cells and hybrids (delta receptor cell lines), opioid agonists were shown to inhibit adenylate cyclase activity via interaction with delta receptors (Klee and Nierenberg, 1974; Sharma et al., 1975; Robson et al., 1983). However, this is of unknown significance as functional roles have not yet been defined for delta receptors in the intact animal. On the other hand, mu receptors have been implicated in antinociceptive effects of opioids but have unknown effects at the second messenger level, in part, because of a lack of available isolated cell systems. Hence the modulation of cyclic nucleotides by mu receptors would assume much importance.

In the SK-N-SH cell line, the prototypical delta (DADL) and mu (morphine) agonists both caused a small but significant degree of inhibition on PGE_1-stimulated cyclic AMP production. Etorphine exhibited the strongest inhibition of cyclic AMP production in both the delta cell line (Law et al., 1983) and SK-N-SH cells. Further, these effects were naloxone reversible, indicating an opiate receptor mediated phenomenon. Maximal inhibitory effects were noted at 10^{-8}, 10^{-7} and 10^{-6} M for etorphine, DADL and morphine, respectively. The kappa agonist, bremazocine, did not affect cyclic AMP production. Hence, opioids that affected cyclic AMP accumulation in rodent neuroblastoma (delta) cells (Law et al., 1983) also did so with SK-N-SH cells. Moreover, the smaller inhibitory signal in SK-N-SH cells might be reflective of the fewer delta sites when compared with NG

108-15 cells. This would suggest a delta receptor mediated action. On the other hand, in comparison to etorphine and DADL, morphine has similar efficacy at inhibiting cyclic AMP production in SK-N-SH cells but is less effective in the delta receptor cell line, NG 108-15 (Law et al., 1983), suggesting the possibility of a mu receptor modulation of cyclic AMP. Nonetheless, quantitative studies with selective agonists and antagonists are needed to fully clarify the question of which receptor type is involved in regulating cyclic AMP. Because of the data variability and small signal inhibition caused by the opioids, such studies should await cell lines/hybrids with stronger opioid signals and/or homogeneous mu opiate receptor populations.

Chapter 5

Human neuroblastoma cell lines as models of catechol uptake

1. SUMMARY

This chapter compares the effect of antidepressants on the accumulation of [^{3}H]catecholamines in 2 human neuroblastoma cell lines, IMR-32, and SK-N-SH, and a rat pheochromocytoma cell line, PC-12. Analysis of the catecholamine uptake system in SK-N-SH cells revealed that both [^{3}H]dopamine and [^{3}H]norepinephrine accumulated via a single competitive, saturable and apparently active process. Similar potencies exhibited by several inhibitors suggest that identical uptake systems exist in all 3 cell lines, with SK-N-SH cells being the most active. Although SK-N-SH and PC-12 cells have been reported to secrete dopamine in preference to other catecholamines, high intracellular concentrations of [^{3}H]norepinephrine were measurable after incubation with [^{3}H]dopamine. Additionally, [^{3}H]norepinephrine/[^{3}H]dopamine ratios increased with longer tracer incubations, in the presence of exogenous ascorbate and when including O-methylated metabolites. Moreover, accumulation of [^{3}H]catechols was competitively inhibited by various antidepressants, with maximal inhibition to 7% of control and Kis consistent with reported noradrenergic uptake$_1$ transport systems. Hence, cultured neuronal cell lines appear to serve as appropriate models for determining a drug's propensity to inhibit catechol reuptake in noradrenergic cells.

