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CHARACTERISATION OF OPIOID RECEPTOR BINDING IN GUINEA-PIG CEREBELLUM

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J.A.CARROLL

A MASTER'S THESIS

A MASTER'S THESIS, SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF M-PHIL OF THE LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY.

APRIL 1990

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All the data presented in this document, its interpretation, and the full text of the thesis, are my own work.

CHARACTERISATION OF OPIOID RECEPTOR BINDING IN GUINEA-PIG CEREBELLUM

J.A.CARROLL

The binding of the non-selective opioid receptor ligand [³H]bremazocine to guinea-pig cerebellum membranes in HEPES buffer was found to be selective for the opioid kappa receptor, as defined in the literature. However, displacement curves to dynorphin A (1-17) and a number of other peptides, suggested the presence of a "dynorphin resistant" binding site, comprising 15-20% of the specific binding. This component was not abolished by high concentrations of the mu/delta selective ligand [D-Ala²,D-Leu⁵]enkephalin, suggesting the presence of an additional binding-site, labelled by [³H]bremazocine, that could not be defined as high affinity mu, delta or kappa.

In Krebs/HEPES buffer containing the stable guanosine triphosphate analogue 5'-guanylylimidodiphosphate (GppNHp), [³H]bremazocine binding in guinea-pig cerebellum membranes was predominantly to a low affinity kappa receptor binding-site. The "dynorphin resistant" component seen in the HEPES buffer system was also present under these conditions. In addition, several selective ICI kappa agonists showed negligible affinity for this binding-site in the Krebs buffer system, an effect not seen in HEPES.

The "dynorphin resistant" component in Krebs buffer was studied, using a suppressed assay system, and revealed an IC_{50} profile that was not kappa-like. IC_{50} values from this assay were therefore compared with data from a low affinity mu receptor binding assay run in the same buffer system. Results from these two tests correlated well.

These results therefore suggest that the "dynorphin resistant" component seen with [³H]bremazocine binding in Krebs/HEPES buffer

containing GppNHp represents a low affinity mu receptor binding site. The nature of this component in HEPES buffer however, given the very different results obtained with certain ICI kappa agonists, remains unclear.

TABLE OF CONTENTS:

	PAGE	NOS
INTRODUCTION:		
1. THE OPIATE RECEPTOR.	1 -	5
2. OPIATE RECEPTOR TYPES.		
A. THE MU AND DELTA RECEPTORS.	5 –	9
B. THE KAPPA RECEPTOR.	9 -	15
C. THE SIGMA AND PCP RECEPTORS.	15 -	16
3. THE EFFECTS OF IONS AND NUCLEOTIDES ON OPIATE RECEPTOR	BINDING	3.
A. THE Na ⁺ EFFECT.	17 –	19
B. THE GUANINE NUCLEOTIDE EFFECT.	19 –	23
C. DIVALENT CATION EFFECTS.	23	
D. MODELS OF AGONIST BINDING.	23 -	30
4. THE AIMS OF THE STUDY.	31	
MATERIALS AND METHODS:	32 -	40

4

•

•

RESULTS:

- 1. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: HEPES BUFFER.
 - A. ASSAY VALIDATION.
 B. EVIDENCE FOR HETEROGENEITY OF BINDING.
 i. [³H]bremazocine binding.
 ii. [³H]bremazocine binding in the presence of DADLE.
 iii. [³H]Bremazocine binding at 0°C in the presence
 of protease inhibitors.
 iv. [³H]EKC binding in presence and absence of DADLE.
 - C. SUMMARY. 56 57
 - FIGS 1.1 1.22 58 79
- 2. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: KREBS/HEPES + GPPNHP.
 - A. ASSAY VALIDATION.
 - i. [³H]bremazocine binding. 80 84
 - ii. Comparison of [³H]bremazocine displacement curves 84 85
 obtained in HEPES buffer and Krebs/HEPES + GppNHp.
- B. EVIDENCE FOR HETEROGENEITY OF BINDING.
 - i. [³H]Bremazocine binding. 85 88
 - ii."Kappa suppressed" [³H]bremazocine binding. 88 91
- 3. [3H]NALOXONE BINDING IN RAT BRAIN MEMBRANES.
 4. SUPPRESSION OF [³H]NALOXONE AND "KAPPA SUPPRESSED"
 94 97
 [³H]BREMAZOCINE ASSAYS BY ICI 174864 AND ICI 204879.
- 5. SUMMARY.

DISCUSSION:

- 1. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: HEPES BUFFER.
- A. ASSAY VALIDATION.

iii	.[³ H]EKC binding.						125	_	126
	of protease inhi	bitors.		·					
ii.	[³ H]Bremazocine	binding	at	$0^{\circ}C$ in	the	presence	123	-	125
i.	[³ H]Bremazocine	binding	at	25°C.			118		123

B. HETEROGENEITY OF BINDING.

i.	Hill Coefficients.	126 -	129
ii.	Dynorphin resistant subsite.	129 -	132

2. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: KREBS/HEPES + GPPNHP:

A. ASSAY VALIDATION.	132 - 135
B. HETEROGENEITY OF BINDING.	135 – 136
C. "KAPPA SUPPRESSED" [³ H]BREMAZOCINE BINDING.	136 - 137
3.1 ³ HINALOXONE BINDING IN RAT BRAIN MEMBRANES.	137 - 141
4. SUCCESSIVE SUPPRESSION EXPERIMENTS.	141 - 144
5. SUMMARY AND CONCLUSIONS.	144 - 147
6. APPENDIX.	148 - 153
REFERENCES.	154 – 172
ABBREVIATIONS	173 – 175

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INTRODUCTION:

The effects of opium and its derivatives have been well known for centuries. However, it is only in the last 80 years that any real understanding has been reached as to their mechanism of action.

Opiates produce a large range of apparently diverse and confusing effects, including analgesia, euphoria, sedation, miosis, respiratory depression, bradycardia, hypothermia, constipation and emesis. In addition, chronic use produces tolerance and dependence. The development of a unified theory, explaining all these effects in terms of actions at three cell surface receptors, has therefore been one of the major achievements of pharmacology in the 20th century.

1. THE OPIATE RECEPTOR:

The first clear indications that the actions of morphine, and other narcotic analgesic drugs were mediated via a specific receptor, came from early in-vitro studies performed by Kosterlitz et al. In the first of these, Gyang and Kosterlitz [53] examined the inhibitory effects of a range of narcotic analgesic drugs on the contractions of the electrically stimulated longitudinal muscle of the guinea-pig ileum (GPI), and found that all the compounds tested were capable of producing both agonist and antagonist effects, irrespective of whether they were considered as agonists in clinical use. This work was extended two years later, in another study by Kosterlitz and Watt [73], also using the GPI preparation, in which the ratio of the agonist IC₅₀ to antagonist affinity value was used as a measure of the antagonist activity of opiate drugs. This allowed the identification of naloxone as the only opiate drug then available showing negligible agonist activity, with only a 2-3% maximum reduction of twitch height in the GPI. The effects of morphine, levorphanol, codeine and nalorphine were competitively antagonised in this study by naloxone, with affinity values between 1 and 1.5nM in all cases, suggesting that these agents were acting through the same receptor site.

The idea of a specific receptor site for morphine also received considerable support in 1972, with the introduction of the mouse vas deferens preparation (MVD). This tissue was shown by Henderson et al. [55] to contain an excitatory adrenergic motor innervation capable of being inhibited by low concentrations of morphine. This effect could be blocked by naloxone with an affinity of 4.5nM, a value similar to that obtained in the GPI, thus indicating that morphine was likely to be acting through the same receptor site in both preparations.

Subsequently, a large number of compounds of differing potencies, chemical type, and lipophilicity were examined in these <u>in-vitro</u> models [76, 74, 75]. In the case of agonist and partial agonist drugs a good correlation was found between the IC_{50} value in GPI and analgesic potency in man, whereas in the case of the antagonists affinity values obtained in this tissue were in good general agreement with their activity in producing an abstinence syndrome in the morphine dependent dog. The affinity values obtained for naloxone as a competitive antagonist in the GPI preparation also showed very little variation over a wide range of agonist potencies and chemical types.

Agonist potency in the MVD preparation correlated well with data from the GPI. However dose-response curves to partial agonists such as nalorphine and levallorphan were very shallow in this tissue, suggesting some differences in the sensitivity of the two models to agonist action at the opiate receptor.

Overall the similarity of the naloxone affinity values, and the good correlations, certainly in terms of the rank order of potency, between the <u>in-vivo</u> and <u>in-vitro</u> preparations, both served to confirm the usefulness of the GPI and MVD preparations as predictive of opiate activity <u>in-vivo</u>, and provided clear evidence that narcotic drugs were acting through a common receptor, in these diverse models.

The final confirmation of the existence of the opiate receptor, came from the early binding studies. The very first of these was published by Goldstein et al. in 1971 [47], and described the binding of

 $[{}^{3}H]$ levorphanol to mouse whole brain homogenates. Stereospecific binding was found to be located mainly in a nuclear membrane fraction, and also to some extent in microsomal and synaptosomal membranes, but comprised only 2% of the total binding of $[{}^{3}H]$ levorphanol. There was also no significant difference in binding between a number of brain areas, including cerebrum and cerebellum. However, given the extremely low specific activity of the $[{}^{3}H]$ levorphanol available at this time, and the very high concentrations needed as a consequence, this equivocal result is perhaps not surprising.

It is likely therefore, that the first successful opiate receptor binding experiments were in fact those published by Pert and Snyder in 1973 [105]. In this study, $[{}^{3}H]$ naloxone binding to rat whole brain homogenates was shown to be both reversible and temperature dependent. Specific binding in the presence of 5nM $[{}^{3}H]$ naloxone comprised approximately 60% of the total and was completely prevented by preheating the homogenates at 55°C for 15 min. Binding was displaced with high affinity by a range of opiate drugs, including levorphanol, morphine and nalorphine, whereas no activity was seen with dextrorphan, the inactive isomer of levorphanol, or with such agents as phenobarbitone, serotonin, noradrenaline, atropine or histamine.

[³H]Naloxone binding levels in minced guinea-pig intestine were approximately half of those obtained in rat brain, and specific binding was completely abolished by the removal of the myenteric nerve plexus from intestinal strips. In addition, no specific binding could be obtained in non-innervated tissues such as erythrocytes or bakers yeast preparations, clearly suggesting that opiate receptors could be localised to nervous tissue.

The IC_{50} values obtained in minced guinea-pig intestinal tissue roughly paralleled those seen in rat brain, although there were some discrepancies between the two tissues, suggesting possible qualitative differences in opiate receptor populations. However, opiate receptor binding was strictly stereoselective, and of high affinity, in both of these preparations, with a close correlation between pharmacological potency and binding IC_{50} , thus confirming that [³H]naloxone was

interacting with a specific and pharmacologically relevant receptor site in both cases.

Pert and Snyder's paper was followed later in the same year by a similar study [132], in which the very high affinity opiate agonist etorphine was used as the radiolabelled ligand. [³H]etorphine binding to rat brain homogenates was saturable, reversible and of high affinity, with a maximum binding level similar to that obtained in the [³H]naloxone binding study. IC₅₀ values in the nanomolar range were obtained for a number of opiate drugs, including levorphanol, naloxone and morphine, whereas dextrorphan was four orders of magnitude weaker.

The properties of the $[{}^{3}H]$ etorphine binding site in rat brain were therefore similar to those of the $[{}^{3}H]$ naloxone site reported by Pert and Snyder, lending further support to the theory of a specific opiate receptor.

Opiate receptor binding was subsequently identified in the brain membranes of all vertebrates examined, including mammals, birds, reptiles, amphibia and teleost fish, but was not detectable in tissues from invertebrate species [107]. The distribution of opiate receptor binding in brain areas from several species, including man, monkey and rat, was also quite distinct. Kuhar et al. for instance [80], obtained variations in [³H]Dihydromorphine (DHM) binding levels of up to 30 fold in different brain areas of the rhesus monkey, with the highest receptor numbers in the anterior amygdala, periaqueductal gray, hypothalamus and caudate nucleus, but low levels in cortex, cerebellum, lower brain stem and spinal cord. In addition, minute intracranial injections of morphine in rhesus monkeys, elicited analgesia only in the medial thalamus, and periventricular and periaqueductal regions [108], and the application of naloxone crystals to sites in rat brain most frequently produced signs of abstinence in opiate tolerant animals when these were localised to the diencephalic/mesencephalic areas, both sites with high opiate receptor binding levels [80]. This suggests that the relative density of opiate receptor sites reflects their role in the pharmacological

action of opiates.

The physical nature of the opiate receptor was also investigated further [99] in a study on the effects of enzymatic treatments on $[{}^{3}\text{H}]$ naloxone binding in rat brain homogenates. Binding levels were markedly reduced by phospholipase A, the proteolytic enzymes trypsin and chymotrypsin and detergents such as deoxycholate and Triton X 100, whereas little effect was seen with neuraminidase, or RNAase and DNAase. This would indicate that the opiate receptor is likely to involve a membrane bound complex, the integrity of which is dependent on both protein and phospholipid molecules.

Overall therefore, these early studies confirmed the existence of the opiate receptor, both as a physical and pharmacological entity. Good correlations were obtained between binding affinities and analgesic potency for a wide range of opiate drugs, and opiate receptors were clearly localised to neuronal pathways involved in the transmission and processing of nociceptive stimuli, in a number of different species.

2. OPIATE RECEPTOR TYPES:

A. THE MU AND DELTA RECEPTORS:

One early indication of multiple opiate receptor types came with the discovery of the enkephalin peptides. The confirmation of the existence of a specific opiate receptor, present in nervous tissue, and capable of high affinity interaction with opiate analgesics, had led naturally to the idea of an endogenous morphine-like substance that would act as the natural agonist at this receptor, and a number of groups began to search for such an agent in brain tissue.

The first endogenous opioids to be discovered were isolated by Hughes et al. in 1975 [60] from pig brain, and identified as a mixture of the two pentapeptides leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and met-enkephalin (Tyr-Gly-Gly-Phe-Met). These agents were potent agonists in both the GPI and MVD preparations, and their effects could

be antagonised by naloxone [59].

The enkephalins were subsequently identified in a range of tissues, including bovine, rabbit and guinea-pig brains, and guinea-pig, mouse, and rat intestine [132, 61]. Regional variations in enkephalin levels were also demonstrated in different areas of guinea-pig and rabbit brain [61], and enkephalin distribution in the brain was found to parallel that of the opiate receptors [131]. Final confirmation of the enkephalins as neuromodulators however, came from release studies performed in slices of guinea-pig striatum and rat globus pallidus [56, 5].

Although the actions of morphine and the enkephalin peptides were similar in many respects, a number of important differences rapidly became apparent. In particular, the enkephalins were shown to be less potent than morphine in the GPI preparation, whilst in the MVD they were more potent. In addition a ten fold higher concentration of naloxone was required to antagonise the effects of the enkephalins in MVD, whereas no such difference was seen in the GPI [86, 87]. These results were explained by Kosterlitz et al., by proposing the existence of two separate opiate receptors, the classical or mu receptor and the enkephalin or delta receptor. Classical opiates such as normorphine were proposed to act only through the mu receptors, with effects that could be reversed by naloxone at low concentrations, whereas the enkephalins were likely to be non-selective, and capable of acting at both receptor types. To explain the differences observed with the enkephalins in the two tissue preparations, they suggested that although these peptides could act through both mu and delta receptors, delta receptors were present only in the MVD. Therefore the action of the enkephalins in the GPI would be mainly through the mu receptors, but in the MVD, these peptides would act through the delta receptor, thus producing a more potent effect, that was relatively resistant to naloxone.

Further support for the hypothesis of separate mu and delta receptors was provided from binding assays performed by Lord et al. [87], and Simantov and Snyder [134]. In these studies, classical opiates such

as morphine, DHM and oxymorphone, were shown to be potent inhibitors of $[{}^{3}H]$ naloxone and $[{}^{3}H]$ DHM binding in both rat and guinea-pig brain but were much weaker as displacers of $[{}^{3}H]$ Leu and Met enkephalin binding, whereas the enkephalin peptides displayed the opposite selectivity.

Final confirmation of the separate existence of the delta receptor however, came from a "protection study" published in 1979 by Robson and Kosterlitz [114]. In this paper, the inactivation of mu and delta opiate receptors in guinea-pig brain homogenates by the irreversible a-antagonist phenoxybenzamine was shown to be selectively prevented by preincubation with selective high affinity ligands. Non-selective ligands were able to protect both receptor types.

In the years following these early studies, some of the problems of instability encountered with the enkephalins were overcome by the substitution of stable groupings into the peptides. The first relatively stable and selective delta ligand to appear was $[^{3}H]$ [D-Ala²,D-Leu⁵]enkephalin (DADLE). Comparison of the affinities obtained in $[^{3}H]DHM$ and $[^{3}H]DADLE$ binding assays [43] showed that the most receptor specific agents then available were normorphine, which was 30 fold selective for the mu receptor, and DADLE, with a 10 fold selectivity for the delta receptor.

More recently the introduction of the very selective delta and mu ligands [³H][D-Pen²,D-Pen⁵]enkephalin (DPDPE) and [³H][D-Ala², (Me)Phe⁴,Gly(ol)⁵] (GLYOL) has allowed detailed binding profiles to be assembled for these two receptor sites, and very distinct profiles have emerged. [³H]DPDPE binding to the delta receptor was displaced with high affinity by delta selective peptides such as DADLE and also by non-selective benzomorphan drugs such as ethylketocyclazocine (EKC) and bremazocine, whereas mu selective agents such as morphine and GLYOL were much less active, with affinities in the range 100-400nM [30]. In standard mu selective receptor binding assays however, relatively high affinities were obtained with the majority of opiate drugs, although some selectivity was seen with classical analgesics such as morphine, and with the

antagonists naloxone and naltrexone [10].

Two stable antagonists, ICI 154129 (N,N-Diallyl-Tyr-Gly-Gly-(CH2S)-Phe-Leu-OH) and ICI 174864 (N,N-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH) [129, 29] have subsequently also became available, with selectivities for the delta receptor in excess of 30 fold. ICI 174864, the most potent and selective of these agents, was shown to be 100 fold selective for the delta receptor in isolated tissue models, with an affinity of 30nM against delta agonists such as DADLE [29]. A lower delta receptor affinity of only 200nM, was however obtained with this agent in binding assays, for reasons that are unclear, although ionic effects may be involved [1]. ICI 174864 has been the most useful tool available for the selective identification of delta receptor effects, for a number of years, although the situation may change with the recent introduction of the non-peptide antagonist naltrindole [112], which is reported to be more than 150 fold selective for the delta receptor.

The regional distribution of mu and delta receptors has also been shown to differ considerably, with higher [3 H]naloxone binding levels in both thalamus and striatum, compared with those seen with [3 H]DADLE, where receptor density was maximal in frontal cortex [14]. Autoradiographic studies of [3 H]DPDPE and [3 H]GLYOL binding in rat brain also showed the mu receptors to be localised mainly in cortical layers 1 and 4, thalamus, amygdala and the peri-aqueductal grey, whereas [3 H]DPDPE binding was heaviest in the other areas of the cortex, the caudate putamen and the amygdala. Distribution within the amydala also varied for these two ligands.

Investigations into the functional effects of delta receptor activation have been hampered by the lack of stable, potent and specific delta agonists. Evidence from a number of studies suggests that delta agonists may mediate analgesia following intrathecal injection [126, 144, 121]. However, efficacy has not been demonstrated, following either systemic or i.c.v. administration, at doses low enough to exclude the possibility of a mu receptor interaction [65, 13], and the physiological role of this receptor

therefore remains unclear.

B. THE KAPPA RECEPTOR:

Although the delta receptor was the first opiate receptor subtype to become a proven entity, the idea of multiple opioid receptors had in fact been proposed almost ten years earlier, by Martin [93], as a result of the properties of nalorphine. This agent was first synthesized in 1941 [88], and was shown both to antagonise the effects of morphine [144], and to possess antinociceptive properties in its own right [54]. Further investigation of the actions of various combinations of morphine and nalorphine, did not however produce the pattern of effects that would have been expected from an agonist/partial agonist mixture. Instead, the dose response curves obtained were biphasic [58, 153]. A similar pattern was also seen with levorphanol/levallorphan combinations. Martin suggested that these effects could best be explained on the basis that nalorphine was acting as an antagonist at the morphine receptor, but possessed agonist activity at a second receptor. He called this effect "receptor dualism", and referred to the compounds involved as agonist-antagonists, to distinguish them from partial agonists. Evidence for receptor dualism continued to accumulate, and in addition it was shown that higher doses of naloxone were needed to block the agonist effect of nalorphine, than of morphine itself.