2. INTRODUCTION

Human neuroblastoma cell lines represent particularly useful models in the study of drug action (Yu et al., 1986). Thus, several neuronal cell lines were screened to test the inhibitory capacity of antidepressants on the uptake of [^{3}H]catecholamines. The catecholamine uptake systems of 2 of the human neuroblastoma cell lines, ie., IMR-32 and SK-N-SH, were compared to a rat

pheochromocytoma cell, PC-12, which has been extensively studied in previous investigations (Green and Rein, 1977; 1978; Green and Tischler, 1976; Chalfie and Perlman, 1976). Under usual incubation conditions, the SK-N-SH and PC-12 cell lines have been reported to produce more dopamine (DA) than norepinephrine (NE) (Green and Rein, 1978; Casper et al., 1983). However, the fact that the cells convert [^{3}H]DA to [^{3}H]NE intracellularly and that the Kis for a number of uptake inhibitors were more consistent with reported noradrenergic uptake$_1$ transport processes suggests that these cell lines derive from a noradrenergic cellular origin. Moreover, while the literature data on the inhibitory potencies of antidepressants vary considerably when measured in different experimental systems, the present report provides rather consistent Ki values for the uptake inhibitors among the 3 cell lines studied.

3. MATERIALS AND METHODS

3.1 Materials

The following drugs were generously donated by the indicated sources: butriptyline, Ayerst Laboratories (New York, NY); nomifensine, Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ); buproprion, Burroughs Wellcome Co. (Research Triangle Park, NC); iprindole, Wyeth Laboratories (Philadelphia, PA); mianserin, Organon Laboratories Ltd. (West Orange, NJ); protriptyline, Merck Sharp and Dohme (West Point, PA); fluoxetine, Eli Lilly and Co. (Indianapolis, IN), maprotiline, Ciba-Geigy Pharmaceutical Co. (Summit, NJ); amoxapine, Lederle Laboratories (Wayne, NJ); bremazocine and etorphine, NIDA (Rockville, MD); naloxone, Endo Laboratories (Garden City, NY); mazindol, Tony Trevor (University of California); catechol-0-methyl transferase, Brian Frazer (University of California). D-Ala-MePhe-Gly-ol enkephalin and D-Ala-D-Leu enkephalin were purchased

from Peninsula Laboratories (San Carlos, CA). The following compounds were purchased from Sigma: benztropine, pargyline, imipramine, nortriptyline, amitriptyline, desipramine, clomipramine, dopamine, (-)norepinephrine, normetanephrine, metanephrine, 3-methoxytyramine, ɣ-amino-butyric acid and serotonin. [^{3}H]Dopamine, [^{3}H](±)norepinephrine and [^{3}H]S-adenosyl-methionine were purchased from Amersham Corp. [^{3}H]Mazindol was purchased from New England Nuclear.

3.2 Catecholamine production

All cell lines were cultured as monolayers at 37°C in RPMI 1640 culture medium supplemented with 10% fetal calf serum and 100units/ml each of penicillin and streptomycin.

Catecholamines were measured in SK-N-SH cells following incubation at confluency in 17 mm culture wells (Falcon Primaria) for 48 hr. The cultures were maintained at 37°C in darkness. Using a radioenzymatic assay (Peuler and Johnson, 1977) with HPLC separation (reverse phase, C_{18}; mobile phase, 10% methanol plus 2:1, 0.05 M citric acid: 0.05 M Na_2HPO_4 with 5 x 10^{-5} M EDTA, pH adjusted to 3.4 with 5 M NaOH) of the O-methylated products, the incubation medium was tested for extracellular catecholamines. Intracellular catecholamines were measured after removing the medium, washing the cells x 2 with phosphate buffered saline, adding 200 ul of 6% trichloroacetic acid, transferring the cells to 1.5 ml polypropylene centrifuge tubes (Eppendorf), sonicating (30 s) and centrifuging (1800 x g, 10 min).

3.3 [^{3}H]Catecholamine uptakes

Cultured cells were transferred to 17 mm culture wells and incubated a minimum of 24 hr prior to experimentation. Before tracer addition, the culture

medium was removed, the cell monolayer washed twice with prewarmed (37°C) Krebs-Ringers-Hepes (KRH) medium (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 25 mM Hepes, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, pH adjusted to 7.4 with 5 M NaOH) and preincubated with 0.5 ml KRH (± inhibitors) for 10 min. [^{3}H]DA and [^{3}H]NE uptake by SK-N-SH cells was linear for at least 5 min and reached steady state at approximately 20 min (data not shown). [^{3}H]Catecholamine uptake also was reported to be linear for 5 min in PC-12 cells (Green and Rein, 1977) Thus, incubations for uptake experiments were terminated after 5 min of [^{3}H]catecholamine exposure by removal of incubation medium and washing with 3 x 0.5 ml aliquots of ice-cold KRH. After addition of 0.5 ml of 6% trichloroacetic acid, the cells were scraped from the culture dish, transferred to microcentrifuge tubes, sonicated (30 s) and centrifuged at 1800 x g for 10 min. The protein content of the pellet was determined colorimetrically (Lowry et al., 1951) and the supernatant radioactivity was measured by liquid scintillation counting.