In 1976 Martin et al. [92] published their classical paper on the effects of opiates in the chronic spinal dog model. Using this preparation they were able to identify 3 distinct syndromes produced by morphine, the benzomorphan drug ketocyclazocine, and SKF-10047, which they attributed to 3 receptor types, mu (morphine), kappa (ketocyclazocine) and sigma (SKF-10047). In a further study [41] this group also demonstrated a lack of cross-tolerance between morphine and ketocyclazocine <u>in-vivo</u>, thus strengthening the case for the involvement of separate receptors.

In parallel with these developments, unusual results had also been obtained with the benzomorphan drugs in the in-vitro models. In two

similar studies Hutchinson et al. [62] and Kosterlitz et al. [74] showed that higher concentrations of naloxone were needed to reverse the effects of agents such as nalorphine, diprenorphine, Mr 2034 and ethylketocyclazocine (EKC), on both the GPI and the MVD preparations. These compounds were also significantly less active on the MVD than would have been predicted from their GPI potencies.

Once the benzomorphan drugs had been identified as possible kappa receptor agonists, investigations into their <u>in-vivo</u> effects rapidly established that they had properties distinct from those of the mu agonists such as morphine. Kappa agonists neither supported dependence, nor precipitated abstinence in the morphine dependent monkey [140], but were nevertheless potent analgesics in a range of animal models [149]. Tolerance to the effects of kappa agonists was shown [41], although the abstinence syndrome was different from that produced by morphine, and could only be precipitated by high doses of naloxone [31]. In addition, kappa agonists produced a specific range of side-effects, including dysphoria, sedation and ataxia [91].

Although the identification of the kappa receptor did not follow directly from the discovery of the relevant natural agonist, the isolation of dynorphin A [48], the first endogenous peptide with selectivity towards the kappa receptor, did a great deal to strengthen the case. This substance was shown to produce potent effects in the GPI, which could be reversed only by high concentrations of naloxone. A number of studies were subsequently performed, using the peptide fragment dynorphin A (1-13), which showed a lack of cross-tolerance with mu and delta agonists such as normorphine, fentanyl and DADLE, in in-vitro models. EKC tolerant tissues however, did show reduced effects in the presence of dynorphin A (1-13) [152,127,17]. In addition, Chavkin and Goldstein [17] showed that both EKC and dynorphin A (1-13) were able to protect a similar site from inactivation by the non-selective opioid receptor alkylating agent B-chlornaltrexamine, in GPI tissues.

These results confirmed that the dynorphin peptides were likely to be acting through the kappa receptor. However, dynorphin A (1-17) and

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PRODYNORPHIN



<u>Figure A</u> Arrangement of the various peptides within the precursor molecule (Epper et al TINS 1986 p464)

the fragment dynorphin A (1-13) have since been shown to be members of a larger group of kappa selective peptides, also including dynorphin B and a-neoendorphin, all of which are contained within a single 265 amino-acid dynorphin precursor protein, first isolated by Kakidini et al. in 1982 [68] (see figure A). <u>in-vitro</u> studies have shown that the shorter fragments, dynorphin A (1-8) and (1-9), although unstable have the greatest selectivity for the kappa receptor [25]. The exact nature of the endogenous ligand for this site is therefore still not entirely clear.

The first benzomorphan drug to be labelled for use as a binding ligand for the kappa receptor was $[^{3}H]EKC$. This compound was used by Kosterlitz's group in 1981-82, in an extensive study characterising kappa receptor binding in guinea-pig whole brain tissue [77,90]. In the first of these papers [³H]EKC binding in guinea-pig whole brain membranes was shown to be heterogeneous, with binding to both high and low affinity sites. Displacement of bound [³H]EKC by DHM. morphine and normorphine resulted in very shallow curves. In the case of the very selective mu agonist GLYOL these were clearly biphasic, and could be separated into two distinct portions, with affinities of 4.6nM and 4960nM respectively. A similar effect was also obtained with the delta peptide DADLE, but not with the non-selective agonist etorphine. The affinities of all these agents for the first phase of [³H]EKC displacement were in line with those obtained against [³H]DHM binding, indicating that this site was likely to represent a mu receptor. However, 30 times more DHM than EKC was required to protect 50% of ³H]EKC binding-sites from the effects of the irreversible ligand phenoxybenzamine, and 4900nM DADLE was able to protect only 20-40% of the binding, although EKC was equipotent with DHM in protecting [³H]DHM sites, and only slightly less potent than DADLE in protecting ³H]DADLE binding. These results showed that EKC was able to bind with high affinity to both mu and delta receptor sites. However, the biphasic displacement curves to GLYOL and DADLE, and the inability of DHM and DADLE to fully protect [³H]EKC binding from inactivation, also provided evidence for the existence of a separate kappa receptor binding site in guinea-pig whole brain tissue.

The non-selective nature of EKC as a opioid receptor binding ligand was confirmed in the second of the guinea-pig whole brain studies, by Magnan et al., with Ki values of 1.00 and 5.5nM respectively against $[^{3}H]$ GLYOL and $[^{3}H]$ DADLE binding. In order to obtain a degree of specificity for the kappa receptor the mu and delta components of ³H]EKC binding were therefore suppressed by the addition of high concentrations of unlabelled mu and delta ligands. This approach, which is dependent on the very low kappa receptor affinities of GLYOL, DADLE and other delta selective peptides, [116] has since been used with considerable success for a number of unselective opioid receptor ligands. Under these conditions [³H]EKC binding was monophasic, with an affinity of 0.62nM, and the maximum number of binding-sites was reduced from 12.7 to 6.0 pmoles/g tissue. The approximate proportions of the 3 opioid receptor subtypes in guinea-pig whole brain were judged by the authors, on the basis of these two papers, to be 25% mu, 45% delta and 30% kappa.

A similar pattern of activity was also obtained with the benzomorphan ligands in rat brain, although the opioid receptor proportions were markedly different. Using the then newly available benzomorphan ligand [3 H]bremazocine, in the presence of 100nM GLYOL and DADLE, to label the kappa receptor, and [3 H]GLYOL and [3 H]DADLE to label the mu and delta receptors respectively, Gillan and Kosterlitz [44] obtained receptor proportions in rat brain of 46% mu, 42% delta and 12.5% kappa. Comparison of [3 H]EKC and [3 H]bremazocine as kappa ligands in this tissue indicated similar levels of cross-reactivity with mu and delta sites, although the kappa receptor affinity of [3 H]bremazocine was higher than that of [3 H]EKC.

Chang and Cuatracasas [15] obtained generally similar results to Kosterlitz et al., using rat brain membranes and the non-selective antagonist ligand [³H]diprenorphine. The simultaneous addition of DADLE and the mu selective peptide morphiceptin, at concentrations occupying 98% of mu and delta receptors, only partially inhibited the specific binding of [³H]diprenorphine, indicating that a third site was likely to be involved. This site had high affinity for several benzomorphan drugs such as cyclazocine, EKC and SKF10047, but the

receptor numbers were however again very low compared with the kappa receptor densities obtained by Kosterlitz et al. in guinea-pig whole brain.

The distribution of the three opiate receptor types across 6 brain regions was also found to differ between the guinea-pig and the rat [117]. In the guinea-pig, the highest levels of mu receptor binding were found in striatum, midbrain and hypothalamus, whereas in the rat, mu binding in the hypothalamus was 40-50% lower than in striatum. Kappa receptor binding levels in all regions of the rat brain except for hypothalamus, were low compared with those seen in the guinea-pig, with the kappa receptor found predominantly in the cortex and striatum of the guinea-pig, and in the midbrain, hypothalamus and striatum of the rat.

Kappa receptor binding levels in human, rabbit and mouse brain have subsequently been shown to be closer to those obtained in the guinea-pig [117] and do not reflect the very low levels seen in the rat, suggesting that this species has unusually low kappa receptor densities. In contrast, a very high proportion of kappa receptors, greater than 84% of the total opioid receptor population, has been discovered in membranes prepared from the cerebellum of the guinea-pig [116], making this the tissue of choice for the study of kappa receptor binding.

Following the development of the first suppressed benzomorphan kappa binding assays, a number of agents were shown to have high affinity for this receptor. In particular, the dynorphin peptides were able to displace suppressed [3 H]bremazocine binding in guinea-pig brain membranes with affinities in the range 0.04 to 9.4nM. The longer fragments (dynorphin A (1-13), dynorphin A (1-17) and a-neoendorphin) were the most active, but the shorter peptides, notably dynorphin A (1-9), were more selective for the kappa receptor [25]. The use of these agents, particularly as labelled ligands has however been quite severely limited by the lack of stability, particularly of the shorter fragments [45]. Assays involving the dynorphin peptides are therefore usually carried out at 0^oC, in the presence of peptidase inhibitors,

in order to limit the breakdown, as far as possible.

Other more promising kappa receptor ligands have emerged over the years. The very selective agonist ligand [³H]U69593 and its analogue [³H]PD117302, recently released by Upjohn and Parke Davis [81,22] are likely to prove very useful in the further study of the kappa receptor, and should remove the need for mu and delta suppressing agents in these binding systems. Two new antagonists, binaltorphamine and nor-binaltorphamine, have also become available. These agents have been shown to be 20-50 fold selective for the kappa receptor, thus facilitating the verification of kappa receptor involvement in both in-vivo and in-vitro assays [111].

C. THE SIGMA AND PCP RECEPTORS:

The existence of the opiate sigma receptor was first postulated by Martin et al. in 1976 [92], and was followed by a further study [41] in which cyclazocine was found to produce tachycardia, mydriasis and delerium in the chronic spinal dog model, effects which were not observed with either morphine or EKC. In addition both morphine and EKC were able to produce analgesia and hypothermia in the rat [149], whereas SKF10047 was not.

The psychotomimetic syndrome seen with cyclazocine and SKF10047 in a number of mammalian species, including humans, was identified as being very similar to that produced by phencyclidine (PCP). This agent was first introduced into clinical practice in 1958 as a potent and fast acting anaesthetic [154]. However it was subsequently withdrawn because of symptoms such as hallucinations, maniacal excitement and "drunkenness" and agitation, experienced by patients coming out of anaesthesia [67]. Similar effects were also seen with the less potent congener ketamine. PCP is now a major drug of abuse.

Early binding studies in brain tissue, in which either [³H]PCP or [³H]SKF10047 were used as the labelled ligands, also suggested that all these agents were acting through the same binding site, and that a specific "sigma" receptor site was responsible for the observed

behavioural effects. Careful analysis of the binding data, and the development of more selective ligands has however led to the identification of two separate binding-sites for these agents, termed respectively PCP and sigma receptors.

The binding profiles obtained for these two sites have been shown to be somewhat similar, with submicromolar affinities for both PCP and the benzomorphans [83]. These agents do however reveal a degree of selectivity, the PCP site showing a higher affinity for PCP than for SKF10047, and the sigma site binding benzomorphans with higher affinity than PCP. The main pharmacological distinction between these two binding sites lies in the high affinity of the sigma receptor for the dopamine D₂ antagonist drug haloperidol, and several other classes of neuroleptic drug, whereas the PCP binding site is insensitive to these agents [82,155].

Although most of the classic opioid drugs, including naloxone, morphine, levorphanol, met-enkephalin and B-endorphin, [154,139,141,146] have negligible affinity for the PCP and sigma binding sites, many of the agents which do interact with these sites also show some cross-reactivity with the classical opioid mu, delta and kappa receptors [141]. However, both these sites show an anatomical distribution and a pharmacological profile that clearly differentiates them, both from each other, and also from the opioid receptors [136]. In particular, most of the compounds with affinity for both sets of sites show reversed stereoselectivity at the PCP and sigma sites, with the (+) enantiomers showing greater activity than the (-) [82,83], whereas the opposite effect is seen at the opioid receptors.

(en'refloring)]

Although the exact nature of the PCP and sigma sites is not yet resolved, it would therefore appear likely that the only reason they were ever included amongst the opioid receptors was due to the cross-reactivity of some racemic benzomorphans. Since these are now mostly available in resolved form, these sites are unlikely to present any further problem in opioid receptor binding assays.

- REDAY

3. THE EFFECTS OF IONS AND NUCLEOTIDES ON OPIATE RECEPTOR BINDING

A. THE Na⁺ EFFECT:

The first report of ionic effects on opiate receptor binding was published by Simon et al. [132] in 1973, as part of the paper describing the binding of [3 H]etorphine to rat brain membranes. Specific [3 H]etorphine binding was shown to be quite sensitive to ionic strength, and was decreased by high concentrations of NaCl and KCl, both of which were apparently equally effective. Pert and Snyder, in the same year however [105] reported no effect of Na⁺ or K⁺ on [3 H]naloxone binding in rat brain at physiological concentrations, although at levels greater than 500mM, both these ions produced a gradual decrease in binding.

These initial papers were followed by a series of much more detailed studies, in which the differential nature of the effects of Na⁺ on agonist and antagonist binding were clearly shown [108]. Incubation of rat brain membranes with 100mM Na⁺ reduced the binding of opiate agonists [³H]levorphanol, [³H]oxymorphone and [³H]DHM by between 30% and 70%, whereas the binding of the antagonists [³H]levallorphan, [³H]naloxone and [³H]nalorphine was increased by 30-140%. These effects were specific to Na⁺, and to some extent Li⁺, but were not elicited by other monovalent or divalent cations, suggesting that Na⁺ may interact with sites on the membrane which can allosterically transform the opiate receptor. Saturation curves to [³H]naloxone and [³H]DHM were interpreted by the authors as indicating that the observed changes in binding levels were due to alterations in receptor number, rather than affinity.

The addition of Na⁺ also had no significant effect on the IC_{50} s of a range of antagonist ligands, as displacers of [³H]naloxone binding. Agonist inhibitory potency was however reduced by a factor of 12-133 in the presence of Na⁺, and only very small shifts were seen with a number of partial agonist ligands. The size of the "Na⁺ shift" seen with the agonists and partial agonists was shown to correlate well with their relative agonist/antagonist properties "<u>in-vivo</u>". Similar

results were also obtained by Simon et al. [133], with the most pronounced effects on both [³H]naloxone and [³H]etorphine binding occurring between 100-200mM Na⁺, whereas KCl produced a small depression in the binding of both ligands. The effects of Na⁺ on both the agonist and antagonist ligands were also shown to be fully reversible, up to 200mM. However, saturation curves to [³H]naloxone in the presence and absence of Na⁺, showed the increase in binding to be due to a higher affinity, rather than the unmasking of new sites as suggested by Pert et al. [³H]etorphine saturation curves obtained in the presence of Na⁺, similarly showed a decrease in affinity in this study, rather than in the number of sites. However, in the presence of 150mM Na⁺, [³H]etorphine was seen to bind to both low and high affinity sites, an effect not seen under control conditions. At very high Na⁺ concentrations, all binding was to the low affinity site, with an affinity of 3-4nM. This shift in affinity from 0.7 to 4nM was also in good agreement with the results of competition experiments involving etorphine as the displacing ligand.

This work was extended by Pasternak and Snyder [101] in another paper published the same year. This group found that the binding of [³H]naloxone could be resolved into two components, with Kd values of 0.4nM and 30nM respectively, both in the presence and absence of Na⁺. The effect of Na⁺ in this case was interpreted as a doubling in the number of high affinity sites, whereas the low affinity sites were unaffected, with no significant change in the Kd values. A similar picture was also obtained with [³H]DHM, with Kd values of 0.3nM and 3nM for the high and low affinity components. In this case Na⁺ appeared to virtually abolished binding to the high affinity site, but again, the low affinity sites were unaffected.

On the basis of these results the authors proposed that the opiate receptor was capable of existing in two distinct but interchangeable conformations, the antagonist or Na⁺ form, for which antagonists have the higher affinity, and the agonist no Na⁺ form to which agonists would preferentially bind. Pharmacological activation was considered to require binding to the agonist form, and Na⁺ was thought to reduce agonist binding by converting receptors to the antagonist state. This

conclusion received considerable support from experiments involving protein modifying agents with effects on sulphydryl groups [100]. These agents, including iodoacetamide, N-ethylmaleimide (NEM) and mercuriacetate, were shown to strongly inhibit [³H]DHM binding to rat brain membranes, at concentrations that did not alter [³H]naloxone binding, an effect that could be prevented by prior treatment of the membranes with opiate drugs. This loss of agonist binding was coupled with an increased sensitivity to the inhibitory effects of Na⁺, suggesting a specific binding site for this ion on the opiate receptor. In addition the rate of inactivation of opiate receptor binding by NEM was markedly slower in the presence of 100mM Na⁺, an effect that was not seen on the alkylation rate of model sulphydryl modifying agents, and that was best explained in terms of a conformational change, in the presence of Na⁺ ions, rendering the SH groups on the receptor less susceptible to alkylation.

B. THE GUANINE NUCLEOTIDE EFFECT:

The first attempts to discover the effects of nucleotides such as guanosine triphosphate (GTP) on cell surface receptor binding systems were prompted by the growing realisation of the importance of the GTP binding proteins as vital links between receptors and a number of second messenger systems, Pincluding cAMP generation, the regulation of intracellular calcium and the gating of ion channels [119). In 1971 Rodbell et al. [118] identified GTP as an essential component in the transduction mechanism leading to the generation of cyclic AMP. The sequence of events involved in this process have since been elucidated by Gilman et al. [46] and appear to involve two separate GTP binding proteins, one inhibitory (Gi) and one stimulatory (Gs), capable of linking the receptor to adenylate cyclase, the enzyme responsible for cAMP production. Binding of an agonist to the cell surface receptor induces a conformational change, which is transmitted to the G-protein, making it reactive to GTP, which approaches from inside the cell. The binding of GTP to Gs or Gi then produces a further conformational change, and constitutes an "on" reaction that allows the G-protein to interact with adenylate cyclase, thus reducing or increasing cAMP synthesis, depending on the nature of the elements

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- Rs Receptor linked to the stimulation of adenyiate cyclase
- As Agonist acting at Rs
- Gs Stimulatory GTP binding protein
- AC Adenylate cyclase

- Ri Receptor linked to the inhibition
 of adenylate cyclase
 Ai Agonist acting at Ri
- GI Inhibitory GTP binding protein

Figure B: Relationship between the various elements of the receptor activated adenylate cyclase system, within the plasma membrane.

involved. The activity of the G-protein-GTP complex is terminated by hydrolysis of the bound GTP molecules to GDP by a GTPase activity present in the G protein itself, and the complex returns to the resting-state (see figure B).

The effects of GTP on opiate receptor binding have been studied both in brain tissue and membranes prepared from a mouse neuroblastoma/rat glioma hybrid cell line (NG108-15). The work was largely prompted by the discovery that morphine was able to inhibit the PGE₁ stimulated accumulation of cAMP in these cells, [128] and also by reports of a similar opiate mediated inhibition of basal and PGE₁ stimulated adenylate cyclase activity in brain tissue [23].

NG108-15 cell membranes were shown to possess a large population of opiate receptors, detectable by both [3 H]DHM and [3 H]naloxone binding [72], and displacement of [3 H]DHM binding was in line with the affinity profile expected for a mu or delta receptor [71]. Opiate mediated inhibition of basal and PGE₁ stimulated adenylate cyclase activity both in homogenates and whole cells was naloxone reversible and the effectiveness of a range of opiates as inhibitors of cyclase activity correlated well with their affinities as displacers of [3 H]naloxone binding in the same preparation [128,96].

The early work on opiate inhibited cyclase activity in brain homogenates [23,147] however, proved difficult to repeat, and a number of conflicting reports appeared in the literature, claiming either that opiates stimulated the enzyme [34,113,] or had no effect at all [69,142]. In two slightly later papers however [84, 4] opiate inhibition of PGE₁ stimulated adenylate cyclase both in rat striatal slices and homogenates was clearly demonstrated. In the slice preparation inhibitory effects were seen with morphine, levorphanol and EKC at concentrations in line with their activity against [³H]etorphine binding in rat brain. These effects could be reversed by naloxone, naltrexone and the benzomorphan Mr2266, and no activity was seen with dextrorphan, the inactive isomer of levorphanol. Similar effects were also seen in the homogenate preparation, using either leu-enkephalin, [23,24] [D-Ala²,Met⁵]enkephalin or morphine as

the inhibitory agent, and the order of potency obtained, (Met-enk. > Leu.enk > B-endorphin) was suggested by the authors to imply an action through the delta receptor. The activity of Leu-enkephalin in the homogenate preparation was also shown to be completely dependent on Na^+ and GTP, an effect not noted in any of the earlier papers, where broken cell preparations had been used, but subsequently confirmed by Cooper et al. [24], again in homogenates of rat striatum, and also by Blume et al. [8] in NG108-15 cell homogenates.