3.4 [^{3}H]Dopamine incubations

The metabolic fate of [2,5,6-^{3}H]dopamine, both intra- and extracellular, was tested in SK-N-SH cells. Cells were transferred to 17 mm culture wells 48 hr prior to experimentation. At 6 hr before tracer incubation, cells were incubated in the absence or presence of 1 mM ascorbate. Medium (± ascorbic acid) was changed every 2 hr during this period. Cells were washed twice with 500 ul of pre-warmed (37°C) KRH buffer, followed by addition of 500 ul KRH, [^{3}H]DA and ascorbic acid where appropriate. After varying incubation periods, medium was aspirated and the cells washed x 2 with 500 ul KRH (0°C). In cases where the KRH fraction was tested for [^{3}H]catecholamines, the cells were washed x 2 with 500 ul KRH (37°C) followed by addition of 200 ul KRH and incubation for an additional 15 min. The KRH buffer was then removed and the cells washed in ice-cold KRH. After washing, the cell monolayer was extracted with 200 ul trichloroacetic acid, transferred to 1.5

ml centrifuge tubes, sonicated and centrifuged (1800 x g, 10 min). The 200 ul KRH or trichloroacetic acid samples were spiked with unlabeled NE, DA, epinephrine and their O-methyl metabolites, normetanephrine, 3-methoxytyramine and metanephrine. Twenty ul aliquots of the extracts were injected onto the HPLC, using identical conditions as for separating O-methylated catechols for the radioenzymatic assay. Radioactivity was determined in the appropriate fractions.

4. RESULTS

4.1 Catecholamine production

Dopamine (as 3-methoxytyramine) was detected via a radioenzymatic assay in both the incubation medium and trichloroacetic acid extracts of SK-N-SH cells (data not shown). Norepinephrine was not detectable in either.

4.2 [^{3}H]Catecholamine uptake

The uptake of [^{3}H]DA by SK-N-SH cells was competitive and saturable, with a V_{max} of 10 pmol/min/mg protein (fig. V-1). [^{3}H]DA uptake was inhibited at 0°C (to 6% control), by co-addition of the metabolic inhibitors, 20 mM potassium cyanide plus 20 mM 2-deoxyglucose (to 40% control), or by replacement of Na^+ with either choline or sucrose (to 30% control).

The Km for dopamine and norepinephrine uptake into SK-N-SH cells was 533 and 1150 nM, respectively (table V-1). [^{3}H]Catecholamine uptake was prevented by known inhibitors, mazindol, benztropine and amphetamine, whereas other neurotransmitters, opioids and most neuroleptics were either weak inhibitors of [^{3}H]DA accumulation or lacked an effect entirely at 10 uM (table V-1).

Desipramine's inhibition of [^{3}H]DA accumulation in SK-N-SH cells was

competitive (fig. V-1) and, as with most other inhibitors, virtually complete (to 7% control). A majority of the antidepressants demonstrated a potent inhibitory effect on the uptake of [^{3}H]catechols (table V-1). Ten uM of either imipramine, desipramine, amitriptyline, nortriptyline or clomipramine did not affect dopamine release; assessed by labeling SK-N-SH cells with [^{3}H]DA and then placing them in tracer free medium in the presence or absence of the antidepressants. However, [^{3}H]catecholamine release neither could be stimulated by the introduction of 56 mM K^+ into the culture medium.