Agonist binding to the opiate receptors present on NG108-15 cells was shown to be influenced by both ions and nucleotides [7]. The binding of $[{}^{3}H]$ Leu-enkephalin in this tissue was decreased by both Na⁺, GTP, GDP and the stable GTP analogue GppNHp. In rat brain membranes Na⁺ and GppNHp were shown to reduce the steady state binding of [³H]DHM [8]. by producing an increase in the dissociation rate of the ligand from its receptor. Similar effects were seen with other agonists, including $[{}^{3}H]$ etorphine, and also with the antagonist $[{}^{3}H]$ naltrexone. Zukin et al. [156], in a study on the effect of GTP on agonist and antagonist binding in rat brain found that GTP alone reduced [³H][D-Ala²-Met⁵]enkephalinamide (DALA) binding by between 28-37% in all regions examined. In the presence of Na⁺ the inhibition increased to approximately 85%. Small reductions in [³H]naloxone binding were also seen in the presence of GTP. However, in contrast to the picture seen with $[{}^{3}H]DALA$, this effect was largely reversed by the addition of Na⁺.

The differential effects of GTP on agonist and antagonist binding were studied in greater detail by Childers et al. [19,20], who confirmed that in the absence of Na⁺, 50 μ M GTP decreased agonist and antagonist binding by 20-60% and 0-20% respectively. The addition of 100mM Na⁺ abolished the effect of GTP on [³H]antagonist binding, in line with the findings of Zukin et al., but led to further reductions in agonist binding, of up to 90% in some cases. Similarly, displacement of [³H]antagonist binding by antagonist ligands was not affected by Na⁺ or GTP, whereas agonist affinities were markedly reduced.

Pretreatment of rat brain membranes with NEM was shown to reduce both

agonist binding and the effect of GTP on binding levels [21]. These effects could only be partially prevented by the addition of either agonist or GTP to the membranes. However, in the presence of agonist ligand and Na⁺ ion, both GTP regulation and the binding site were fully protected from NEM mediated inactivation. It appeared that although the GTP and agonist binding sites were clearly separate, some kind of allosteric interaction occurred between the two following either agonist or nucleotide binding, and that Na⁺ was involved in the coupling of this process.

C. DIVALENT CATION EFFECTS:

The effects of divalent cations, particularly Mn^{2+} and Mg^{2+} , on opioid receptor binding have also been studied. Mn^{2+} ion enhanced the binding of [³H]opiate agonists in rat brain membranes, whereas antagonist binding was unaffected. Similar but smaller effects were also seen with Mg^{2+} and Ni^{2+} [102]. Mn^{2+} also increased the ability of unlabelled agonists to displace [³H]antagonist binding, suggesting an effect on ligand affinity. These actions were most pronounced in the presence of Na^+ .

 Mg^{2+} ions were also found to increase steady-state binding levels in NG108-15 cell membranes [7], and Mn^{2+} ions appear both to decrease the dissociation rate of a range of ligands in brain tissue, and to reverse the effects of Na⁺ [8]. Mg^{2+} and Mn^{2+} ions have been shown to be involved in the regulation of adenylate cyclase [124], and it is likely therefore that they play some part in the allosteric regulation of agonist receptor binding.

D. MODELS OF AGONIST BINDING:

No clear picture has yet emerged, as to the mechanism through which Na⁺, the divalent cations and the guanine nucleotides act to regulate the coupling between the agonist-receptor complex, and the relevant G-proteins. However a number of theories have been proposed to account for the various affinity states of the receptor produced by these agents.

Studies involving the B-adrenergic receptor have led to the development of a model explaining the interaction of GTP with the binding site [138]. Many of the effects of GTP were similar to those seen at the opiate receptor, in that antagonist displacement of [³H]antagonist binding was steep and monophasic and could be described according to the law of mass action. These curves were unaffected by GTP or its stable analogues. Agonist displacement however, was shallow and complex, and best fitted by a model defining two classes of binding site, with high and low affinities respectively, for the agonist ligand. In the presence of GTP, agonist displacement curves_ were steepened and shifted to the right, apparently due to the conversion of all the receptors to the low affinity form. The binding characteristics of a series of partial agonists were similar to those of full agonists, but less pronounced, with the proportion of receptors in the high affinity state, and the differences in affinity for the two states, increasing with the degree of intrinsic activity.

On the basis of these results the authors suggested that "the unique property of agonists and partial agonists was their ability to induce, stabilise, or recognise a high affinity form of the receptor", which was in some way assumed to be involved in the activation of adenylate cyclase. They proposed a simple model capable of explaining their findings:



Activation of cyclase.

H - Hormone

- R Receptor
- X G Protein

K, M, K' and L are the dissociation constants of the various equilibria.

In this scheme, the initial formation of a low affinity HR complex was thought to be followed by a further interaction with X, to form the high affinity ternary complex HRX, depending on the agonist activity of the ligand. This intermediate complex was proposed to be a transient but necessary step in the coupling of the receptor to adenylate cyclase, and was destabilised by GTP, in association with the activation of the enzyme.

Evidence obtained from a number of sources supported this conclusion. For instance Ross et al. in 1977 [123] demonstrated that wild-type S49 lymphoma cells possessed B-adrenergic receptors coupled to adenylate cyclase and that agonist binding showed the characteristic shallow displacement curves. In a variant of the cell line deficient in G-protein however, agonist competition curves were steep, and were not affected by guanine nucleotides. These cells also did not show receptor linked adenylate cyclase activity, suggesting that the G-protein was involved in the formation of some kind of complex necessary for the activation of the enzyme. In another study the size of the solubilised B-receptor complex was shown to be larger when the receptors were first labelled with an agonist ligand, and formation of the agonist-receptor complex before solubilisation was associated with the co-elution of the G-protein with the HR complex on further purification [85].

A separate model was proposed in 1978 by Birdsall et al. [6], to explain the complexities of agonist binding at the muscarinic receptor. Flattened agonist/[³H]antagonist competition curves were obtained at the muscarinic receptor by this group, in membrane preparations both from the brain and smooth muscle of several species, and from cloned neuroblastoma cells. These results were interpreted in terms of two major populations of binding sites, H and L, with high and low affinities respectively for agonist ligands, which did not interconvert during the binding experiments, and had the same affinity for antagonists. The possibility of co-operative interactions between
these sites was ruled out by receptor occlusion experiments in which blockade of the majority of the binding-sites by an irreversible antagonist was shown to have no effect on the slope of agonist competition curves.

Based on quantitative correlations suggesting that the L receptor sites were likely to be the most relevant to contractile responses in smooth muscle, the following scheme was proposed, in which the L receptors were considered to have their effective affinity constants reduced by conformational coupling to an effector grouping, whereas binding to the H receptors was not constrained in this way:

 $Kh K_{2}$ $A + R \geqslant AR \geqslant AR' H-SITES$

K1 QK_2 A + RE \geq ARE \geq AR'E* L-SITES

A = LIGAND

R = RECEPTOR

E = EFFECTOR GROUP

@ <<1

Kh, Kl, K_2 and $@K_2$ = Dissociation constants of the various equilibria.

K2 > 0 for agonists and = 0 for antagonists.

Muscarinic receptor activation has been shown to inhibit adenylate cyclase activity in a number of systems, including NG108-15 cells and myocardial homogenates, and in all cases where the question has been investigated, this effect has been shown to be dependent on GTP [36]. In initial studies on the influence of guanine nucleotides on

muscarinic receptor binding in rat myocardium, the affinities of the agonists oxotremorine and carbachol as displacers of $[{}^{3}H]$ antagonist binding were decreased 10-12 fold in the presence of GTP or GppNHp [146,120]. This effect of the guanine nucleotides was shown by experiments on EDTA treated membranes to be Mg²⁺ dependent, and similar modulations of muscarinic binding were also seen in smooth muscle and various brain regions, although the effects were less marked than in heart tissue.

The magnitude of the guanine nucleotide effect on agonist binding was found to be directly related to the efficacy of the ligand involved, consistent with the idea that in the presence of the guanine nucleotides a selective conversion of H to L sites was occurring. Thus for highly efficacious agonists, with large differences between their Kh and Kl values, the guanine nucleotide induced shift from H to L produced a relatively larger increase in IC₅₀ values, whereas for partial agonists the reduction was smaller. It is likely therefore that the guanine nucleotides are involved in the coupling of the effector unit E (possibly Gi) to the AR complex, although this is not explicitly stated in the description of the model.

This model fits the observed binding data better than that of Leftowitz et al., in that it does not propose agonist induced co-operative changes in receptor conformation, which, in the absence of GTP, do not appear to occur during standard equilibrium binding assays. It does not however provide any explanation for the role of the high affinity site in the cycle of activation. The H binding sites may simply represent a pool of inactive or uncoupled receptors, however the evidence from the ß-adrenergic receptor studies does suggest that high affinity agonist binding is a necessary step in the coupling of the receptor to its effector unit, although <u>in-vivo</u> it may represent a transient high energy state.

The question of which of these receptor conformations is the most physiologically relevant, or best represents the affinity state seen by an agonist ligand <u>in-vivo</u>, has been addressed at the opiate receptor in a number of ways. Creese and Snyder, in an early opiate

receptor binding study [32], compared the binding affinities of a wide range of opiate agonists and antagonists in membranes prepared from GPI muscle, with their ability to inhibit electrically induced contractions in the same tissue. They obtained an excellent correlation between these two parameters when the binding assay was run in Krebs buffer containing a high concentration of Na^+ ions, suggesting that the low agonist affinity conformation was most likely to be the state involved in the pharmacological action of opiate drugs in this assay. The potency of opiates in an <u>in-vivo</u> test, the measurement of antidiarrhoeal potency, also showed a significant correlation with opiate receptor binding to brain membranes when Na^+ ions were included in the incubation medium [137]. However in both these and other studies, agonist binding affinities have been compared with pharmacological potency, rather than <u>in-vitro</u> measurements of affinity, making genuine comparison difficult.

Because of the presence of "spare receptors" [70] in many tissue preparations, full agonists frequently produce their maximal pharmacological effects at only very low levels of receptor occupancy, thus complicating the estimation of their affinities. These problems have however been overcome, in a paper by Carroll et al. [10] in which agonist affinity constants at the opioid mu receptor were obtained, in the GPI preparation, by the receptor occlusion technique of Furchgott [38]. The affinities of a wide range of partial agonists were also determined by antagonism of the mu agonist GLYOL in the rat vas deferens preparation, a tissue known to possess an extremely insensitive population of mu receptors, in which only full agonists are able to produce a pharmacological effect. The values obtained from these two preparations correlated extremely well with those from a mu specific binding assay using the antagonist ligand [³H]naloxone, and run in Krebs buffer containing GppNHp. There was however no correlation between either of these parameters and a high affinity mu specific assay using the high affinity agonist [³H]GLYOL, run in a non-ionic buffer system, suggesting that in the case of the mu receptor, the low agonist affinity conformation is likely to be the most physiologically relevant. The same conclusion was also reached by Birdsall et al. in their studies on the muscarinic receptor. IC₅₀

values for contractile responses on smooth muscle were similar to binding affinities at the H site of the receptor. However where data was available on the activity of agonists on smooth muscle after the elimination of spare receptors, the affinity values obtained correlated reasonably well with the affinities of these agents at the muscarinic L site [6] in binding assays. The ratio of IC_{50} /Ke value, taken as a measure of efficacy, also correlated with the ratio of the affinities at H and L receptor sites, suggesting that the high affinity binding site might be in some way connected with intrinsic activity, as suggested in the Leftowitz et al. *B*-adrenergic receptor model.

Much of the work on the effects of ions and nucleotides on opiate receptor binding does not take account of the existence of the separate opiate receptor subtypes. Where mu, delta and kappa receptors have been studied separately, regulation of binding of the type described here has been found to occur at all three sites, although some differences do emerge.

Kouakou et al. [79] found that Na⁺ ions produced similar inhibitory effects on the binding of both mu and delta agonists to rat brain membranes. Binding of [³H]DHM to the mu receptor was however inhibited by divalent cations such as Mn^{2+} , whereas that of [³H]DADLE to the delta receptor was enhanced. The steady state binding of [³H]GLYOL to the mu receptor was shown to be decreased by GppNHp, whereas that of the delta ligand [³H]DSLET was unaffected [156]. In the presence of Na⁺ however, GppNHp reduced the specific binding of both ligands in a concentration specific manner. In rabbit cerebellum, a tissue where greater than 80% of the opioid receptors are of the mu type the binding of mu agonists was again inhibited by Na⁺, but in this study MnCl₂ produced a potentiation [78]. Inhibition of agonist binding by Na⁺ at all three opioid receptor sites was demonstrated by Paterson et al. [103] using a number of selective agonist ligands, in both guinea-pig whole brain and guinea-pig cerebellum tissue. Binding at the kappa site was inhibited by MnCl, and MgCl, in this study, whereas delta binding was enhanced, and a biphasic effect was seen at the mu receptor, with inhibition at

concentrations above 1mM. Maximum potentiation of delta binding was seen at 2mM MnCl₂ and 1-2 mM MgCl₂.

Overall the ions and nucleotides studied produced broadly similar effects on agonist binding to the mu and kappa receptors, although divalent cations were able to potentiate mu receptor binding at low concentrations. At the delta receptor however, the consistent potentiation of agonist binding seen in the presence of Mg^{2+} and Mn^{2+} suggests that the regulation of agonist binding at this site may be different, certainly from that of the kappa receptor. This is interesting, in view of the fact that the opiate receptor which mediates the inhibition of adenylate cyclase in the NG108-15 cell line has been identified as a delta receptor [42], and some studies have also suggested that the opiate receptor linked effects on adenylate cyclase in brain homogenates are mediated through the delta receptor [24]. Certainly agonist binding in NG108-15 cells is clearly potentiated by Mg^{2+} and Mn^{2+} [7].

There is no clear evidence however of a mu or kappa receptor mediated effect on adenylate cyclase activity or cAMP levels. North et al. [98] have shown that the activation of mu or delta receptor types produced an increase in a potassium conductance of the neuronal membrane, which was possibly identical to that activated by alpha 2 adrenoceptors, also negatively coupled to adenylate cyclase. Activation of kappa receptors however produced a reduction in a voltage dependent Ca²⁺ conductance. Receptors of the kappa type were shown to coexist with either mu or delta receptors, and in both cases stimulation led to a reduction in the rate of neuronal discharge and in the amount of transmitter released by each action potential. Opiate receptor stimulation has also been shown to lead to a reduction in intracellular Ca²⁺ [149], which may account for the reduced neurotransmitter release, however the exact mechanisms of the effects, and the nature of the second messenger linkages of the mu and kappa receptors remains unclear.

4. AIMS OF THE STUDY:

The aim of this study was to establish the effect of a physiological buffer system, containing the full range of salts usually present in the extracellular medium, on the binding of opioid ligands at the kappa receptor. The study took the form of a detailed investigation of kappa receptor binding, including an assessment of the contribution of both low and high agonist affinity receptor conformations to the binding profile, in both "standard" and "ionic" buffer systems.

Conditions have therefore been chosen that would minimise interference from the other opioid receptor types, without the need for the addition of suppressing agents. The binding of an antagonist ligand to the kappa receptor in HEPES buffer has been compared with a Krebs/HEPES buffer system containing the stable GTP analogue GppNHp. Displacement curves have been generated to a wide range of opioid ligands, in both buffer systems, and the effects of the different buffers on the affinity and complexity of both agonist and antagonist binding analysed in terms of the IC₅₀ values and Hill coefficients obtained under both sets of conditions.

MATERIALS:

1. RADIOCHEMICALS:

[D-Ala²,N-methyl-Phe⁴,Glyol⁵][tyrosyl-3,5-³H]enkephalin ([³H]GLYOL) 30-60Ci/mmol. Amersham International plc.

[D-Ala²-[tyrosyl-3,5-³H]-(5-D-Leucine) enkephalin, ([³H]DADLE) 30-60Ci/mmol. Amersham International plc.

[N-Ally1-2,3-³H]Naloxone 40-60 Ci/mmol. Amersham International plc.

(-)-[9-³H(N)]-bremazocine 15-30Ci/mmol. New England Nuclear.

(-)-[9-³H(N)]-Ethylketocyclazocine 15-30 Ci/mmol. New England Nuclear.

2. OPIATE DRUGS:

(±) Ethylketocyclazocine Methane Sulphonate (EKC) - Gift from Sterling Winthrop.

(±) Bremazocine - Gift from Sandoz.

Naloxone HCl - Dupont.

Naltrexone HCl - Dupont.

Diprenorphine - Gift from Reckitt and Colman

Levallorphan Tartrate - Roche.

Nalbuphine (Nubain R) - Dupont.

(±)Tifluadom HCL - ICI Pharmaceuticals.

N-Methyl-Tifluadom Chloride salt (Q. tifluadom) - ICI Pharmaceuticals.

Pentazocine - Gift from Sterling Winthrop.

Nalorphine Hydrobromide - Gift from Wellcome.

N-Methyl-Nalorphine Chloride salt (Q. nalorphine) - ICI Pharmaceuticals.

Mr2034 - (-)-(1R,5R,9R,2"S)-5,9-dimethyl-2'-hydroxy-2--tetrahydrofurfuryl-6,7-benzomorphan. Boehringer Ingelheim.

U50488 - Trans-3,4-dichloro-N-Methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide Methane Sulphonate. ICI Pharmaceuticals.

U69593 - (-)-(5a,7a,8b)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro (4,5)dec-8-yl] benzeneacetamide. - Gift from Upjohn.

Morphine HCl- MacFarlane Smith.

Etorphine HCL - Gift from Reckitt and Colman.

DMPEA - [D-Met², Pro⁵]enkephalinamide. ICI Pharmaceuticals.

ICI174864 - (N,N-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH). ICI Pharmaceuticals.

ICI154129 - (N,N-Diallyl-Tyr-Gly-Gly-(CH₂S)-Phe-Leu-OH) Arginine salt ICI Pharmaceuticals

ICI200940 - 2-(4-nitrophenyl)-N-methyl-N-[(IRS)-1-(4- hydroxyphenyl)--2-(1-pyrrolidinyl)ethyl]acetamide HCl. ICI Pharmaceuticals.

ICI197067 - 2-(3,4-dichlorophenyl)-N-methyl-N-[(IS)-1-(1-methylethyl)--2-(1-pyrrolidinyl)ethyl]acetamide HCl. ICI Pharmaceuticals.

ICI204448 - 2-(3,4-dichlorophenyl)-N-methyl-N-[(IRS)-1-(3-(carboxymethoxy)phenyl)-2-(1-pyrrolidinyl)ethyl] acetamide HCl. ICI Pharmaceuticals.

ICI204879 - 2-(3,4-dichlorophenyl)-N-methyl-N-[(IRS)-1-(3,4-dimethoxyphenyl) 2-(1-pyrrolidinyl)ethyl]acetamide HCl. ICI Pharmaceuticals. GLYOL - [D-Ala²,N-methyl-Phe⁴,Glyol⁵]enkephalin. ICI Pharmaceuticals. DADLE - [D-Ala²,D-Leu⁵]enkephalin - SIGMA Chemicals. Dynorphin A (1-17) - Cambridge Research Biochemicals. Dynorphin A (1-13) - Cambridge Research Biochemicals. B-Endorphin - Cambridge Research Biochemicals. a-Neoendorphin (Porcine) - Peninsula Laboratories Inc. B-Neoendorphin - Peninsula Laboratories Inc.

MEAGLE - [Met⁵]enkephalin (Arg-Gly-Leu). Cambridge Research Biochemicals.

MEAP - [Met⁵]enkephalin (Arg-Phe). Peninsula Laboratories Inc.

3.OTHER REAGENTS:

(N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) HEPES - SIGMA Chemical Company.

5'-Guanylylimidodiphosphate sodium salt (GppNHp) - SIGMA Chemical Company.

Bestatin HCl - Sigma Chemical Company.

Captopril - Squibb Institute for Medical Research.

NaCl, KCl, KH₂PO₄, MgSO₄.7H₂O, NaHCO₃, NaOH - BDH Analar Grade.

Concentrated NH₄OH (0.91g/ml) - BDH General Purpose Reagents.

Dimethyl Sulphoxide (DMSO) - BDH General Purpose Reagents.

Calcium Chloride Solution (1M) - BDH Analar grade.

Beckmann Ready-solv HP Scintillation counting cocktail.

4.SOLUTIONS:

20mM HEPES buffer, adjusted to pH 7.4 with concentrated ammonia solution and stored at 4° C.

Krebs/HEPES buffer containing NaCl 140mM, KCl 5.5mM, KH₂PO₄ 1.4mM, MgSO₄.7H₂O 1.4mM, NaHCO₃ 30mM, HEPES 20mM, CaCl₂ 3mM, and adjusted to pH7.4 with 2M NaOH.

Standard drug solutions were prepared as 1 or 10mM stock solutions, made up in either distilled water or DMSO and stored at -20°C. Serial dilutions in assay buffer were prepared on the day of test.

Peptide stock solutions were made up in distilled water, divided into small aliquots to prevent repeated freeze-thawing of the same samples, and stored at -20° C. Serial dilutions were made up in assay buffer on the day of test. Contact with glass was minimised and the solutions were kept cold at all times.