The accumulation of [^{3}H]NE and [^{3}H]DA was blocked with approximately equal Kis by each of several compounds possessing a wide range of inhibitory potencies: i.e., mazindol, desipramine, imipramine, benztropine, fluoxetine and iprindol (tables V-1 and V-2; fig. V-2). Moreover, based on the Kis of these same ligands, [^{3}H]catecholamine uptakes in IMR-32 and PC-12 cells were similar in character to SK-N-SH cells (table V-2).

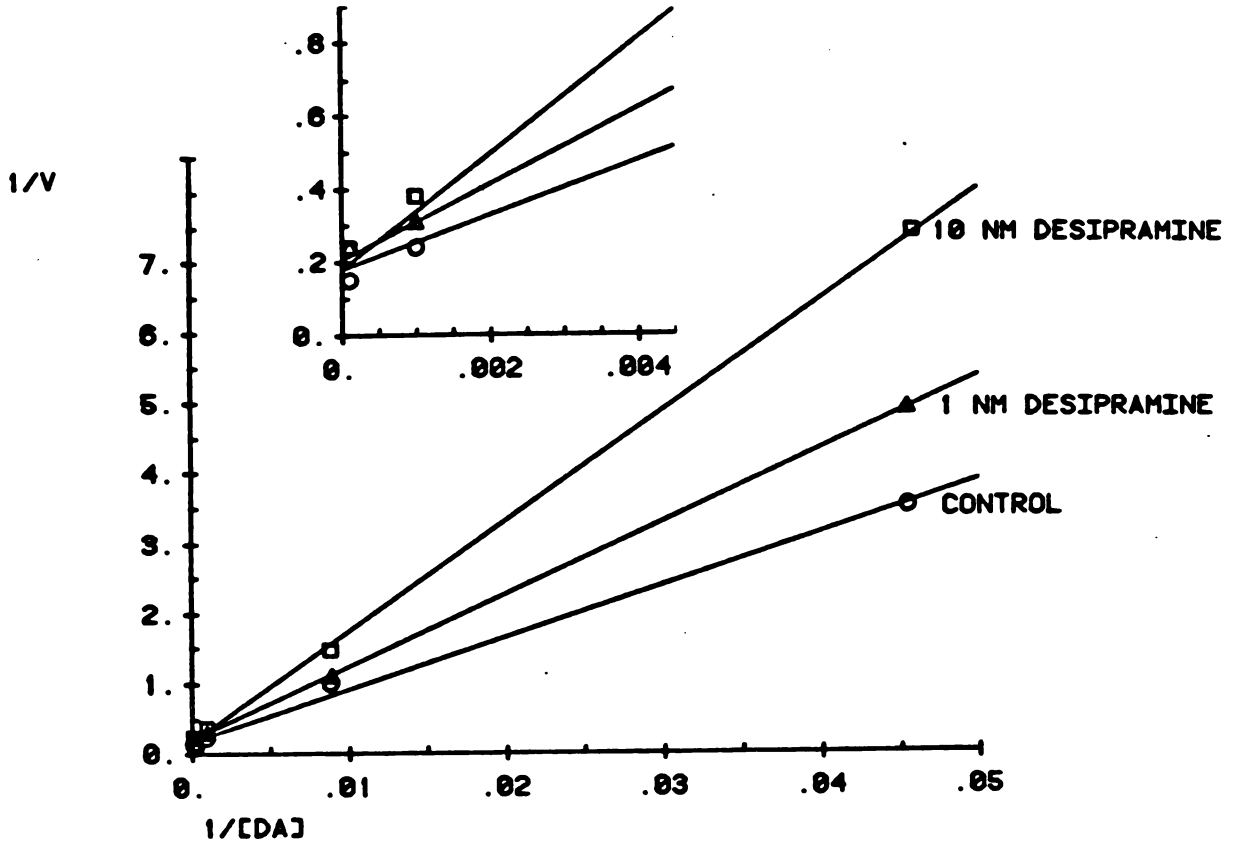


Figure V-1. Velocity of [^{3}H]dopamine uptake as a function of dopamine and desipramine concentration. Vmax (pmol/min/2 mg protein) was determined by adding labeled (10 nM) and unlabeled DA to culture wells containing a confluent monolayer of SK-N-SH cells and incubated for 1, 3 and 5 min (in each of 3 experiments). Double reciprocal plot reveals y-intercepts of 0.180, 0.208 and 0.182 for the linear transformations of dopamine uptake velocity in the absence and presence of 1 and 10 nM desipramine, respectively. Linear correlation coefficients are $\geq$ .997. The similar y-intercepts suggest a competitive mechanism of dopamine uptake inhibition by desipramine. Inset. The data for the higher dopamine concentrations are shown in more detail.