High speed centrifuge - Beckman Model J2-21M/E with JA-20 rotor.
Polytron Homogeniser PCU-2 Kinematica.
Glass/Teflon Homogeniser Potter-S Braun.
Millipore Entonnoir and Manifold - 2.4 cm.
Whatman GF/C filter discs - 2.4cm diameter.

1216 LKB Rack Beta II Scintillation Counter.

6.ANIMALS:

Rats - Male, Alderley Park Strain (180-250g)

Guinea-pigs - Male or female Dunkin Hartley strain bred at ICI Pharmaceuticals.

METHODS:

1.MEMBRANE PREPARATIONS:

Beckmann high-speed centrifuge held at 4°C. The supernatant was discarded and the pellet resuspended in the same volome of buffer using a motor driven glass/teflon homogeniser. The preparation was incubated at 37°C for 40 min with occasional stirring, in order to break down or dissociate any endogenous peptides that might otherwise interfere with the binding. The centrifugation step was then repeated and the final pellet resuspended in 10 volumes of ice-cold buffer, as previously described. The membrane preparations were then rapidly frozen down in liquid nitrogen and stored in liquid nitrogen until the day of use.

2. [³H]BREMAZOCINE BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES:

The binding of 0.2nM [³H]bremazocine to guinea-pig cerebellar membranes was studied in either HEPES buffer or Krebs/HEPES + 10µM GppNHp. All determinations were in triplicate, with each tube containing 200µl of membrane suspension (20mg tissue original wet weight). 50µl [³H]ligand and 100µl of naloxone solution, displacing drug, and/or GppNHp, as appropriate, made up to 1ml with buffer. Incubations were started by the addition of tissue, and run for 40 min at 25°C, in a final volume of 1ml. Non-specific binding was defined using 10µM naloxone, and assay tubes measuring both total and non-specific binding were included at the beginning and end of each displacement curve. Assays were terminated by the addition of 5ml of ice cold HEPES or Krebs/HEPES buffer to each reaction tube, followed by filtration through 2.4cm GFC filters, using a millipore single filtration manifold. Each filter was washed twice with a further 5ml of cold buffer, and transferred to a scintillation vial. 4 ml scintillation fluid was added to each vial and the radioactivity bound to the filters measured by liquid scintillation counting using an LKB rack-beta counter, with a counting efficiency of approximately 45%.

3. [³H]BREMAZOCINE BINDING AT 4°C.

[³H]bremazocine binding assays at 4^oC were run for 60 min in HEPES buffer only, using an ice-bath. The breakdown of unstable peptides

was further minimised by the addition of 30μ M bestatin and 300μ M captopril [45]. Where peptide ligands were involved, plastic assay tubes were used. The methods were otherwise similar to those for the [³H]bremazocine binding assay run at 25^oC.

4. [³H]EKC BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES:

 $[^{3}H]EKC$ binding assays were run in HEPES buffer in presence of 0.5nM $[^{3}H]EKC$. The methods were identical to those for $[^{3}H]$ bremazocine binding.

5. SUPPRESSED BINDING ASSAYS.

In these assays high concentrations (defined as appropriate) of the mu/delta ligand DADLE or the kappa agonist ICI 204879 were added to the $[{}^{3}H]$ bremazocine or $[{}^{3}H]$ EKC assays, in order to suppress a proportion of the binding. These agents were also present in the tubes defining the control binding between the displacement curves. Total binding in the absence of suppressing agent was however measured at the beginning and end of each incubation, in order to ascertain the proportion of the specific binding displaced in each case.

In the successive suppression experiments described in the Results, in which $[{}^{3}\text{H}]$ naloxone and $[{}^{3}\text{H}]$ bremazocine binding assays in Krebs/HEPES were run in presence of high concentrations of ICI 204879 and /or ICI 174864, the degree of suppression of binding produced by these agents both separately and together, was defined at the beginning and end of each incubation, and the appropriate additions made to the control binding tubes between the displacement curves.

6. [³H]GLYOL BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES.

 $[^{3}H]GLYOL$ binding assays were run for 40 min at 25°C in HEPES buffer, using 1nM $[^{3}H]GLYOL$. Non-specific binding was defined using 10µM naloxone. Other methodology was similar to that for $[^{3}H]$ bremazocine binding.

7. [³H]DADLE BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES.

 $[^{3}H]DADLE$ binding assays were run for 40 min at 25°C, as described for $[^{3}H]$ bremazocine binding, using 1nM $[^{3}H]DADLE$. Binding to the mu receptor was suppressed by the addition of 100nM GLYOL.

8. [³H]NALOXONE BINDING TO RAT WHOLE BRAIN MEMBRANES.

 $[{}^{3}\text{H}]$ naloxone binding assays were performed in Krebs/HEPES buffer containing 10µM GppNHp in rat whole brain membranes, using 0.2nM $[{}^{3}\text{H}]$ naloxone. Incubations were allowed to run for 40 min at 25°C, and the reaction terminated by the addition of 5ml of ice-cold Krebs/HEPES buffer. All other methods were the same as those for the $[{}^{3}\text{H}]$ bremazocine assay.

9. CALCULATION OF RESULTS.

Overall control specific binding was defined as the mean of the total minus non-specific binding for each incubation. The % inhibition of control binding due to the addition of displacing drug or suppressing agent was calculated for each triplicate according to the formula:

$$100 - (\frac{D - NS}{T - NS} \times 100)$$

Where T = Total counts bound in the absence of displacing agent. D = counts bound in the presence of displacing agent. NS = counts bound non-specifically.

For any given displacing drug the T and NS cpm values immediately preceding and following the relevant displacement curve cpm values were used in the calculations.

In the suppressed binding assays the total binding cpm values used in calculating the % inhibition due to the displacing drug, were determined in the presence of the relevant suppressing agents. However the % reduction in control binding resulting from the presence of the

suppressing agents was calculated using the unsuppressed total binding values included in each incubation.

More detailed statistical analysis of the displacement curve data was performed by linear regression of the Hill plots of the data [57], using a statistical package produced by ICI pharmaceuticals computing department, and run on an IBM p.c. microcomputer.

All the % inhibition values for a particular set of displacement curves were analysed together, and the data converted to the form:

according to the formula of Hill et al. The line of best fit obtained by linear regression analysis was then used to obtain the IC_{50} value (y = 0), Hill coefficient (slope) and confidence limits.

The displacement curves shown in the figures were presented as the % inhibition of binding v.log displacer concentration, with the curves of best fit derived from the Hill plot linear regression lines.

Where data was normalised to a maximum of less than 100 % inhibition of specific binding, this correction was made to the untransformed % inhibition data before the linear regression function was performed.

RESULTS

1. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: HEPES BUFFER SYSTEM:

A: ASSAY VALIDATION:

[³H]Bremazocine binding assays were run in HEPES buffer at pH 7.4, using 0.2nM labelled ligand. The methods used were similar to those previously described in the literature for the binding of [³H]bremazocine to guinea-pig cerebellar membranes. Under these conditions this ligand has been shown to bind saturably and with high affinity to an apparently homogeneous population of binding-sites, having the characteristics of the kappa opioid receptor [116].

The data in Figure 1.1 shows the displacement of $[{}^{3}H]$ bremazocine binding from guinea-pig cerebellar membranes by naloxone and (\pm) bremazocine.

Of the total [3 H]bremazocine bound 93% was displaced by 1µM naloxone, or 30nM (±)bremazocine. No further displacement was seen with levels of naloxone as high as 100µM, and a concentration of 10µM was therefore chosen to define the non-specific binding. Using this definition, IC₅₀ values and Hill coefficients (N) of 13nM (N=0.9) and 0.78nM (N=1.1) were obtained for naloxone and (±)bremazocine respectively (Table 1.1). These values are in line with the published kappa receptor affinities of these compounds [116] and the Hill coefficients obtained are consistent with the displacement of [3 H]bremazocine from a single population of binding-sites.

A range of opioid standards were examined as displacers of specific [³H]bremazocine binding in the guinea-pig cerebellum. The IC₅₀ values and Hill coefficients are shown in Table 1.1.

Low IC_{50} values, in the range 1-50nM, were obtained for the standard kappa agonists EKC, tifluadom, U50488 and U69593, and also for the selective ICI kappa agonists 204879, 204448 and 197067, part of a

.

COMPOUND	<u>IC₅₀(nM)</u>	HILL COEFFICIENT	
ANTAGONISTS			
NALOXONE	13.0 (10.3-16.3)	0.93+0.041	
NALTREXONE	6.14 (4.96-7.61)	$0.88 \pm 0.054 *$	
DIPRENORPHINE	1.01 (0.82-1.24)	0.98 ± 0.045	
PARTIAL AGONISTS			
BREMAZOCINE	0.78 (0.62-0.99)	1.12±0.046*	
LEVALLORPHAN	1.93 (1.42-2.62)	1.07 <u>+</u> 0.075	
PENTAZOCINE	53.1 (43.9-64.4)	0.93±0.934	
NALORPHINE	25.1 (20.3-31.0)	0.92 <u>±</u> 0.051	
Q.NALORPHINE	413 (260-656)	0.66±0.043*	
KAPPA AGONISTS			
MR2034	1.17 (0.97-1.41)	0.91 <u>+</u> 0.041*	
EKC	3.87 (2.73-5.48)	0.74 <u>+</u> 0.047*	
TIFLUADOM	4.14 (3.04-5.65)	0.79±0.050*	
Q.TIFLUADOM	89.4 (61.3-130)	0.82±0.054*	
U50488	15.7 (11.0-22.3)	0.64 <u>+</u> 0.024*	
U69593	9.54 (6.20-14.7)	0.54 <u>+</u> 0.020*	
DYN A (1-13)	2.34 (1.14-4.78)	0.42±0.033*	
DYN A (1-17)	1.76 (0.97-3.11)	0.41±0.037*	
DYNORPHIN B	9.74 (3.41-27.8)	0.31±0.016*	
ß ENDORPHIN	374 (258–542)	0.55±0.021*	
ICI200940	3.25 (1.89-5.61)	0.71±0.058*	
ICI197067	1.19 (0.66-2.16)	0.48 <u>+</u> 0.021*	
ICI204448	33.2 (23.1-47.9)	0.69±0.017*	
ICI204879	6.82 (4.35-10.7)	0.77 <u>+</u> 0.077*	
MU/DELTA AGONISTS			
ETORPHINE	4.16 (3.06-5.67)	1.16±0.088	
GLYOL~	1040 (668-1610)	0.64 <u>+</u> 0.020*	
MORPHINE	215 (158–294)	0.69±0.040*	
DADLE~~	15900(7050-35900)	0.42 <u>+</u> 0.042*	
DMPEA~~~	1620 (879-2970)	0.54 <u>+</u> 0.051*	
ICI174864	> 50000		
ICI154129	14000(7730-25200)	0.93 <u>+</u> 0.140	
FENTANYL	449 (276–729)	0.66 <u>+</u> 0.043*	

Table 1.1:

 $[^{3}H]$ bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in HEPES buffer at 25^oC. IC₅₀ values with confidence limits and Hill coefficients (±S.E.M) obtained for a range of displacing agents.

~ [D-Ala²,(Me)Phe⁴,Gly(ol)⁵]enkephalin.

~~ [D-Ala²,D-Leu⁵]enkephalin.

~~~[D-Met<sup>2</sup>, Pro<sup>5</sup>]enkephalinamide.

\* Significantly different from one, P</=0.05.

series of benzeneacetamide structures [28]. The mu selective agents GLYOL, morphine, fentanyl and DMPEA were however much weaker displacers of [<sup>3</sup>H]bremazocine binding in guinea-pig cerebellum, whilst the mu/delta agonist DADLE, and the delta selective antagonist ICI 174864 were almost inactive, with IC<sub>50</sub> values greater than 10µM.

Figures 1.2-1.4 show the displacement curves obtained for GLYOL, DADLE and ICI 174864 against [<sup>3</sup>H]bremazocine binding in the guinea-pig cerebellum.

There was no significant displacement of [<sup>3</sup>H]bremazocine binding by the mu selective agonist GLYOL (Figure 1.2) at concentrations below 10nM, and only 15.2% displacement at 100nM. Together with the IC<sub>50</sub> value of 1.04µM, this is consistent with the interaction of GLYOL at the kappa receptor, and does not suggest any significant mu receptor binding component. However this possibility cannot be altogether excluded. Similarly, the mu/delta selective agonist DADLE (Figure 1.3) produced only 17.5% inhibition of [<sup>3</sup>H]bremazocine binding at 1µM, with no significant displacement at lower concentrations. These data support the results obtained in the GLYOL displacement curve, and also suggests the absence of any delta component, for which DADLE would be expected to have a very high affinity. This conclusion also receives further support from the displacement curve to the selective delta antagonist ICI 174864, which shows no significant inhibition of specific binding (Figure 1.4) at concentrations below 3µM.

In order to test further for the presence of mu and delta receptors in the guinea-pig cerebellum, specific binding values were obtained (HEPES buffer) for the mu selective ligand  $[{}^{3}H]GLYOL$  and the mu/delta selective ligand  $[{}^{3}H]DADLE$  as described in the methods section. The results are shown in Table 1.2.

Very low levels of specific binding were obtained in the presence of  $1nM [{}^{3}H]GLYOL$  (mean of 66 spp.cpm), compared with 1112 specific cpm for 0.2nM  $[{}^{3}H]$ bremazocine. The total specific binding obtained with  $1nM [{}^{3}H]$ DADLE was also very low and in the presence of 100nM GLYOL to suppress any binding to the mu receptor, was reduced by more than 50%,

## Specific binding (cpm/20mg tissue wet weight)

[<sup>3</sup>H]GLYOL(1nM)

93 38 MEAN 65(n=2)

## Total specific binding (cpm/20mg tissue wet weight)

[<sup>3</sup>H]DADLE(1nM)

86 62 MEAN 74(n=2)

## Delta specific binding (+100nM GLYOL)

23 22 MEAN 22(n=2)

## Table 1.2:

Specific binding of [<sup>3</sup>H]GLYOL (1nM), and [<sup>3</sup>H]DADLE (1nM) in the presence and absence of 100nM GLYOL, to guinea-pig cerebellar membranes. HEPES buffer at 25<sup>o</sup>C. Results expressed as counts per minute (cpm).

to 22 specific cpm. The  $[{}^{3}H]GLYOL$  results therefore suggest that small numbers of mu receptors may be present in guinea-pig cerebellar membranes, although these are not readily detectable in the GLYOL and DADLE displacement curves. The  $[{}^{3}H]DADLE$  figures are in agreement with the displacement curves to ICI 174864 and DADLE, and do not suggest any significant delta component to the binding of  $[{}^{3}H]$ bremazocine under these conditions.

The possibility of sigma or PCP receptor binding in this system can also be excluded. [ ${}^{3}$ H]bremazocine binding was fully displaced by 1µM naloxone, a compound with no reported sigma receptor affinity [89], and the same level of displacement was achieved by other ligands, such as morphine, which also does not bind to either the sigma or PCP sites [139,146]. In addition there was no significant displacement of [ ${}^{3}$ H]bremazocine binding by haloperidol, a compound with high affinity for the sigma receptor [139], at concentrations up to 1µM.

### B: EVIDENCE FOR HETEROGENEITY OF BINDING:

# i. [<sup>3</sup>H]Bremazocine Binding.

The  $IC_{50}$  values obtained for the standards tested, the displacement curves to GLYOL, DADLE and ICI 174864, and the low levels of [<sup>3</sup>H]GLYOL and [<sup>3</sup>H]DADLE binding, clearly confirm the finding of other groups, that the binding of [<sup>3</sup>H]bremazocine in guinea-pig cerebellar membranes is apparently selective for the kappa receptor. However, although Hill coefficients close to 1.0 were obtained for the antagonists naloxone and diprenorphine, the partial agonists bremazocine, levallorphan, nalorphine and pentazocine, the non-selective agonist etorphine, and the selective delta antagonist ICI 154129, Hill coefficients between 0.7 and 0.95, and significantly different from one were obtained for the antagonist naltrexone, the kappa agonist Mr2034 and the kappa agonists EKC, tifluadom, Q.tifluadom, ICI 200940 and ICI 204879. Hill coefficients of less than 0.7, and significantly different from one, were obtained for all the other compounds tested, including the mu/delta selective ligands GLYOL, DADLE, morphine,

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| COMPOUND         | <u>IC<sub>50</sub>(nM)</u> | HILL COEFFICIENT     |  |
|------------------|----------------------------|----------------------|--|
| DYNORPHIN B      | 4.94(2.89-8.42)            | 0.71±0.060*          |  |
| DYNORPHIN A (1-1 | 7) 0.68(0.46-1.01)         | 0.69±0.057*          |  |
| DYNORPHIN A (1-1 | 0.76(0.46-1.26)            | 0.67 <u>+</u> 0.078* |  |
| B-ENDORPHIN      | 123 (84.8-180)             | 0.79±0.059*          |  |

## Table 1.3:

 $[^{3}H]$  bremazocine (0.2nM) binding to guinea-pig cerebellar membranes in HEPES buffer at  $25^{\circ}$ C. Recalculation of the IC<sub>50</sub> values with confidence limits and Hill coefficients (± S.E.M.) for certain peptide displacers, assuming a maximum of 85% inhibition of specific binding.

\*Significantly different from one, P</=0.05.

fentanyl and DMPEA, and the kappa agonists U50488, U69593, ICI 197067 and ICI 204448. The Hill coefficients obtained for the peptides B-endorphin, dynorphin A (1-13), dynorphin B, and dynorphin A (1-17) were very low, less than 0.6.

Dynorphin A (1-13) (Figure 1.5) displaced [<sup>3</sup>H]bremazocine binding over a very wide concentration range (0.1nM-10µM), with an IC<sub>50</sub> of 2.34nM, and a Hill coefficient of 0.42. The displacement curve was clearly biphasic, with approximately 80% of the [<sup>3</sup>H]bremazocine binding displaced by low concentrations of dynorphin A (1-13) (50nM), and the remaining 20% only displaceable by very high concentrations. This pattern is not consistent with the displacement of [<sup>3</sup>H]bremazocine from a single binding site population. Dynorphin B (Figure 1.6) displaced [<sup>3</sup>H]bremazocine binding over the concentration range 1nM-10 $\mu$ M, with an IC<sub>50</sub> of 9.74nM and a Hill coefficient of 0.31. Only 87% of the specific binding was displaced at 10µM. An IC<sub>50</sub> of 374 nM was obtained with ß-endorphin (Figure 1.7), again with only 85% displacement of specific binding at 10µM and a Hill coefficient of 0.55. In the case of Dynorphin A (1-17) (fig 1.8), 85% of the specific binding of [<sup>3</sup>H]bremazocine was displaceable with high affinity ( $IC_{50}=1.76nM$ ). The remaining 15% of the binding however was not displaced by concentrations up to 1µM, leading to a clear "plateau" in the displacement curve. The results presented above, both for the mu and kappa receptor standards, are not consistent with the displacement of [<sup>3</sup>H]bremazocine from a single binding-site population, with Hill coefficients significantly less than one for most of the compounds tested. In addition, the biphasic displacement curves obtained with the peptides suggest the presence of a "dynorphin resistant" component, comprising approximately 15% of the specific binding, which is not kappa-like in profile. Table 1.3 shows the IC<sub>50</sub> values and Hill coefficients obtained for the peptides, recalculated assuming a maximum of 85% inhibition of specific binding, (see methods section for details).

The exclusion of the "dynorphin resistant" component in these displacement curves decreased the IC<sub>50</sub>s obtained by an average of 2-3 fold. The Hill coefficients were also higher, and although all were

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| COMPOUND     |      | NO ADDITIONS          |              |      | +3µM DADLE            |             |
|--------------|------|-----------------------|--------------|------|-----------------------|-------------|
|              |      | IC <sub>50</sub> (nM) | N            |      | IC <sub>50</sub> (nM) | N           |
| GLYOL        | 1040 | (668–1610)            | 0.64+0.20*   | 2940 | (1690-5100)           | 0.60±0.07*  |
| U50488       | 15.7 | (11.0-22.3)           | 0.64+0.024*  | 18.5 | (14.9-22.9)           | 0.93+0.04   |
| U69593       | 9.54 | (6.20-14.7)           | 0.54+0.020*  | 21.4 | (15.6-29.3)           | 0.75+0.03*  |
| MORPHINE     | 215  | (158-294)             | 0.68+0.040*  | 485  | (342-687)             | 0.74+0.03*  |
| DYN A (1-17) | 0.68 | (0.46 - 1.01)         | 0.69+0.057*@ | 3.65 | (2.43 - 5.49)         | 0.69+0.08*# |
| DYN A (1-13) | 0.76 | (0.46 - 1.26)         | 0.67±0.078*@ | 2.46 | (1.7-3.54)            | 0.63+0.05*# |

## Table 1.4:

 $[^{3}\text{H}]$ Bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in HEPES buffer at 25°C. IC<sub>50</sub> values with confidence limits and Hill coefficients (N) (± S.E.M) for a range of displacing agents, obtained in the presence and absence of 3µM DADLE [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>]enkephalin.

- @ Results calculated assuming an 85% maximum inhibition of specific binding.
- # Results calculated assuming an 80% maximum inhibition of specific binding.
- \* Significantly different from one, P</= 0.05.

still significantly lower than one, they were similar to those obtained with the non-peptide kappa agonists.

# ii. [<sup>3</sup>H]Bremazocine binding in the presence of DADLE.

In order to investigate further the possibility that this heterogeneity of binding might be due to a mu component, displacement curves were generated to a range of standards in the presence of a high concentration of the selective mu/delta agonist DADLE. The IC<sub>50</sub> values and Hill coefficients achieved are summarised in Table 1.4 and compared with the data obtained in HEPES buffer alone. The displacement curves obtained in the presence and absence of DADLE are shown in Figures 1.9-1.14.

Specific [<sup>3</sup>H]bremazocine binding was inhibited by 27% in the presence of 3 $\mu$ M DADLE, in good agreement with the IC<sub>50</sub> value of 15.9 $\mu$ M obtained in this study. The IC<sub>50</sub> values and Hill coefficients obtained for GLYOL and morphine were only slightly increased in the presence of 3 $\mu$ M DADLE, and no change was seen in the U50488 IC<sub>50</sub> value obtained under these conditions. However the displacement curve was steeper, with a Hill coefficient not significantly different from 1. In the case of U69593 there was both a small increase in the IC<sub>50</sub> value obtained in the presence of DADLE and some steepening of the slope, although this value was still significantly different from one.

The displacement curves to dynorphin A (1-13) and dynorphin A (1-17) were shifted to the right 3 and 5 fold respectively in the presence of DADLE. There was no significant change in the Hill coefficients obtained. In the presence of DADLE, only 80% displacement of  $[^{3}H]$  bremazocine binding was seen with 1µM dynorphin A (1-17), as opposed to 85% in the absence of DADLE. For this reason a maximum of 80% inhibition of specific binding for dynorphin A (1-13) and (1-17) was assumed in calculating the IC<sub>50</sub> values and Hill coefficients obtained for these peptides in the presence of DADLE (see Table 1.4).

There was no indication of any reduction in the size of the "dynorphin

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| COMPOUND          | 0°C+INHIBITORS |                            |                           | <u>25</u> ° <u>C</u> |                            |                                            |
|-------------------|----------------|----------------------------|---------------------------|----------------------|----------------------------|--------------------------------------------|
|                   |                | <u>IC<sub>50</sub>(nM)</u> | N                         |                      | <u>IC<sub>50</sub>(nM)</u> | N                                          |
| EKC<br>U69593     | 27.0<br>1090   | (14.9-48.8)<br>(475-2480)  | 0.93±0.14<br>0.46±0.07*   | 3.87<br>9.54         | (2.73-5.48)<br>(6.20-14.7) | 0.74 <u>+</u> 0.05*<br>0.54 <u>+</u> 0.02* |
| NALOXONE<br>DYN B | 9.56           | (6.34-14.4)<br>(9.70-18.1) | 0.93±0.09*<br>0.74±0.05*# | 13.0<br>4.94         | (10.3-16.3)<br>(2.89-8.42) | $0.94\pm0.04$<br>$0.71\pm0.06*@$           |
| DYN A (1-13)      | 3.58           | (2.11-6.09)                | 0.4/±0.04*#               | 0./6                 | (0.46 - 1.26)              | 0.66±0.08*@                                |

### Table 1.5:

 $[^{3}H]$ Bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in HEPES buffer. A comparison of the IC<sub>50</sub> values with confidence limits and Hill coefficients (N) (± S.E.M.) obtained for a range of displacers at 25°C, and at 0°C in the presence of protease inhibitors.

# Results calculated assuming an 80% maximum inhibition of specific binding.

@ Results calculated assuming an 85% maximum inhibition of specific binding.

\* Significantly different from one, P</=0.05.

resistant" component, or the slopes of the displacement curves to the dynorphin peptides, in the presence of DADLE, confirming that this effect is not due to a mu or delta binding component.

iii. [<sup>3</sup>H]Bremazocine binding at 0<sup>°</sup>C in the presence of protease inhibitors:

In order to investigate further this "dynorphin resistant component" it was decided to test a wider range of kappa opioid peptides in this binding system. Because of the instability of many of these peptides,  $[^{3}H]$  bremazocine binding assays were run for 60 min at 0°C in the presence of proteolytic enzyme inhibitors, as described in the methods. Under these conditions the specific binding of 0.2nM  $[^{3}H]$  bremazocine was reduced by 980 cpm. A small proportion of this loss (approximately 150 specific cpm) was due to the presence of the inhibitor cocktail.

Table 1.5 compares the  $IC_{50}$  values and Hill coefficients obtained for a number of peptide and non-peptide standards assayed both at 25°C and at 0°C in the presence of inhibitors.

The naloxone displacement curve was unaffected by the alterations in the assay conditions, with no significant difference in either the  $IC_{50}$  value or Hill coefficient obtained. The U69593  $IC_{50}$  value however, was increased approximately 100 fold from 9.54nM to 1090nM. The displacement curves obtained for dynorphin B (Figure 1.15), dynorphin A (1-13) (Figure 1.16), and EKC were also shifted to the right, but to a lesser extent, with  $IC_{50}$  values increased 2.7, 4.7 and 7.0 times respectively. With the exception of EKC, there was no significant change in the Hill coefficients obtained. The large apparent reduction in the affinities of the kappa standards in particular, could explain the loss of [<sup>3</sup>H]bremazocine specific binding seen under these conditions.

Both the dynorphin B and dynorphin A (1-13) displacement curves showed significant rightward shifts under the altered assay conditions. In addition the percentage inhibition values achieved at the highest

| COMPOUND              | <u>IC<sub>50</sub>(nM)</u> | HILL COEFFICIENT     |  |
|-----------------------|----------------------------|----------------------|--|
| a-NEOENDORPHIN        | 8.50 (5.14-14.1)           | 0.604±0.07*          |  |
| <b>B-NEOENDORPHIN</b> | 10.8 (7.36-16.0)           | 0.670 <u>+</u> 0.06* |  |
| MEAGLE~               | 206 (136-313)              | 0.728±0.08*          |  |
| MEAP~~                | 32.2 (20.5-50.5)           | 0.589±0.05*          |  |

## Table 1.6:

 $[^{3}$ H]bremazocine binding (0.2nM) to guinea-pig cerebellar membranes at 0<sup>o</sup>C in the presence of protease inhibitors. IC<sub>50</sub> values with confidence limits and Hill coefficients (± S.E.M.) obtained for a range of displacers.

Maximum of 80% inhibition of specific binding assumed for all compounds.

\* Significantly different from one, P</=0.05.

~ [Met<sup>5</sup>]enkephalin(Arg-Gly-Leu). ~~ [Met<sup>5</sup>]enkephalin(Arg-Phe). concentration tested (10µM) were in both cases lower than those obtained at  $25^{\circ}$ C, with a maximal displacement of 80% of specific binding. An 80% binding maximum was therefore used in calculating the IC<sub>50</sub> values and Hill coefficients of the peptides at 0°C, as shown in Table 1.5. The altered position of the plateau may be a result of the lowered affinities of the peptides under these conditions, or may reflect differences in the population of receptors labelled by [<sup>3</sup>H]bremazocine at 0°C. Unfortunately it was not possible to look at the effect of DADLE suppression under these conditions, because of the low number of specific counts. However, the overall shape of the displacement curves was the same under both sets of conditions, suggesting that these peptides are likely to be displacing [<sup>3</sup>H]bremazocine from a similar receptor population.

Table 1.6 shows the  $IC_{50}$  values and Hill coefficients obtained for four peptides tested at 0°C in the presence of inhibitors. Although 80% of specific [<sup>3</sup>H]bremazocine binding was displaced by these compounds with high affinity, the curves were clearly biphasic (Figures 1.17-1.20). In the case of a-neoendorphin, ß-neoendorphin and [Met<sup>5</sup>]enkephalin (Arg-Phe) (MEAP), approximately 20% of the specific binding was resistant to displacement at concentrations up to 10µM, leading to the formation of clear "plateaux" in these curves. A very low Hill coefficient was also seen in the [Met<sup>5</sup>]enkephalin (Arg-Gly-Leu) (MEAGLE) displacement curve, although no clear plateau was formed, possibly due to the lower affinity of this peptide. A maximum of 80% inhibition of specific binding was therefore assumed in calculating the IC<sub>50</sub> values and Hill coefficients shown in Table 1.6.

Care is therefore needed in the interpretation of this data, due to the altered conditions and the effects on the affinities of the ligands. However, biphasic displacement curves were obtained for dynorphin A (1-13) and dynorphin B, similar to those seen in the  $25^{\circ}$ C assay. In addition, the four new peptides tested were only able to displace 80% of the specific binding at concentrations up to 10µM. These results therefore provide further support for the conclusion reached in the  $25^{\circ}$ C system, that there is a "dynorphin resistant" component to the binding of [<sup>3</sup>H]bremazocine in the guinea-pig

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NO ADDITIONS

### +10µM DADLE

| COMPOUND     | IC <sub>50</sub> (nM) | N                   | IC <sub>50</sub> (nM) | N           |
|--------------|-----------------------|---------------------|-----------------------|-------------|
| ICI204879    | 8.15(4.79-13.9)       | 0.60 <u>+</u> 0.05* |                       |             |
| DADLE~       | 12500(7280-21600)     | 0.53±0.05*          |                       |             |
| GLYOL~~      | 696 (351-1380)        | 0.54±0.05*          | 3270(1340-7990)       | 0.54±0.07*  |
| DYN A (1-17) | 1.99(1.24 - 3.19)     | 0.61±0.04*#         | 2.11(1.45-3.06)       | 0.73±0.04*@ |
| DYN A (1-13) | 1.26(0.91-1.74)       | 0.76±0.06*#         | 4.43(2.57-7.64)       | 0.59±0.06*@ |
| U69593       | 16.4(9.63-27.8)       | 0.57±0.03*          | 21.0(15.3-28.9)       | 0.77±0.03*  |

### Table 1.7:

 $[^{3}H]$ EKC binding (0.5nM) to guinea-pig cerebellar membranes in HEPES buffer at 25<sup>o</sup>C. IC<sub>50</sub> values with confidence limits and Hill coefficients (N) (± S.E.M.) for a range of displacers, obtained in the presence and absence of 10µM DADLE [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>]enkephalin.

# Results calculated assuming a maximum of 90% inhibition of specific binding.

@ Results calculated assuming a maximum of 85% inhibition of specific binding.

- \* Significantly different from one, P</=0.05.
- ~ [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>]enkephalin.
- ~~ [D-Ala<sup>2</sup>, (Me)Phe<sup>4</sup>, Gly(ol)<sup>5</sup>]enkephalin.
cerebellum.

### iv. [<sup>3</sup>H]EKC binding in presence and absence of DADLE.

In order to investigate whether this "dynorphin resistant component" was unique to  $[{}^{3}H]$  bremazocine binding in guinea-pig cerebellum membranes, the kappa agonist  $[{}^{3}H]$  EKC was used to generate displacement curves to a number of opioid standards, both in the presence and absence of 10µM DADLE.  $[{}^{3}H]$  EKC binding assays were run in HEPES buffer in the presence of 0.5nM  $[{}^{3}H]$  EKC, as described in the methods. 10µM naloxone was used to define the non specific binding, resulting in a mean of 946 specific counts bound. In the presence of 10µM DADLE, the specific binding was reduced by 49%. The IC<sub>50</sub> values and Hill coefficients obtained for the standards tested are shown in Table 1.7.

The IC<sub>50</sub> values and Hill coefficients obtained for the kappa standards U69593 and ICI204879, and also for the mu selective agonist GLYOL, were similar to those achieved in the  $[{}^{3}H]$ bremazocine binding assay, suggesting that under these conditions  $[{}^{3}H]$ EKC predominantly labels a kappa receptor. Only 11% of specific  $[{}^{3}H]$ EKC binding was displaced by GLYOL at 30nM, and 9.5% with DADLE at 100nM, suggesting no significant mu/delta receptor contamination, under these conditions. Small rightward shifts were however seen in the displacement curves to GLYOL and U69593, in the presence of DADLE.

The IC<sub>50</sub> values obtained for dynorphin A (1-17) and (1-13) (Figures 1.21-1.22) in the absence of DADLE were very close to those achieved in the [ ${}^{3}$ H]bremazocine binding assay. Approximately 10% of the specific binding was not displaced at concentrations up to 10µM, and a maximum of 90% inhibition of specific binding was assumed in calculating the IC<sub>50</sub>s and Hill coefficients shown in Table 1.7. These results are similar to those obtained in the [ ${}^{3}$ H]bremazocine assay, in that they show the presence of a "dynorphin resistant component" to the binding. This component appears to represent a slightly lower proportion of the specific binding in the [ ${}^{3}$ H]EKC assay. There was very little difference in the IC<sub>50</sub>s obtained for the dynorphin

peptides in the presence and absence of DADLE (Figures 1.21-1.22). The maximum inhibition of binding seen with these peptides was however slightly reduced in the presence of DADLE, and a value of 85% was used in calculating the  $IC_{50}$  values and Hill coefficients, as shown in Table 1.7. There was no indication of any reduction in the size of the "dynorphin resistant" component in the presence of DADLE, for either of the peptides, confirming that it is not likely to represent a mu or delta component.

The binding of  $[{}^{3}H]EKC$  to guinea-pig cerebellum therefore appears to be selective for the kappa receptor, with no evidence of any significant mu or delta contamination. The results obtained with the dynorphin peptides are in line with those achieved in the  $[{}^{3}H]$ bremazocine binding assay and support the concept of a "dynorphin resistant component" which is not high affinity mu or delta.

#### C. SUMMARY:

[<sup>3</sup>H]bremazocine binding to guinea-pig cerebellar membranes in HEPES buffer was selective for the kappa receptor as previously defined. There was no evidence for a significant mu, delta or sigma component to the binding, under the assay conditions used. However the possibility of a small mu receptor component, comprising less than 10% of the specific binding, cannot be excluded.

The majority of compounds tested displaced [ ${}^{3}$ H]bremazocine binding with Hill coefficients significantly less than 1.0. In the case of the dynorphin peptides the displacement was clearly biphasic, with a 15-20% "dynorphin resistant component" of specific binding not displaceable by dynorphin A (1-13) and A (1-17) at concentrations up to 10 $\mu$ M.

This "dynorphin resistant component" was not abolished in the presence of  $3\mu$ M DADLE, and was therefore not due to a mu or delta binding component. Similar results were also obtained in a [<sup>3</sup>H]EKC binding assay performed under the same conditions, and in a [<sup>3</sup>H]bremazocine assay run at 0<sup>o</sup>C in the presence of protease inhibitors

The data obtained from the guinea-pig cerebellum [<sup>3</sup>H]bremazocine and [<sup>3</sup>H]EKC binding assays, run in HEPES buffer, therefore clearly support the presence of an additional binding site or binding site conformation, for which the dynorphin peptides have negligible affinity, and which cannot be defined as high affinity mu or delta. The exact contribution of this binding-site to the low Hill coefficients seen with the majority of the displacing ligands is unclear, and would require further work.

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Displacement of total  $[{}^{3}H]$  bremazocine binding (0.2 nM) to guinea-pig cerebellum membranes in HEPES buffer by naloxone ( $\blacksquare$ ) and (±) bremazocine (+---+).

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by [D-Ala<sup>2</sup>, (Me)Phe<sup>4</sup>, Gly(ol)<sup>5</sup>]enkephalin (GLYOL): % inhibition of control binding vs. log GLYOL concentration. • .

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin (DADLE): % inhibition of control binding vs. log DADLE concentration.

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by ICI 174864: % inhibition of control binding vs. log ICI 174864 concentration.

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-13): % inhibition of control binding vs. log dynorphin concentration.

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin B: % inhibition of control binding vs. log dynorphin concentration.

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Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by ß-endorphin: % inhibition of control binding vs. log ß-endorphin concentration.



Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-17): % inhibition of control binding vs. log dynorphin concentration.



Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by  $[D-Ala^{2}, (Me)Phe^{4}, Gly(ol)^{5}]$  enkephalin (GLYOL) in the presence ( $\blacksquare --\blacksquare$ ) and absence ( $\blacksquare --\blacksquare$ ) of  $3\mu M$   $[D-Ala^{2}, D-Leu^{5}]$  enkephalin: % inhibition of control binding vs. log displacer concentration. .

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Figure 1.10

Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by morphine in the presence ( $\blacksquare$ --- $\blacksquare$ ) and absence ( $\blacksquare$ --- $\blacksquare$ ) of 3 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log morphine concentration.

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Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by U50488 in the presence ( $\blacksquare$ --- $\blacksquare$ ) and absence ( $\blacksquare$ --- $\blacksquare$ ) of 3 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log U50488 concentration.

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Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by U69593 in the presence ( $\blacksquare --\blacksquare$ ) and absence ( $\blacksquare --\blacksquare$ ) of 3 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log U69593 concentration.



Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) to guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-13) in the presence ( $\blacksquare - - - \blacksquare$ ) and absence ( $\blacksquare - - \blacksquare$ ) of 3 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>] enkephalin: % inhibition of control binding vs. log dynorphin concentration.



Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-17) in the presence ( $\blacksquare$ --- $\blacksquare$ ) and absence ( $\blacksquare$ --- $\blacksquare$ ) of 3 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log dynorphin concentration.



Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin B at 25°C (IIII) and at 0°C in the presence of proteolytic enzyme inhibitors (IIIII): % inhibition of control binding vs. log dynorphin concentration.



Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-13) at 25°C ( ) and at 0°C in the presence of proteolytic enzyme inhibitors ( ): % inhibition of control binding vs. log dynorphin concentration.

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#### Figure 1.17

Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by alpha-neoendorphin at 0°C in the presence of proteolytic enzyme inhibitors: % inhibition of control binding vs. log displacer concentration.

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## Figure 1.18

Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by ß-neoendorphin at 0°C in the presence of proteolytic enzyme inhibitors: % inhibition of control binding vs. log displacer concentration.

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# Figure 1.19

Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by [Met<sup>5</sup>]enkephalin(Arg-Gly-Leu) at 0°C in the presence of proteolytic enzyme inhibitors: % inhibition of control binding vs. log displacer concentration.

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# Figure 1.20

Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES buffer by [Met<sup>5</sup>]enkephalin(Arg-Phe) at 0°C in the presence of proteolytic enzyme inhibitors: % inhibition of control binding vs. log displacer concentration.

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# Figure 1.21

Displacement of specific  $[{}^{3}H]EKC$  binding (0.5 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-17) in the presence ( $\blacksquare$ -----) and absence ( $\blacksquare$ ------) of 10 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log displacer concentration. ·

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## Figure 1.22

Displacement of specific  $[{}^{3}H]EKC$  binding (0.5 nM) from guinea-pig cerebellum membranes in HEPES buffer by dynorphin A(1-13) in the presence ( $\blacksquare - - - \blacksquare$ ) and absence ( $\blacksquare - - \blacksquare$ ) of 10 µM [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin: % inhibition of control binding vs. log displacer concentration. 2. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: KREBS/HEPES BUFFER + GppNHp

A: ASSAY VALIDATION

# i. [<sup>3</sup>H]Bremazocine binding:

In order to investigate the effects of Na<sup>+</sup> ions and GTP on kappa receptor binding in guinea-pig cerebellum, and also to discover whether the heterogeneity of binding observed in HEPES buffer would be preserved under altered ionic conditions, a [<sup>3</sup>H]bremazocine binding assay was set up in guinea-pig cerebellum membranes, in a Krebs/HEPES buffer system containing 0.15M Na<sup>+</sup> ions and 10µM GppNHp, a stable analogue of GTP.

Figure 2.1 shows the displacement curves obtained for naloxone and  $(\pm)$  bremazocine under these conditions.

In this assay 91% of the total binding of  $[^{3}H]$  bremazocine was displaced by 10µM naloxone. 100µM naloxone did not produce significant further displacement, and a concentration of 10µM naloxone was therefore chosen to define the non-specific binding.

Under these conditions 934 specific cpm bound were obtained with 0.2nM [ ${}^{3}$ H]bremazocine. This compares with 1112 cpm (93% specific), in the HEPES assay. IC<sub>50</sub> values and Hill coefficients (N) of 25.4nM (N=0.82) and 1.09nM (N=0.90) were obtained for naloxone and bremazocine respectively, in Krebs/HEPES +GppNHp, compared with values of 13.0nM (N=0.93) and 0.78nM (N=1.13) in HEPES. The bremazocine data was therefore very close to that obtained in HEPES, suggesting that the Krebs buffer system, and the presence of GppNHp, had no major effect on the affinity or binding capacity of [ ${}^{3}$ H]bremazocine. The naloxone IC<sub>50</sub> however, was higher than the HEPES value, and the Hill coefficient was significantly lower than one.

Table 2.1 shows the  $IC_{50}$  values and Hill coefficients obtained for a range of opioid standards as displacers of [<sup>3</sup>H]bremazocine binding in

| COMPOUND                    | <u>IC<sub>50</sub>(nM)</u> |                            | HILL COEFFICIENT                         |  |
|-----------------------------|----------------------------|----------------------------|------------------------------------------|--|
| ANTAGONISTS                 |                            |                            |                                          |  |
| NALOXONE                    | 25.4                       | (20.2-32.0)                | 0.82±0.03*                               |  |
| NALTREXONE<br>DIPRENORPHINE | 10.7                       | (8.29–13.7)<br>(1.25–1.87) | 0.93 <u>+</u> 0.07<br>1.07 <u>+</u> 0.05 |  |
| PARTIAL AGONISTS            |                            |                            |                                          |  |
| BREMAZOCINE                 | 1.09                       | (0.91-1.29)                | 0.89±0.03*                               |  |
| LEVALLORPHAN                | 5.51                       | (4.26-7.11)                | 0.94 <u>+</u> 0.07                       |  |
| PENTAZOCINE                 | 408                        | (284–586)                  | 1.30±0.07*                               |  |
| NALORPHINE                  | 186                        | (148-232)                  | $1.08 \pm 0.05$                          |  |
| Q.NALORPHINE                | 1260                       | (749-2120)                 | 0.59 <u>+</u> 0.02*                      |  |
|                             |                            |                            |                                          |  |
| KAPPA AGONISIS              |                            |                            |                                          |  |
| MR2034                      | 11.4                       | (9.66-13.5)                | 1.07±0.04                                |  |
| EKC                         | 121                        | (89–164)                   | 0.83±0.05*                               |  |
| TIFLUADOM                   | 137                        | (93.9-200)                 | 0.69 <u>+</u> 0.04*                      |  |
| Q.TIFLUADOM                 | 1870                       | (1290–2700)                | 0.89 <u>+</u> 0.06                       |  |
| U50488                      | 331                        | (211-519)                  | 0.68±0.08*                               |  |
| U69593                      | 972                        | (687–1380)                 | 0.65 <u>+</u> 0.03*                      |  |
| DYN A (1-13)                | 81.2                       | (47.0-140)                 | $0.52 \pm 0.04 *$                        |  |
| DYN A (1-17)                | 414                        | (262-653)                  | $0.51 \pm 0.04 *$                        |  |
| ICI200940                   | 53.8                       | (30.1-93.4)                | 0.57 <u>+</u> 0.04*                      |  |
| ICI197067                   | 35.8                       | (16.9-76.2)                | $0.49\pm0.03*$                           |  |
| ICI204448                   | 197                        | (96./-402)                 | $0.40\pm0.03*$                           |  |
| 101204879                   | 24.2                       | (9.45-62.0)                | 0.32 <u>+</u> 0.03*                      |  |
| MU/DELTA LIGANDS            |                            |                            |                                          |  |
| ETORPHINE                   | 26.9                       | (18.3-39.6)                | 1.03±0.10                                |  |
| GLYOL~                      | 50900                      | (26100-99600)              | 0.56±0.05*                               |  |
| MORPHINE                    | 6030                       | (3940-9230)                | 0.74±0.07*                               |  |
| DADLE~~                     | >>50000                    |                            |                                          |  |

| DMPEA~~~  | 33700   | (20300-55900) | 0.61±0.08*         |
|-----------|---------|---------------|--------------------|
| ICI174864 | >>50000 | -             |                    |
| ICI154129 | 18100   | (14700-22300) | 0.89 <u>+</u> 0.05 |
| FENTANYL  | 5030    | (3590-7040)   | 0.74±0.07*         |
|           |         | _ :           |                    |

#### Table 2.1:

 $[^{3}H]$ bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in Krebs/HEPES +10  $\mu$ M GppNHp. IC<sub>50</sub> values with confidence limits and Hill coefficients (± S.E.M.) obtained for a range of displacers.

- ~ [D-Ala<sup>2</sup>,(Me)Phe<sup>4</sup>,Gly(ol)<sup>5</sup>]enkephalin
- ~~ [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>]enkephalin
- ~~~ [D-Met<sup>2</sup>, Pro<sup>5</sup>]enkephalinamide
- \* Significantly different from one, P</=0.05.