Table V-1. Ki estimates for inhibition of [^{3}H]DA and [^{3}H]NE uptakes in SK-N-SH cells and reported Ki or IC_{50} values for [^{3}H]NE accumulation in isolated heart (Iversen, 1976) and synaptosomes from occipital cortex (Randrup and Braestrup, 1977), cerebral cortex (Lee et al., 1982) and whole brain (Richelson, 1984) of the rat. Control uptakes were determined in the absence of inhibitors. Non-specific uptake/binding (6 to 8% control) was determined by measuring uptake either at 0°C or in the presence of excess inhibitor. This value was subtracted from all measurements. The Kis are obtained by computer estimation using a model based on Michaelis Menten kinetics. Thus, Ki represents 50% inhibition of [^{3}H]catecholamine uptake after subtraction of nonspecific uptake/binding. The model allowed for variation in the slope (mathematically equivalent to the Hill coefficient), which ranged from 0.7 to 1.3. The deviations from unity predicted by the law of mass action probably reflect data variability. The following drugs did not affect the uptake of [^{3}H] DA at 10 uM: serotonin, GABA*, acetylcholine, epinephrine, DAGO, DADL, bremazocine, etorphine and naloxone. Tracer concentrations: [^{3}H]DA = 10 nM; [^{3}H]NE = 30 nM.

	Ki (S.E.) nM			
	SK-N-SH cells		literature sources	
inhibitor	[^{3}H]DA	[^{3}H]NE	[^{3}H]NE	ref.†
mazindol	0.30 (.03)	0.59 (.03)	3	a
benztropine	377 (38)	268 (37)	170 - 290	a, b
amphetamine	219 (22)		90 - 320	a, c
dopamine	533 (65)	206 (24)		
norepinephrine	2,840 (470)	1150 (310)		
trifluoperazine	4,570 (720)			
perphenazine	555 (43)			
thioridazine	1,840 (190)			
promazine	37 (6)		76	d

Table V-1. (cont'd)

	Ki (S.E.) nM			
	SK-N-SH cells		literature sources	
inhibitor	[^{3}H]DA	[^{3}H]NE	[^{3}H]NE	ref.†
desipramine	4.5 (0.4)	5.2 (0.5)	0.9-20	a, b, d, e
imipramine	52 (4)	21 (3)	13-79	a, b, d, e
amitriptyline	29 (5)		20-130	a, b, d, e
nortriptyline	9.1 (0.5)		4-24	a, b, d, e
clomipramine	76 (7)		28-255	a, b, e
nomifensine	5.8 (0.6)		5-7	b, e
iprindole	1890 (240)	1040 (92)	640-5000	a, b, e
amoxapine	11 (1.0)		4.4	e
maprotiline	27 (5)		7.4-20	b, e
buproprion	1450 (400)		2260	e
protriptyline	4.2 (0.6)		1-13	a, b, e
mianserin	42 (0.6)		42-410	a, b, e
butriptyline	866 (130)		990-1700	b, e
fluoxetine	527 (64)	411 (35)	280	e

*Abbreviations: S.E., standard error; GABA, ϑ-aminobutyric acid; DADL, D-Ala-D-Leu enkephalin; DAGO, D-Ala-MePhe-Gly-ol enkephalin.

†References: a, Lee et al., 1982; b, Randrup and Braestrup; c, Langer et al., 1981; d, Iversen, 1976; e, Richelson, 1984.