#### Krebs/HEPES buffer + 10µM GppNHp.

The majority of the standards tested in HEPES buffer were also active in the Krebs/HEPES buffer system. However the affinities of the agonists were much reduced, whereas those of the antagonists were not greatly affected.

Under these non-standard conditions it is clearly much more difficult to establish the kappa specificity of the assay, particularly as most of the mu and delta standards are agonists. Excluding the non selective antagonists and partial agonists, the highest affinities obtained ( $IC_{50}$  values 24-54nM) were those for the ICI kappa agonists 204879, 197067, 200940 and the non selective agonist etorphine.  $IC_{50}$ values of 81.2nM and 414nM were achieved for dynorphin A (1-13) and (1-17) respectively, and the remaining kappa standards (EKC, tifluadom, U50488 and U69593) all had  $IC_{50}$ s below 1µM. These results suggest that under these conditions [<sup>3</sup>H]bremazocine is still predominantly labelling the kappa receptor.

Very low affinities were also obtained for the mu agonists tested in this assay system, with  $IC_{50}$  values of 6µM and 50µM respectively for morphine and GLYOL. In addition, only 10-13% of specific [<sup>3</sup>H]bremazocine binding was displaced by morphine and DMPEA at 1µM, and 12% with GLYOL at 3µM. This does not suggest any binding to the high agonist affinity conformation of the mu receptor. However these agents are all agonist ligands, and their affinities at the mu receptor might be reduced under these buffer conditions. These results are therefore difficult to interpret and any mu receptor component to the binding of [<sup>3</sup>H]bremazocine in this buffer system would be hard to identify.

The displacement curves to DMPEA, morphine and GLYOL are shown in Figures 2.2-2.4.

The results obtained with the selective ICI antagonists 154129 and 174864, the affinities of which should not be decreased by the altered conditions, were similar to those seen in HEPES buffer, and suggest no

.

| COMPOUND          | HEPES                 |        | KREBS/HEPES           |       | SHIFT |  |
|-------------------|-----------------------|--------|-----------------------|-------|-------|--|
| ANTAGONISTS       | IC <sub>50</sub> (nM) | N      | IC <sub>50</sub> (nM) | N     |       |  |
| NALOXONE          | 13.0                  | 0.93   | 25.4                  | 0.82* | 1.9   |  |
| NALTREXONE        | 6.14                  | 0.88*  | 10.7                  | 0.93  | 1.7   |  |
| DIPRENORPHINE     | 1.01                  | 0.98   | 1.53                  | 1.1   | 1.5   |  |
| PARTIAL AGONISTS  |                       |        |                       |       |       |  |
| BREMAZOCINE       | 0.78                  | 1.13   | 1.09                  | 0.90* | 1.4   |  |
| LEVALLORPHAN      | 1.93                  | 1.07   | 5.51                  | 0.94  | 2.8   |  |
| PENTAZOCINE       | 53.1                  | 0.93   | 408                   | 1.30* | 7.7   |  |
| NALORPHINE        | 25.1                  | 0.93   | 186                   | 1.10  | 7.4   |  |
| Q.NALORPHINE      | 413                   | 0.66*  | 1260                  | 0.59* | 3.0   |  |
| KAPPA AGONISTS    |                       |        |                       |       |       |  |
| MR2034            | 1.17                  | 0.91*  | 11.4                  | 1.07  | 9.7   |  |
| EKC               | 3.87                  | 0.74*  | 121                   | 0.83* | 31.3  |  |
| TIFLUADOM         | 4.14                  | 0.79*  | 137 .                 | 0.69* | 33.0  |  |
| Q.TIFLUADOM       | 89.4                  | 0.83*  | 1870                  | 0.89  | 20.9  |  |
| U50488            | 15.7                  | 0.64*  | 331                   | 0.68* | 21.0  |  |
| U69593            | 9.54                  | 0.54*  | 972                   | 0.65* | 101.8 |  |
| DYN A (1-13)      | 0.76                  | 0.67*# | 81.2                  | 0.52* | 106.8 |  |
| DYN A (1-17)      | 0.68                  | 0.70*# | 414                   | 0.51* | 608.8 |  |
| ICI200940         | 3.25                  | 0.72*  | 53.8                  | 0.57* | 16.3  |  |
| ICI197067         | 1.19                  | 0.49*  | 35.8                  | 0.48* | 30.0  |  |
| ICI204448         | 33.2                  | 0.69*  | 197                   | 0.39* | 5.9   |  |
| ICI204879         | 6.82                  | 0.77*  | 24.2                  | 0.32* | 3.5   |  |
| MU/DELTA AGONISTS |                       |        |                       |       |       |  |
| ETORPHINE         | 4.16                  | 1.16   | 26.9                  | 1.03  | 6.5   |  |
| GLYOL             | 1040                  | 0.64*  | 50900                 | 0.56* | 48.6  |  |
| MORPHINE          | 215                   | 0.69*  | 6030                  | 0.74* | 26.9  |  |
| DADLE             | 15900                 | 0.43*  | >50000                |       |       |  |
| DMPEA             | 1620                  | 0.54*  | 33700                 | 0.61* | 20.8  |  |
| ICI174874         | >50000                |        | >50000                |       |       |  |
| ICI154129         | 14000                 | 0.93   | 18100                 | 0.89  | 1.3   |  |
| FENTANYL          | 449                   | 0.66*  | 5030                  | 0.74* | 11.2  |  |
|                   |                       |        |                       |       |       |  |

## Table 2.2:

 $[^{3}\text{H}]$ Bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in HEPES buffer and Krebs/HEPES + 10µM GppNHp. A comparison of the IC<sub>50</sub> values and Hill coefficients (N) obtained for a range of standards, with a measure (SHIFT) of the difference in IC<sub>50</sub> between the two buffer systems (ratio of IC<sub>50</sub> values in Krebs/HEPES and HEPES buffers).

- # Results calculated assuming an 85% maximum inhibition of specific binding.
- \* Significantly different from one, P</=0.05

detectable delta component in this assay. In addition the mu/delta agonist ligand DADLE was almost inactive in this system ( $IC_{50}$ >50µM).

There is little evidence available as to the possible effects of Krebs +GppNHp on any  $[{}^{3}$ H]bremazocine sigma binding component. Haloperidol did not displace  $[{}^{3}$ H]bremazocine binding in this buffer system, at concentrations up to 1µM, and all the specific binding was displaced by 1-10µM naloxone.

[<sup>3</sup>H]bremazocine binding in Krebs/HEPES buffer +GppNHp therefore appears to show some selectivity for the kappa receptor. The results with the delta antagonists ICI 174864 and ICI 154129, and with haloperidol, suggest that there is no delta or sigma component to the binding. The lack of selective mu receptor antagonists makes the exclusion of a mu component under these conditions very difficult. However, the data with the mu receptor standards is consistent with displacement predominantly from a kappa receptor.

ii. Comparison of the [<sup>3</sup>H]bremazocine displacement curves obtained in HEPES buffer and Krebs/HEPES + GppNHp:

Table 2.2 compares the  $IC_{50}s$  and Hill coefficients obtained for the standards tested in the HEPES and Krebs/HEPES + GppNHp assays, and gives a measure of the rightward shift in  $IC_{50}$  due to the Krebs buffer system.

The largest increases in  $IC_{50}$  value were seen with the peptides dynorphin A (1-17) (x609), dynorphin A (1-13) (x107) and the kappa agonist U69593 (x102). Increases in  $IC_{50}$  value of between 10 and 50 fold were seen with most of the kappa agonists tested, and also with the mu agonists GLYOL, morphine, DMPEA and fentanyl. Smaller shifts, in the range 2 to 9 fold, were seen with the partial agonists, such as pentazocine and nalorphine. An  $IC_{50}$  difference of only 1 to 2 fold was obtained with the antagonists naloxone, naltrexone and diprenorphine, and also with bremazocine. Displacement curves to

U69593 run in the presence of  $50\mu$ M GppNHp, showed no further change in  $IC_{50}$  (587nM), although the slope obtained was shallower (Hill coefficient: 0.42).

**B: EVIDENCE FOR HETEROGENEITY OF BINDING** 

# i. [<sup>3</sup>H]Bremazocine binding:

Despite the apparent kappa selectivity of the binding in Krebs/HEPES buffer + GppNHp, the majority of the compounds tested displaced <sup>3</sup>H]bremazocine with Hill coefficients significantly less than one. Hill coefficients not significantly different from one were obtained only for naltrexone, diprenorphine, levallorphan, nalorphine, Mr2034, etorphine, ICI 154129 and Q.tifluadom. These compounds also had slopes that were fairly steep or not significantly less than one in HEPES buffer. Hill coefficients significantly different from one and between 0.8 and 0.9 were obtained for naloxone, bremazocine, and EKC. Values of between 0.6 and 0.8 were seen with tifluadom, U50488, U69593, DMPEA, morphine and fentanyl, and between 0.5 and 0.6 for Q.nalorphine, ICI 200940 and GLYOL. Clearly biphasic lines were achieved with the dynorphin peptides, and also with the ICI kappa agonists 204448, 197067 and 204879. In the case of ICI 200940, 204448 and 204879, the slopes were markedly shallower than those obtained in the HEPES buffer system. Biphasic displacement curves were obtained in HEPES for the dynorphin peptides, but not for the three ICI kappa agonists mentioned above.

Figures 2.5-2.9 show the displacement curves obtained for dynorphin A (1-17), dynorphin A (1-13), ICI204879, ICI204448 and ICI197067 in both HEPES and Krebs/HEPES + GppNHp.

Biphasic displacement curves for dynorphin A (1-17) were obtained in both buffer systems. In Krebs/HEPES + GppNHp this ligand displaced a maximum of 70% of the specific binding of  $[{}^{3}$ H]bremazocine at concentrations of 1 to 10 µM. This compares with a maximum displacement of 85% at 1µM in HEPES buffer. The dynorphin A (1-17)

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|----|----|-----|-------|--|
|    | mr | UN  | INU   |  |

IC<sub>50</sub>(nM)

#### HILL COEFFICIENT

## Table 2.3:

 $[^{3}$ H]Bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in Krebs/HEPES + 10µM GppNHp. Recalculation of the IC<sub>50</sub> values with confidence limits and Hill coefficients (± S.E.M.) obtained for certain compounds, assuming a maximum of 70% inhibition of specific binding.

\* Significantly different from one P</=0.05

displacement curve was also markedly shifted to the right in the Krebs buffer system. A similar effect was seen with dynorphin A (1-13), although the binding "plateau" was not as clear.

In the case of the kappa agonist ICI 204879, 70% of the specific binding in Krebs buffer was displaced in the concentration range 1 to 100nM, whereas the remainder of the binding was not displaceable at concentrations up to  $10\mu$ M, leading to the formation of a clear plateau in the binding curve. In the HEPES buffer system ICI 204879 displaced 100% of the specific binding between 1 and 100nM, with a Hill coefficient of 0.77.

This pattern was also seen with ICI 204448, and to a lesser extent with ICI 197067. No significant further displacement of  $[^{3}H]$ bremazocine binding was seen with 3µM dynorphin A (1-17) or ICI 204448, in the presence of 1µM ICI 204879, suggesting that all three agents lacked affinity for the same component of the binding.

The data for dynorphin A (1-17), dynorphin A (1-13), ICI 204448, ICI 197067 and ICI 204879 was therefore recalculated assuming a maximum of 70% displacement of specific binding. Table 2.3 shows the corrected IC<sub>50</sub> values and Hill coefficients.

The recalculation of the data obtained for these four compounds, assuming a maximum of 70% inhibition of specific binding, produced a clear decrease in the  $IC_{50}$  values. The Hill coefficients were also much higher, with values not significantly different from one for all the compounds except dynorphin A (1-17). Comparison with the HEPES data showed a marked reduction in the size of the "Na<sup>+</sup> shifts" seen with dynorphin A (1-17), ICI 197067 and dynorphin A (1-13), and no remaining shift in the displacement curves to ICI 204879 and ICI 204448.

The apparent heterogeneity of binding seen with [<sup>3</sup>H]bremazocine in HEPES buffer has therefore not been resolved by the alterations in the ionic conditions of the assay, suggesting that it is not likely to be due to a low affinity kappa receptor conformation. The "dynorphin

,

| COMPOUND                                                                                                                                          | <u>1C<sub>50</sub>()</u>                                                                            | nM)                                                                                                                                   | HILL COEFFICIENT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ANTAGONISTS                                                                                                                                       |                                                                                                     |                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| NALOXONE<br>NALTREXONE                                                                                                                            | 5.76<br>1.27                                                                                        | (4.54-7.31)<br>(1.02-1.59)                                                                                                            | 1.12 ± 0.065<br>0.94 ± 0.069                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| PARTIAL AGONISTS                                                                                                                                  |                                                                                                     |                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| BREMAZOCINE<br>LEVALLORPHAN<br>PENTAZOCINE<br>NALORPHINE<br>Q.NALORPHINE<br>NALBUPHINE                                                            | 1.42<br>5.65<br>478<br>38.9<br>298<br>40.3                                                          | (1.08-1.88)<br>(4.72-6.75)<br>(399-572)<br>(30.2-50.2)<br>(236-376)<br>(33.8-48.0)                                                    | $\begin{array}{r} 1.08 \pm 0.08 \\ 1.00 \pm 0.064 \\ 1.01 \pm 0.067 \\ 0.85 \pm 0.046 \\ 0.72 \pm 0.045 * \\ 0.86 \pm 0.041 * \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| KAPPA AGONISTS                                                                                                                                    |                                                                                                     |                                                                                                                                       | -                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| MR2034<br>EKC<br>TIFLUADOM<br>Q.TIFLUADOM<br>U50488<br>U69593<br>DYN A (1-13)<br>DYN A (1-17)<br>ICI200940<br>ICI197067<br>ICI204448<br>ICI204879 | 22.1<br>203<br>1790<br>3540<br>17000<br>39500<br>742<br>>50000<br>5170<br>20800<br>>50000<br>>50000 | (14.6-33.5)<br>(163-254)<br>(1380-2320)<br>(2360-5310)<br>(12800-22600)<br>(18900-82500)<br>(565-974)<br>(4500-5870)<br>(17400-24900) | $1.09 \pm 0.12 \\ 1.12 \pm 0.062 \\ 1.03 \pm 0.076 \\ 1.16 \pm 0.131 \\ 0.91 \pm 0.078 \\ 1.25 \pm 0.258 \\ 0.96 \pm 0.101 \\ 0.90 \pm 0.042 \\ 0.91 \pm 0.063 \\ 0.91 \pm 0.063 \\ 0.063 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.002 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\ 0.012 \\$ |
| MU/DELTA LIGANDS<br>ETORPHINE<br>GLYOL<br>MORPHINE<br>DADLE<br>DMPEA<br>ICI174864                                                                 | 33.6<br>4570<br>1630<br>9270<br>787<br>33800                                                        | (27.1-41.7)<br>(3260-6420)<br>(1220-2170)<br>(6660-12900)<br>(598-1030)<br>(18800-60700)                                              | $\begin{array}{r} 0.83 \pm 0.051*\\ 0.70 \pm 0.034*\\ 1.01 \pm 0.086\\ 0.71 \pm 0.046*\\ 0.67 \pm 0.028*\\ 0.59 \pm 0.175*\\ \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |

#### (598-1030) DMPEA 787 ICI174864 33800 (18800-60700) FENTANYL 921 (752-1130)

#### Table 2.4:

"Kappa suppressed" [<sup>3</sup>H]bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in Krebs/HEPES buffer + 10uM GppNHp and 1uM  $IC_{50}$  values with confidence limits, and Hill ICI204879. coefficients ( $\pm$  S.E.M.) obtained for a range of standards.

 $0.94 \pm 0.058$ 

\* Significantly different from one, P</=0.05.

resistant" site seen in HEPES is still present in the Krebs buffer system, and appears to comprise a larger proportion of the specific binding. In addition a number of ICI kappa agonists which showed no selectivity in HEPES show negligible affinity for the "dynorphin resistant" site in Krebs/HEPES + GppNHp.