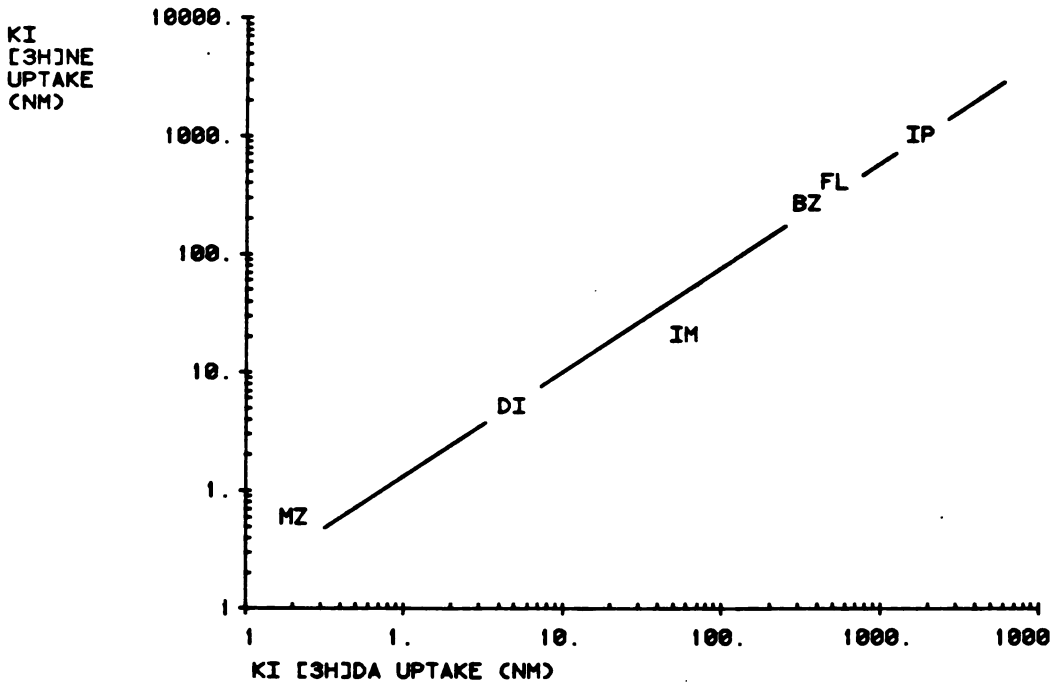


Figure V-2. Relationship between [^{3}H]DA uptake versus [^{3}H]NE uptake inhibition in SK-N-SH cells. Kis for mazindol (MZ), desipramine (DI), imipramine (IM), benztropine (BZ), fluoxetine (FL) and iprindol (IP) are taken from table V-1; slope = 1.73, r^2 = .992.

Table V-2. Comparison of [^{3}H]DA (0.2 uCi, 10 nM) and [^{3}H]NE (0.2 uCi, 3 nM) uptakes in 3 cultured cell lines. Experiments are performed as described in the methods and the legend of table V-1.

	SK-N-SH	IMR-32	PC-12
[^{3}H]dopamine			
	% [^{3}H]DA uptake/5 min/mg protein		
	12.47	2.31	3.33
		Ki (± S.E.) nM	
mazindol	0.30 (0.03)	0.81 (0.11)	0.24 (.05)
desipramine	4.5 (.4)	2.0 (0.2)	6.2 (1.3)
imipramine	52 (4)	13 (1.7)	41 (8)
benztropine	377 (38)	405 (49)	289 (71)
iprindole	1890 (240)	2630 (730)	3610 (710)
[^{3}H]norepinephrine			
	% [^{3}H]NE uptake/5 min/mg protein		
	9.61	1.77	2.60
		Ki (± S.E.) nM	
desipramine	3.8 (0.4)	4.6 (2.7)	4.5 (0.5)

Table V-3. Fate of [^{3}H]DA accumulated in SK-N-SH cells. Values represent the mean of duplicate measurements. Post-tracer incubations represent the period following replacement of tracer with tracer-free KRH medium.

		[^{3}H]NE/[^{3}H]DA	
		SK-N-SH cells	
tracer incubation	post-tracer incubation	intracellular	extracellular
5	0	0.28	
30	0	1.23	
30	15	2.3	0.62 0.92[a]
30[b]	15	3.5	0.57 1.32[a]
60	0	2.2	

[a]represents the ratio, (NE + normetanephrine)/(DA + 3-methoxytyramine)

[b]cells were incubated with 1 mM ascorbic acid for six hours prior to experimentation

4.3 Tracer incubations

In SK-N-SH cells, the intracellular ratio of [^{3}H]NE/[^{3}H]DA increased with time of incubation (table V-3). Two to 5 fold lower levels of radioactivity co-eluted with epinephrine than with NE. In SK-N-SH cells, similar results were demonstrated in the presence and absence of 100 uM pargyline, a monoamine oxidase inhibitor. Increased intracellular [^{3}H]NE/[^{3}H]DA ratios were noted when SK-N-SH cells were allowed an additional incubation of 15 min in tracer-free medium following a 30 min tracer incubation. Moreover, this was further increased if cells were incubated in the presence of 1 mM ascorbate. After 30 min tracer incubations, [^{3}H]NE/[^{3}H]DA ratios within the cell were higher than in the medium. However, the extracellular levels of the O-methyl metabolites exceeded the parent catechol by factors of 2.0 (DA) to 3.6 (NE), giving rise to a doubling of the [^{3}H]NE/[^{3}H]DA ratios for SK-N-SH cells when including the O-methylated metabolites.

5. DISCUSSION

Two human neuroblastoma cell lines were found to accumulate exogenous [^{3}H]catecholamines: SK-N-SH and IMR-32. The catecholamine transport process in SK-N-SH cells was saturable, competitive and apparently active. Further, the uptake was temperature and Na^+ sensitive. These features are characteristic of catecholamine transport processes that have been described in rodent brain preparations (Iversen, 1976; Horn, 1978). Moreover, accumulation of DA in SK-N-SH cells was inhibited by mazindol, amphetamine, benztropine and neuroleptics with Kis that are consistent with DA or NE uptake systems (Lee et al., 1982; Hyttel, 1978; Friedman et al., 1977; Coyle and Snyder, 1969).

As evidenced by the similarity of inhibitory potencies demonstrated by several

ligands for [^{3}H]DA and [^{3}H]NE uptake (table V-2, fig. V-2), the catecholamine transport system described for SK-N-SH cells appears to be duplicated in IMR-32 and PC-12 cells. The uptake data for IMR-32 cells were more highly discordant compared with other cell lines. However, IMR-32 cells are subject to clumping and do not attach well to incubation containers. Thus, the Kis were partially dependent on changes of uptake kinetics, due to cell clumping, and variable number of uptake sites per culture well because of cell loss during washing procedures.

The Kis for [^{3}H]catecholamine uptake inhibition did not bear any relationship to [^{3}H]mazindol binding inhibition in SK-N-SH cells (data not shown). The Kis for [^{3}H]mazindol binding (500 - 2000 nM) were consistent despite use of a variety of incubation conditions. [^{3}H]Mazindol has been reported to label both dopamine and norepinephrine uptake sites (Javitch et al., 1983). Although the results suggest that the site labeled by [^{3}H]mazindol on SK-N-SH cells is different from the uptake site, a Scatchard transform of the binding saturation isotherm indicates the presence of at least two populations of sites labeled by [^{3}H]mazindol. Moreover, the 270 pmol of binding sites per mg protein 50- to 1000-fold higher in concentration than reported for catecholamine uptake sites in rat brain synaptosomes (Lee et al., 1982; Rehavi et al., 1981; Langer et al., 1981). Thus, [^{3}H]mazindol appears to label more than just the catecholamine uptake site on SK-N-SH cells.