# ii. "Kappa suppressed" [<sup>3</sup>H]bremazocine binding assay:

In order to investigate further the nature of the "dynorphin resistant" site in Krebs/HEPES buffer + GppNHp, [<sup>3</sup>H]bremazocine binding assays were run in the presence of 1µM ICI 204879, to suppress out the binding to the classical kappa binding-site. The displacement curve (see Figure 2.7) obtained for this compound showed a clear plateau at 70-75% inhibition of specific binding over the concentration range 300nM-10µM, suggesting that a concentration of 1µM would be sufficient to suppress all kappa receptor binding without preventing binding to the "dynorphin resistant" site. All other methods were unchanged. An average of 72.5% inhibition of specific binding, equivalent to a binding level of 204 specific cpm, was achieved in the "kappa suppressed" assay.

The IC<sub>50</sub> values and Hill coefficients obtained for a range of standards under these conditions are shown in Table 2.4.

Naloxone, naltrexone and bremazocine displaced [ ${}^{3}$ H]bremazocine binding in this "kappa suppressed" system with IC<sub>50</sub> values in the nanomolar range. Much lower affinities were obtained with the kappa agonists, with IC<sub>50</sub> values of 17 and 39µM for U50488 and U69593 respectively. Dynorphin A (1-17), ICI 204448 and ICI 204879 had IC<sub>50</sub> values greater than 50µM, in good agreement with the biphasic displacement curves obtained for these ligands in the unsuppressed assay. Hill coefficients not significantly different from one were obtained for the majority of the compounds tested. Exceptions to this were the partial agonists nalorphine, Q.nalorphine and nalbuphine, the kappa agonist ICI 200940, the mu/delta agonists etorphine, GLYOL, DADLE and DMPEA and the selective delta antagonist ICI 174864. Only DADLE,

| COMPOUND                                                                                                                                                              | <u>1µМ 204879</u>                                                                                   |                                                                                | NO ADDITIONS                                                                             |                                                                                                                    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| ANTAGONISTS                                                                                                                                                           | IC <sub>50</sub> (nM)                                                                               | N                                                                              | 1c <sub>50</sub>                                                                         | <b>N</b> .                                                                                                         |
| NALOXONE<br>NALTREXONE                                                                                                                                                | 5.76<br>1.27                                                                                        | 1.115<br>0.944                                                                 | 25.4<br>10.7                                                                             | 0.816*<br>0.930                                                                                                    |
| PARTIAL AGONISTS                                                                                                                                                      |                                                                                                     |                                                                                |                                                                                          |                                                                                                                    |
| BREMAZOCINE<br>LEVALLORPHAN<br>PENTAZOCINE<br>NALORPHINE<br>Q.NALORPHINE                                                                                              | 1.42<br>5.65<br>478<br>38.9<br>298                                                                  | 1.080<br>1.000<br>1.010<br>0.846<br>0.715*                                     | 1.09<br>5.51<br>408<br>186<br>1260                                                       | 0.898*<br>0.941<br>1.29*<br>1.08<br>0.587*                                                                         |
| KAPPA AGONISTS                                                                                                                                                        |                                                                                                     |                                                                                |                                                                                          |                                                                                                                    |
| MR2034<br>EKC<br>TIFLUADOM<br>Q.TIFLUADOM<br>U50488<br>U69593<br>DYN A (1-13)<br>DYN A (1-17)<br>IC1200940<br>IC1197067<br>IC1204448<br>IC1204879<br>MU/DELTA LIGANDS | 22.1<br>203<br>1790<br>3540<br>17000<br>39500<br>742<br>>50000<br>5170<br>20800<br>>50000<br>>50000 | 1.094<br>1.115<br>1.032<br>1.161<br>0.907<br>1.255<br>0.964<br>0.899*<br>0.913 | 11.4<br>121<br>137<br>1870<br>331<br>972<br>12.6<br>59.7<br>53.8<br>5.91<br>25.3<br>3.96 | 1.07<br>0.827*<br>0.687*<br>0.893<br>0.680*<br>0.650*<br>1.005#<br>0.779*#<br>0.565*<br>1.090#<br>0.847#<br>1.264# |
| ETORPHINE<br>GLYOL<br>MORPHINE<br>DADLE<br>DMPEA<br>ICI174874<br>FENTANYL                                                                                             | 33.6<br>4570<br>1630<br>9270<br>787<br>33800<br>921                                                 | 0.832*<br>0.696*<br>1.014<br>0.713*<br>0.666*<br>0.588*<br>0.944               | 26.9<br>50900<br>6030<br>>50000<br>33700<br>>50000<br>5030                               | 1.031<br>0.565*<br>0.736*<br>0.605*<br>0.737*                                                                      |

## Table 2.5:

 $[^{3}$ H]bremazocine binding (0.2nM) to guinea-pig cerebellar membranes in Krebs/HEPES buffer + 10µM GppHNp. A comparison between the IC<sub>50</sub> values and Hill coefficients (N) obtained in the presence, ("kappa suppressed") and absence, of 1µM ICI 204879.

- # Results calculated assuming a 70% maximum inhibition of specific binding.
- \* Significantly different from one, P</=0.05.

GLYOL, DMPEA, ICI 174864 and Q.nalorphine had Hill coefficients of less than 0.8.

Figures 2.10-2.13 show the curves obtained for a range of displacers, under these conditions.

Table 2.5 compares the  $IC_{50}$  values and Hill coefficients obtained in these experiments with those from the unsuppressed Krebs assay.

There was very little correlation between the IC<sub>50</sub> values obtained in these two systems. Most notably, the affinities obtained for the kappa agonists were greatly reduced in the suppressed assay, greater than 1000 fold in the case of ICI 204879.

The affinities of the mu/delta agonists however, were increased, up to 40 fold in the case of DMPEA, compared to the unsuppressed system. Similarly, 4 and 8 fold increases in affinity were seen for naloxone and naltrexone respectively under these conditions. The improvement in the Hill coefficients in the suppressed assay compared with the unsuppressed Krebs system, is also very marked.

In view of the high affinities obtained in the suppressed assay with naltrexone and naloxone, it was decided to further investigate the possibility that the "dynorphin resistant site" seen in the Krebs/HEPES buffer system might represent a low affinity mu receptor component.  $IC_{50}$  values from the "kappa" suppressed assay were therefore compared with data from a mu receptor binding assay run in Krebs/HEPES + GppNHp

## 3. [<sup>3</sup>H]NALOXONE BINDING IN RAT BRAIN MEMBRANES:

A low affinity mu receptor binding assay was therefore developed in rat whole brain membranes, using Krebs/HEPES buffer +  $10\mu$ M GppNHp, and 0.2nM [<sup>3</sup>H]naloxone, as described in the methods section.

Table 2.6 shows the IC<sub>50</sub> values and Hill coefficients obtained for a

| COMPOUND         | <u>IC<sub>50</sub> (nM)</u> |               | HILL COEFFICIENT     |  |
|------------------|-----------------------------|---------------|----------------------|--|
| ANTAGONISTS      |                             |               |                      |  |
| NALOXONE         | 3.00                        | (2.68-3.53)   | 1.05±0.025           |  |
| NALTREXONE       | 0.76                        | (0.58 - 1.01) | 0.90±0.068           |  |
| DIPRENORPHINE    | 0.78                        | (0.55-1.11)   | $1.23 \pm 0.116$     |  |
| PARTIAL AGONISTS |                             |               |                      |  |
| BREMAZOCINE      | 2.36                        | (1.69-3.32)   | 0.87±0.086           |  |
| LEVALLORPHAN     | 1.68                        | (1.18-2.40)   | 1.02 <u>+</u> 0.085  |  |
| PENTAZOCINE      | 270                         | (220–332)     | 1.05 <u>+</u> 0.061  |  |
| NALORPHINE       | 19.5                        | (15.5-24.5)   | 0.88±0.059           |  |
| Q.NALORPHINE     | 137                         | (85.0-221)    | 0.65±0.040*          |  |
| NALBUPHINE       | 16.7                        | (14.2-19.6)   | 0.89 <u>+</u> 0.021* |  |
| KAPPA AGONISTS   |                             |               |                      |  |
| MR2034           | 7.25                        | (4.74-11.1)   | 1.09±0.098           |  |
| EKC              | 224                         | (160-313)     | 0.83 <u>+</u> 0.079* |  |
| TIFLUADOM        | 1960                        | (1170-3290)   | 1.14 <u>+</u> 0.141  |  |
| Q.TIFLUADOM      | 2320                        | (1590-3390)   | 0.96 <u>+</u> 0.106  |  |
| U50488           | 14200                       | (12100-16700) | 0.86 <u>+</u> 0.029  |  |
| U69593           | >50000                      |               |                      |  |
| DYN A (1-13)     | 376                         | (246-576)     | 0.98±0.094           |  |
| DYN A (1-17)     | 6990                        | (3520-13900)  | 0.43±0.042*          |  |
| ICI200940        | 2070                        | (1550–2760)   | 0.83 <u>+</u> 0.046* |  |
| ICI197067        | 10500                       | (8980-12200)  | 0.90 <u>+</u> 0.037* |  |
| ICI204448        | >50000                      |               |                      |  |
| ICI204879        | 6410                        | (4320–9490)   | 0.65 <u>+</u> 0.042* |  |
| MU/DELTA LIGANDS |                             |               |                      |  |
| ETORPHINE        | 10.8                        | (8.11-14.5)   | 0.81±0.072*          |  |
| GLYOL            | 966                         | (646-1450)    | 0.76±0.051*          |  |
| MORPHINE         | 416                         | (306-568)     | 0.99 <u>+</u> 0.087  |  |
| DADLE            | 3190                        | (1860–5470)   | 0.56 <u>+</u> 0.054* |  |
| DMPEA            | 276                         | (187-406)     | 0.63±0.029*          |  |
| TCT174864        | 4570                        | (3300-6350)   | 0.77+0.067*          |  |

## Table 2.6:

ICI154129

FENTANYL

 $[^{3}$ H]Naloxone binding (0.2nM) to rat whole brain membranes in Krebs/HEPES +GppNHp. IC<sub>50</sub> values with confidence limits and Hill coefficients (± S.E.M.) obtained for a range of standards.

4930 (3640-6680) 214 (162-282)

\* Significantly different from one, P</=0.05.

0.86±0.055\*

 $0.86\pm0.059*$ 

#### range of standards in this assay

The displacement curves for naloxone, ICI 174864 and U50488 in this assay are shown in Figure 2.14.

The antagonists naloxone and naltrexone displaced the binding of  $[{}^{3}\text{H}]$ naloxone with IC<sub>50</sub>s in the low nanomolar range. Much lower affinities were however obtained for the mu selective agonists, with IC<sub>50</sub> values of 406nM and 966nM for morphine and GLYOL respectively.

The kappa agonists tifluadom, U50488 and U69593 also displaced [ ${}^{3}$ H]naloxone binding with IC<sub>50</sub> values 10-50 fold higher than those seen in the unsuppressed [ ${}^{3}$ H]bremazocine binding assay in Krebs. U69593 in particular was barely active, with an IC<sub>50</sub> of approximately 60 $\mu$ M. The Hill coefficients obtained with these agents were close to one, consistent with displacement from a single binding site. These results do not suggest any significant kappa component in this assay.

Hill coefficients significantly less than one were seen with a number of agents in the [<sup>3</sup>H]naloxone assay, in particular the mu/delta agonists GLYOL, DADLE, DMPEA and fentanyl, and the delta selective antagonists ICI 174864 and ICI 154129. The reasons for this are unclear, but the possibility of a small delta component cannot be excluded.

Displacement curves to GLYOL and DADLE appear in Figures 2.15 and 2.16.

A good correlation was obtained between the  $IC_{50}$  values obtained in the [ ${}^{3}$ H]naloxone binding assay and the "kappa suppressed" [ ${}^{3}$ H]bremazocine assay run in the presence of 1µM 204879, with a correlation coefficient of 0.979 and a slope of 1.003. This is represented graphically in Figure 2.17. No correlation was seen between the  $IC_{50}$  values obtained in the unsuppressed [ ${}^{3}$ H]bremazocine assays and either the [ ${}^{3}$ H]naloxone or suppressed [ ${}^{3}$ H]bremazocine systems (see Figure 2.18). The excellent correlation achieved between the  $IC_{50}$  values obtained in the [<sup>3</sup>H]naloxone and suppressed [<sup>3</sup>H]bremazocine binding assays, and the lack of correlation between either of these two systems and the unsuppressed [<sup>3</sup>H]bremazocine assay in Krebs, clearly suggests that the "dynorphin resistant" component investigated in the suppressed [<sup>3</sup>H]bremazocine assay represents a low-affinity mu receptor binding-site.

However the correlation was not complete and some differences were seen between the  $IC_{50}$  values obtained in the two assays. The majority of the suppressed [<sup>3</sup>H]bremazocine  $IC_{50}$ s were approximately 2-4 fold higher than those seen in the [<sup>3</sup>H]naloxone system. In the case of dynorphin A (1-17), ICI 204879, and ICI 174864 however, the suppressed [<sup>3</sup>H]bremazocine  $IC_{50}$  values were increased 7 fold over those seen in the [<sup>3</sup>H]naloxone assay. The reasons for this are unclear.

The majority of the Hill coefficients were greater than 0.8 in both assays, with the exception of Q.nalorphine, dynorphin A (1-17), ICI 204879, and a number of the mu/delta ligands, notably ICI 174864, GLYOL, DADLE and DMPEA, which showed markedly shallow slopes in both systems. These results suggest some correlation between the Hill coefficients achieved in the two systems, and also some remaining heterogeneity of binding in both assays, possibly of the same type.

4. SUPPRESSION OF [<sup>3</sup>H]NALOXONE AND "KAPPA SUPPRESSED" [<sup>3</sup>H]BREMAZOCINE ASSAYS BY ICI 174864 AND ICI 204879:

In order to investigate the possibility that the low Hill coefficients seen in the [<sup>3</sup>H]naloxone and suppressed [<sup>3</sup>H]bremazocine assays might be due to the presence of kappa or delta binding sites in these systems, displacement curves were generated to the mu selective agonist DMPEA in the presence of high concentrations of ICI 204879 and/or ICI 174864. DMPEA was chosen as the displacing ligand both because of its selectivity for the mu receptor, and the low Hill coefficients it displayed in both assays.

For this study, the concentration of ICI 204879 used in the suppressed

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|               | SUPPRESSED [               | SUPPRESSED [ <sup>3</sup> H]BREM. |                            | OXONE               |
|---------------|----------------------------|-----------------------------------|----------------------------|---------------------|
| ADDITIONS     | <u>IC<sub>50</sub>(nM)</u> | <u>N</u>                          | <u>IC<sub>50</sub>(nM)</u> | <u>N</u>            |
| NONE          | 787 (598-1030)             | 0.67±0.04*                        | 276 (187-406)              | 0.63±0.03*          |
| 1/10µM 204879 | 3240 (2400-4360)           | 0.72 <u>+</u> 0.05*               | 293 (195-441)              | 0.63±0.02*          |
| 1µM 174864    | 1120 (790-1600)            | 0.73±0.04*                        | 315 (212-467)              | 0.66±0.03*          |
| BOTH          | 3250 (2510-4220)           | 0.84 <u>+</u> 0.08*               | 492 (331-730)              | 0.69 <u>+</u> 0.05* |

#### Table 2.7:

"Kappa suppressed"  $[{}^{3}$ H]bremazocine binding (0.2nM) to guinea-pig cerebellar membranes and  $[{}^{3}$ H]naloxone binding (0.2nM) to rat whole brain membranes, in Krebs/HEPES + 10µM GppNHp. IC<sub>50</sub> values with confidence limits and Hill coefficients (N) (± S.E.M.) obtained for the displacer DMPEA ([D-Met<sup>2</sup>,Pro<sup>5</sup>]enkephalinamide) in the presence of 1 or 10µM ICI 204879, 1µM ICI 174864 or both these agents.

\* Significantly different from one, P</=0.05.

 $[^{3}$ H]bremazocine assay was raised from 1 to 10 µM, in order to block any possible remaining kappa binding. However, because of the lower IC<sub>50</sub> value obtained for ICI 204879 in the  $[^{3}$ H]naloxone assay (6.41µM) a concentration of 1µM ICI 204879 was used to suppress any kappa receptor binding component in this system. A 1µM concentration of the selective ICI antagonist ICI 174864 was chosen to block any binding to the delta receptor, in both assays.

The IC<sub>50</sub> values and Hill coefficients obtained with DMPEA in the two assays, under these various conditions are shown in Table 2.7. The displacement curves are shown in Figures 2.19 and 2.20.

In the [ ${}^{3}$ H]naloxone binding assay a mean of 16.4% inhibition of binding was achieved in the presence of 1µM ICI 204879, 21.2% in the presence of 1µM ICI 174864, and 32.9% with both ligands together. These values are in good agreement with the IC<sub>50</sub>s obtained for these ligands in the [ ${}^{3}$ H]naloxone binding system. The DMPEA displacement curves run under these conditions were superimposable, with no changes in either IC<sub>50</sub> value or Hill coefficient, suggesting that the displacement seen with ICI 174864 and ICI 204879 was likely to be from the mu receptor.

In the suppressed [<sup>3</sup>H]bremazocine assay the addition of 10µM ICI 204879 and/or 1µM ICI 174864 had no further effect on the percentage inhibition of binding obtained with 1µM ICI 204879. This is in line with  $IC_{50}$  values of 50 and 33.8µM respectively for these two compounds in this assay. The  $IC_{50}$  values obtained for DMPEA were increased up to 5 fold in the presence of 10µM ICI 204879, whereas ICI 174864 appeared to have no effect. The Hill coefficients were unchanged. The reason for this increase in  $IC_{50}$  value, in the absence of any change in the level of suppression, is unclear.

The successive suppression of these assay systems with high concentrations of kappa and delta ligands therefore does not appear to have any clear effect on the binding of the mu selective agonist DMPEA. This suggests that delta or kappa contamination is not likely to be responsible for the shallow slopes seen with this ligand.

However, the possibility, particularly of a delta component, cannot be completely excluded on this basis.

#### 5. SUMMARY:

[<sup>3</sup>H]bremazocine binding to guinea-pig cerebellar membranes in Krebs/HEPES buffer + GppNHp was predominantly to a low affinity conformation of the kappa receptor. There was no evidence for either a delta or sigma component to the binding under these conditions. However, due to the lack of selective mu receptor antagonists the possibility of a low affinity mu receptor component could not be completely excluded.

Most of the compounds tested displaced [ ${}^{3}$ H]bremazocine binding with Hill coefficients significantly less than one. In the case of the dynorphin peptides and the ICI kappa agonists 204879 and 204448 the displacement curves obtained were clearly biphasic, with 30% of the specific binding not displaceable at concentrations up to 10  $\mu$ M.

This heterogeneity of binding was similar to the "dynorphin resistant" component seen in HEPES buffer, with the exception of the ICI kappa agonists 204448 and 204879, which did not produce biphasic displacement curves in the HEPES assay.

 $IC_{50}$  values from a kappa suppressed [<sup>3</sup>H]bremazocine assay were therefore compared with data from a low affinity mu receptor binding assay, using the same buffer system. An excellent correlation was found between  $IC_{50}$  values achieved in these two systems, suggesting that the "dynorphin resistant" component in the [<sup>3</sup>H]bremazocine assay might represent a low affinity mu receptor binding-site. There was no correlation between the  $IC_{50}$ s obtained in the [<sup>3</sup>H]naloxone and unsuppressed [<sup>3</sup>H]bremazocine binding assays.

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Displacement of total  $[{}^{3}H]$ bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10  $\mu$ M GppNHp, by (±)bremazocine (+----+) and by naloxone (=----==).

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Displacement of specific  $[{}^{3}H]$ bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10  $\mu$ M GppNHp, by [D-Met<sup>2</sup>, Pro<sup>5</sup>]enkephalinamide (DMPEA): % inhibition of control binding vs. log displacer concentration.



Displacement of specific  $[{}^{3}$ H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10  $\mu$ M GppNHp by morphine: % inhibition of control binding vs. log morphine concentration.





Displacement of specific [<sup>3</sup>H]bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10 µM GppNHp by [D-Ala<sup>2</sup>, (Me)Phe<sup>4</sup>, Gly(ol)<sup>5</sup>]enkephalin (GLYOL): % inhibition of control binding vs. log displacer concentration. 

Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES ( $\blacksquare$ ) and Krebs/HEPES buffer containing 10 µM GppNHp ( $\blacksquare$ --- $\blacksquare$ ) by dynorphin A(1-17): % inhibition of control binding vs. log dynorphin concentration.

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Displacement of specific  $[{}^{3}H]$  bremazocine binding (0.2 nM) from guinea-pig cerebellum membranes in HEPES ( $\blacksquare$ ) and Krebs/HEPES buffer containing 10 µM GppNHp ( $\blacksquare$ --- $\blacksquare$ ), by dynorphin A(1-13): % inhibition of control binding vs. log displacer concentration.