Under usual incubation conditions, PC-12 (Green and Tischler, 1976) and SK-N-SH (Casper et al., 1983) cells have been reported to produce DA in preference to NE or epinephrine, and this was verified for SK-N-SH cells using a radioenzymatic assay. Although the apparent secretion of dopamine might imply a dopaminergic origin for these clonal cell lines, the appropriate enzymes/coenzymes necessary for metabolizing catecholamines might be altered in these "transformed" cells. SK-N-SH, Neuro-2a and PC-12 cells cultured in vitro apparently secrete more DA than NE (Green and Tischler, 1976; Casper et al., 1983; Narotzky and Bondareff, 1974) despite containing high levels of dopamine-β-hydroxylase (Green and Tischler, 1976; Chalfie and Perlman, 1976;

Biedler et al., 1973). However, if PC-12 or Neuro-2a cells are grown in vivo, they secrete more norepinephrine than dopamine (Green and Rein, 1978; Green and Tischler, 1976; Anagnoste et al., 1974). Similarly, when PC-12 cells are incubated in vitro with ascorbate, a putative cofactor for dopamine-β-hydroxylase (Kaufman, 1966) (which is absent from culture medium), much of the accumulated [^{3}H]DA is converted to [^{3}H]NE (Green and Rein, 1978). Hence, similar strategies were employed to determine the fate of [^{3}H]DA in SK-N-SH cells in vitro. Results indicate that, as with the PC-12 cells, ascorbate caused an increase of NE relative to DA (table V-3). Moreover, intracellular [^{3}H]DA was increasingly converted to [^{3}H]NE with time. Extracellular [^{3}H]NE/[^{3}H]DA ratios generally were 0.5 to 0.75, but not as low as expected based on measurements of endogenous catechols (Casper et al., 1983). This might be accounted for by the short incubations here (< 1 hr) versus the 48 hr incubations used to obtain endogenous catechols. At least for SK-N-SH cells, the catechol-O-methyl transferase appears to prefer NE over DA as a substrate based on the higher NE/DA ratios when including the O-methoxy metabolites.

Hence, it is evident that the SK-N-SH, IMR-32 and PC-12 lines are derived from noradrenergic cell origins. Additionally, the high potencies of selected antidepressants for [^{3}H]DA uptake inhibition are in line with the findings for NE uptake inhibition in synaptosomes or slices from cortical or hypothalamic regions of rodent (Richelson, 1984; Langer et al., 1981; Lee et al., 1982) or human (Harms, 1983) brain (table V-1). Potencies of most tricyclic antidepressants at inhibiting [^{3}H]DA uptake in SK-N-SH cells are 2 or more orders of magnitude higher than for striatal (dopaminergic) membrane preparations (Randrup and Braestrup, 1977; Ross and Renyi, 1967). This confirms the noradrenergic nature of SK-N-SH cells. Moreover, inhibitory potencies versus catecholamine uptake are similar whether measured in human neuroblastoma or rat pheochromocytoma cell lines, or noradrenergic membrane preparations from brain (Richelson, 1984; Langer et al., 1981; Lee et al., 1982; Harms, 1983).

Hence, cultured cell lines provide a simple, homogeneous model systems for catecholamine uptake processes in human noradrenergic cells. Because SK-N-SH cells are easily maintained and accumulate catecholamines more efficiently, they appear to be most suited for studying noradrenergic uptake; whereas IMR-32 cells are the least desirable for this purpose.

CONCLUSIONS

A novel ex vivo labeling method was developed for measuring opioid occupancy in rat brain that compared well with an in vivo labeling approach developed earlier in this laboratory. Using in vivo fractional occupancies of opioid antagonists, it was established that the kappa opiate receptor site was responsible for mediating the increased urine output caused by bremazocine in rats. Accordingly, buprenorphine was found to be an antagonist at kappa sites by virtue of it's inhibition of bremazocine-associated diuresis in both naive and morphine (mu) tolerant rats.

The blocking action of opioids on PGE_1-stimulated adenylate cyclase was documented in SK-N-SH human neuroblastoma cells. However, the receptor site(s) responsible for this effect could not be determined. Opioid drugs do not affect the accumulation of catecholamines via noradrenergic $uptake_1$ systems in 2 human neuroblastoma cells, SK-N-SH and IMR-32.

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