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Displacement of specific [<sup>3</sup>H]bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in HEPES (\_\_\_\_\_) and Krebs/HEPES buffer containing 10µM GppNHp (\_\_\_\_\_) by ICI 204879: % inhibition of control binding vs. log ICI 204879 concentration. •



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Figure 2.9



Displacement of specific  $[{}^{3}H]$  bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp and 1µM ICI 204879 by: (±) bremazocine  $\blacksquare$ , naloxone  $\blacktriangle$ , EKC  $\blacklozenge$ --- $\diamondsuit$  and U69593  $\blacksquare$  : % inhibition of control binding vs. log displacer concentration. •





Displacement of specific [<sup>3</sup>H]bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp and 1µM ICI 204879 by [D-Ala<sup>2</sup>, (Me)Phe<sup>4</sup>, Gly(ol)<sup>5</sup>]enkephalin (GLYOL): % inhibition of control binding vs. log GLYOL concentration.



Figure 2.12

Displacement of specific [<sup>3</sup>H]bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp and 1µM ICI 204879 by [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin (DADLE): % inhibition of control binding vs. log DADLE concentration. ·



Displacement of specific [<sup>3</sup>H]bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp and 1µM ICI 204879 by [D-Met<sup>2</sup>, Pro<sup>5</sup>]enkephalinamide (DMPEA): % inhibition of control binding vs. log DMPEA concentration.

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Displacement of specific [<sup>3</sup>H]naloxone (0.2nM) binding from rat whole brain membranes in Krebs/HEPES buffer containing 10µM GppNHp by naloxone , ICI 174864 and U50488 : % inhibition of control binding vs. log displacer concentration.

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Displacement of specific  $[{}^{3}H]$ naloxone (0.2nM) binding from rat whole brain membranes in Krebs/HEPES buffer containing 10µM GppNHp by  $[D-Ala^{2}, (Me)Phe^{4}, Gly(ol)^{5}]$ enkephalin (GLYOL): % inhibition of control binding vs. log GLYOL concentration. ,



Displacement of specific  $[{}^{3}H]$ naloxone (0.2nM) binding from rat whole brain membranes in Krebs/HEPES buffer containing 10µM GppNHp by  $[D-Ala^{2}, D-Leu^{5}]$ enkephalin (DADLE): % inhibition of control binding vs. log DADLE concentration.

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Correlation between the  $IC_{50}$  values (nM) obtained from [ ${}^{3}$ H]bremazocine binding to guinea-pig cerebellum membranes in Krebs/HEPES buffer (containing 10µM GppNHp and 1µM ICI 204879) and [ ${}^{3}$ H]naloxone binding binding to rat whole brain membranes in Krebs/HEPES buffer (containing 10µM GppNHp). Slope = 1.003, correlation coefficient = 0.979.



Correlation between the IC<sub>50</sub> values (nM) obtained from [<sup>3</sup>H]bremazocine binding to guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp, and A: [<sup>3</sup>H]naloxone binding to rat whole brain membranes in Krebs/HEPES buffer (containing 10µM GppNHp); B: [<sup>3</sup>H]bremazocine binding to guinea-pig cerebellum membranes in Krebs/HEPES buffer (containing 10µM GppNHp and 1µM ICI 204879).

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 $[^{3}$ H]Naloxone (0.2nM) binding to rat whole brain membranes in Krebs/HEPES buffer containing 10µM GppNHp. Displacement by  $[D-Met^{2}, Pro^{5}]$ enkephalinamide (DMPEA) in the absence of suppressing agents . , and in the presence of 1µM ICI 204879 . , 1µM ICI 174864 . , and both 1µM ICI 204879 and ICI 174864 . % inhibition of control binding vs. log DMPEA concentration. .

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### Figure 2.20

Displacement of specific [<sup>3</sup>H]bremazocine (0.2nM) binding from guinea-pig cerebellum membranes in Krebs/HEPES buffer containing 10µM GppNHp by [D-Met<sup>2</sup>, Pro<sup>5</sup>]enkephalinamide (DMPEA) in the presence of 1µM ICI 204879 . 1µM ICI 204879 plus 1µM ICI 174864 . 10µM ICI 204879 . and 10µM ICI 204879 plus 1µM ICI 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 174864 . 17488 . 17488 . 17488 . 17488 . 17488 . 1748 . 17488 . 17488 . 17488 .

#### **DISCUSSION:**

## 1. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: HEPES BUFFER SYSTEM

#### A. ASSAY VALIDATION:

Since its introduction in 1980 [122] bremazocine has been shown to be a potent and long acting kappa agonist, both <u>in-vivo</u> and <u>in-vitro</u>, with no evidence of any agonist action at the mu receptor. Miller et al. [95], in their study of the effects of a range of opioid standards in different isolated tissue models, also showed that although bremazocine had antagonist activity at the mu and delta receptors, its agonist actions were mainly through the kappa receptor. Data from the MVD studies performed by this group however, suggested that bremazocine was a partial agonist at the kappa receptor, compared with the full agonist EKC.

 $(-)[{}^{3}H]$ bremazocine has since become established as a useful, universal opioid receptor ligand. As a result of its low opioid receptor selectivity, this compound is usually reported as binding to an apparently homogeneous population of binding sites, with an affinity of 0.2-0.6nM [44]. Following the suppression of mu and delta binding components, values of 0.04-0.07nM are given for the kappa receptor affinity of  $[{}^{3}H]$ bremazocine in a number of tissues, including guinea-pig cerebellum, guinea-pig whole brain and rat whole brain membranes [44, 45, 116]. Displacement of mu and delta selective labelled ligands such as  $[{}^{3}H]$ GLYOL and  $[{}^{3}H]$ DPDPE, by (-)bremazocine yields affinity values of 0.3-0.6nM and 0.78nM at the mu and delta receptors respectively [30, 77, 44]. Bremazocine therefore binds with high affinity to all three opioid receptor types, but shows approximately 10 fold selectivity for the kappa receptor.

i. [<sup>3</sup>H]Bremazocine Binding at 25<sup>o</sup>C:

In this study, the binding of 0.2nM (-)[<sup>3</sup>H]bremazocine to guinea-pig

cerebellar membranes was displaced by unlabelled (±)bremazocine with an IC<sub>50</sub> of 0.78nM and a Hill coefficient of 1.1. (+)Bremazocine has little opioid receptor affinity [116], therefore assuming a value of approximately 0.06nM for the kappa affinity of (-)[<sup>3</sup>H]bremazocine, a Ki (Affinity constant obtained by displacement) of 0.09nM can be calculated for the (-) isomer of (±)bremazocine in this study, using the Cheng-Prusoff equation [18]. This is in reasonable agreement with the literature values and also suggests that the IC<sub>50</sub>s obtained for the kappa standards tested in this assay should be about 4-5 fold higher than their actual Ki values at the kappa receptor. This is in fact the case, with reported affinities of 2.9 and 5.4nM for U50488 and U695693 respectively [116, 81] and 0.7nM for EKC [116], compared with calculated Ki values of 4.8nM, 2.9nM and 1.20nM respectively for these 3 agents in this study. No comparative data is available for the ICI kappa standards. However, approximate Ki values of 7.6 and 1.6nM can be obtained from this study for ICI 204448 and ICI 204879 respectively, in line with their high potency as kappa agonists, both in-vivo and in-vitro [28].

The high affinity kappa peptides dynorphin A (1-13), dynorphin A (1-17) and dynorphin B, and also the non-selective peptide  $\beta$ -endorphin were tested as displacers in the [<sup>3</sup>H]bremazocine system run at 25°C. The Ki values obtained compared well with data obtained by other groups in kappa receptor binding assays [25, 154, 110, 77]. As these literature values were all obtained in assay systems run at 0°C, there is also no suggestion of any stability problem with these longer peptides in the 25°C assay used in this study.

The results obtained for the standards tested in the guinea-pig cerebellum in HEPES assay therefore suggest that despite its lack of selectivity [ ${}^{3}$ H]bremazocine predominantly labels the kappa opioid receptor type, under the conditions used. This is confirmed by the low affinities obtained for the mu/delta selective agents GLYOL, DADLE, morphine and fentanyl, with IC<sub>50</sub> values 10-100 fold higher than their quoted mu/delta Ki values, [29, 10, 66] and is in line with considerable literature evidence suggesting that guinea-pig cerebellum contains mainly kappa opioid receptors, with very little mu or delta

#### receptor contamination.

The high proportion of kappa receptors in guinea-pig cerebellum was first reported by Robson et al. in 1984 [116]. Of the opioid binding-sites in this tissue 84% were found to be of the kappa type, as defined by [<sup>3</sup>H]bremazocine in the presence of mu and delta suppressing agents. Itzhak et al. [63], in the same year, compared the binding of solubilised and membrane-bound opioid receptors from guinea-pig whole brain and cerebellum preparations. The binding of the mu and delta ligands [<sup>3</sup>H]GLYOL and [<sup>3</sup>H]DADLE to the cerebellar membrane preparation was only 4% of that seen in the guinea-pig whole brain membranes, compared with 55% for the binding of [<sup>5</sup>H]etorphine, [<sup>3</sup>H]EKC and [<sup>3</sup>H]bremazocine. Following digitonin solubilisation of the receptors from these two tissues, sucrose density centrifugation of the guinea-pig whole brain extract produced two peaks of differing molecular weight, identified as mu/delta and kappa respectively. Only one peak however, was found in the extract from guinea-pig cerebellum, corresponding to the kappa receptor. Frances et al., [37] were also unable to detect any specific binding with the mu/delta ligands <sup>3</sup>H]GLYOL and <sup>3</sup>H][D-Ser<sup>2</sup>, Leu<sup>5</sup>, Thr<sup>6</sup>]enkephalin (DSLET) in guinea-pig cerebellar membranes. These studies, plus a number of others, [64, 78] confirm the original finding, and suggest that the guinea-pig cerebellum can be considered as a virtually pure source of kappa opioid receptors.

In the present study, the binding of the mu/delta selective ligands  $[{}^{3}\text{H}]\text{GLYOL}$  and  $[{}^{3}\text{H}]\text{DADLE}$  to guinea-pig cerebellar membranes was very low. The levels obtained for  $[{}^{3}\text{H}]\text{GLYOL}$  were less than 10% of those seen with  $[{}^{3}\text{H}]\text{bremazocine}$ , even following correction for the differing receptor occupancies at the concentrations used, and no significant binding was seen with  $[{}^{3}\text{H}]\text{DADLE}$  following suppression of the mu receptor component. The total absence of delta receptor binding was also confirmed in the  $[{}^{3}\text{H}]\text{bremazocine}$  assay by the displacement curve to the delta selective antagonist ICI 174864, in which no significant inhibition of binding was seen at concentrations below 3µM. This data therefore suggests that although low levels of "non-kappa" opioid receptor binding may be present in the guinea-pig cerebellum, they are

likely to be due to mu rather than delta receptor contamination.

This conclusion receives further support from the displacement curves to GLYOL and DADLE. The  $IC_{50}$  values obtained for these agents in the  $[{}^{3}H]$ bremazocine assay were very high. However in both cases a small proportion of the binding, (approximately 10%) was displaced at much lower concentrations. GLYOL is approximately 3000 fold selective for the mu receptor (Ki = 1.86nM), and DADLE, although it has a lower Ki at the mu receptor (9.32nM), also has very little kappa receptor affinity [26]. This data would therefore be in line with the presence of a small mu receptor component in this system.

Similarly, opioid receptors other than kappa have also been detected by other groups, in this tissue, using suppressing agents. For instance Robson et al. [116] obtained 13% inhibition of [<sup>3</sup>H]bremazocine binding in guinea-pig cerebellum, in the presence of 1µM DADLE, and a 15% reduction in the binding of [<sup>3</sup>H]etorphine, [<sup>3</sup>H]EKC and [<sup>3</sup>H]bremazocine to cerebellar membranes was also seen by Itzhak et al. [63], in the presence of 100nM GLYOL and DADLE. In this study a larger reduction of 27% in [<sup>3</sup>H]bremazocine binding to guinea-pig cerebellar membranes was seen in the presence of 3µM DADLE. This is however a high concentration, which in addition to the suppression of any mu and delta component, would also be expected to reduce kappa receptor binding.

The presence of  $3\mu$ M DADLE in the [ ${}^{3}$ H]bremazocine binding system in HEPES buffer had varied effects on the binding profiles of the agents tested. The IC<sub>50</sub> values and Hill coefficients obtained for GLYOL and morphine were only slightly altered, suggesting that any mu component, if present, is small. The displacement curves to the kappa selective agonists U50488 and U69593 were however steeper in the presence of DADLE, which would be consistent with mu receptor blockade. No change was seen in the shape of the dynorphin A (1-13) and (1-17) displacement curves.

The IC<sub>50</sub> values obtained for U69593, and the dynorphin peptides against [<sup>3</sup>H]bremazocine binding were also increased in the presence of

3µM DADLE, and the proportion of dynorphin resistant binding rose from 15 to 20% of the specific binding. These changes in  $IC_{50}$  value are not likely to be due to the removal of a mu component, as this would be expected to have the opposite effect, if any. It is more likely that the suppression of some kappa binding, by the high concentration of DADLE used, is causing a decrease in the apparent kappa affinity of the displacing agents, in line with the predictions of the Langmuir equation [70]. The loss of kappa binding would also explain the lowering of the binding plateau seen with dynorphin A (1-13) and (1-17).

It would appear therefore, that [<sup>3</sup>H]bremazocine labels a small number of mu receptors in guinea-pig cerebellum, under the assay conditions used. The exact proportion is difficult to assess, but is likely to represent 5-10% of the specific binding of [<sup>3</sup>H]bremazocine. There is no evidence of any delta receptor contamination.

Both sigma and PCP binding sites have been identified in neural tissues from several species, including rat, guinea-pig and human [155, 135, 139]. Only one study however, refers to guinea-pig cerebellum [139]. In this paper the distribution of "etorphine inaccessible" binding-sites, considered to represent sigma receptors, was studied in different areas of the guinea-pig brain, and compared with [<sup>3</sup>H]naloxone binding in the same regions. The highest levels of "etorphine inaccessible" binding were found in midbrain and brainstem, followed by cerebellum and striatum. [<sup>3</sup>H]naloxone binding by contrast, was highest in midbrain, and low in cerebellum. This suggests that there may be sigma sites present in guinea-pig cerebellar membranes.

There is no evidence however, that  $(-)[{}^{3}H]$  bremazocine is likely to bind to either the sigma or the PCP sites, at the concentrations used – in this assay system. McLawhon et al., [89] in a study in a neuroblastoma Chinese hamster brain clonal hybrid cell line (NCB-20), obtained IC<sub>50</sub> values of 25 and 200nM for (±)bremazocine, against  $[{}^{3}H]$ EKC and  $[{}^{3}H]$ SKF10047, under conditions in which naloxone, morphine and etorphine were completely inactive. It is in addition quite

possible that this activity resides largely in the (+) isomer of bremazocine, in line with the reverse stereoselectivity commonly seen for opioid ligands at the sigma binding site.

In this study 100% of the specific binding of  $[{}^{3}H]$  bremazocine was displaced by 1µM naloxone, and the same degree of displacement was seen with morphine. Neither of these agents show any affinity for the sigma or PCP binding sites [146, 139]. In addition, no displacement was seen with haloperidol, which has high affinity for the sigma binding site, at concentrations up to 1µM. This therefore confirms that  $[{}^{3}H]$  bremazocine does not bind to either the sigma or PCP sites, under the conditions used in the HEPES assay in guinea-pig cerebellum.

# ii. [<sup>3</sup>H]Bremazocine binding at 0<sup>°</sup>C in the presence of protease inhibitors:

Peptide stability in binding incubations is dependent on a number of factors, including the source of the tissue homogenate, the length of the peptide, and the presence of particular cleavage sites. Garzon et al. [40], using a [<sup>3</sup>H]naloxone binding assay run in mouse brain homogenates, found that although dynorphin A (1-17) was reasonably stable over a period of 20 min at 37°C, both the shorter dynorphin peptides (up to 1-10) and a and ß neoendorphin were rapidly degraded. This has also been shown as part of a study by Gillan et al. [45] in which only 2-4% of added [<sup>3</sup>H]dynorphin A (1-8) and (1-9) were recovered as unchanged peptide following a 30 min incubation at 37°C with guinea-pig whole brain membranes. However, much higher levels of unchanged peptide were recovered after incubation at  $0^{\circ}C$  for 120 min in the presence of the peptidase inhibitors bestatin and captopril. Under these conditions the maximal binding capacities of [<sup>3</sup>H]dynorphin A (1-8) and (1-9) in guinea-pig whole brain membranes were also comparable to that of [<sup>3</sup>H]bremazocine, again suggesting no significant breakdown of the peptides.

Therefore, in order to study a wider range of peptides,  $[^{3}H]$  bremazocine binding assays were run for 60 min at 0<sup>°</sup>C, in the

presence of 30µM bestatin and 300µM captopril. Under these conditions  $[{}^{3}\text{H}]$ bremazocine binding was very much reduced, an effect that was not due to the inhibitor cocktail. This is in line with the results of Gillan et al., [45] who obtained a kappa receptor affinity of only 0.255nM for  $[{}^{3}\text{H}]$ bremazocine in guinea-pig whole brain membranes, following incubation for 150 min at 0°C, compared with a value of 0.06nM at 25°C, and also a 40% reduction in receptor numbers. As a consequence, this group found  $[{}^{3}\text{H}]$ bremazocine to be less selective for the kappa receptor under these conditions, with only 27% of the specific binding at 0°C resistant to mu/delta suppression, compared with 49% at 25°C.

Although in this study, the altered assay conditions had no significant effect on either the  $IC_{50}$  or the Hill coefficient obtained for the non-selective antagonist naloxone, the affinities of the kappa agonists EKC and U69593 were markedly reduced in the 0°C assay system, with increases in the  $IC_{50}$  values of 7 and 100 fold respectively. This provides further support for the possibility that kappa receptor affinities may be selectively decreased at 0°C, thus leading to an altered binding profile for [<sup>3</sup>H]bremazocine, and lowered affinities for kappa selective displacing agents.

However, considerable care must be taken in the interpretation of these results, due to the rather short incubation time used for the  $0^{\circ}$ C assay. Although no detailed information is available on the time taken for [<sup>3</sup>H]bremazocine binding to come to equilibrium, either at  $25^{\circ}$ C or  $0^{\circ}$ C, most of the studies in the literature quote incubation times of 120-150 min for this ligand at  $0^{\circ}$ C. After 60 min [<sup>3</sup>H]bremazocine binding may therefore not have reached equilibrium, and this could explain both the low levels of specific binding and the altered displacement curves seen in this study.

Despite these problems, the displacement curves to dynorphin A (1-13) and dynorphin B obtained in the  $[{}^{3}\text{H}]$ bremazocine assay at  $0^{\circ}\text{C}$ , were similar in shape to those seen at 25 $^{\circ}\text{C}$ , with only small shifts in the IC<sub>50</sub> values. The maximum inhibition of binding seen with these compounds was slightly reduced, under these conditions, again possibly

due to a reduction in  $[{}^{3}H]$  bremazocine binding to the kappa receptor. However this was adjusted for in the calculation of the IC<sub>50</sub> values and slopes.

The majority of  $[{}^{3}H]$  bremazocine specific binding under these conditions was displaced by the unstable peptides MEAP, MEAGLE and a and ß neo-endorphin with IC<sub>50</sub> values in line with their kappa receptor affinities, as quoted in the literature [97, 25]. It was not possible to compare the binding of the peptides in the presence and absence of DADLE, due to the low number of specific counts at  $0^{\circ}C$ .

# iii. [<sup>3</sup>H]EKC Binding:

The benzomorphan ligand EKC has been found to be a full agonist at the kappa receptor, although it also has high affinity for the mu receptor in isolated tissue preparations [95]. The binding data reported for this compound shows a fairly non-selective profile, with mu and delta affinities of 1.80nM and 6.24nM respectively, and a kappa receptor affinity of 0.73nM [90].

The opioid receptor affinity profile of  $[{}^{3}H]EKC$  is therefore very similar to that of  $[{}^{3}H]$ bremazocine, and appears to vary only in the higher kappa agonist efficacy of EKC. This is borne out by the results from this study, which show almost identical binding profiles for these two ligands in guinea-pig cerebellar membranes. Displacement of  $[{}^{3}H]EKC$  binding by low concentrations of GLYOL and DADLE was less than 10%, suggesting no significant mu or delta receptor contamination.

A higher concentration of DADLE (10µM), was used in this assay, to suppress any possible mu/delta binding. This produced 45% inhibitionof specific binding, compared with the 27% reduction seen in the  $[^{3}H]$ bremazocine system in the presence of 3µM DADLE. However 45.22% inhibition of  $[^{3}H]$ bremazocine binding was obtained by 10µM DADLE in the full displacement curve, in close agreement with the value from the  $[^{3}H]$ EKC assay. The effect of DADLE on the displacement curves was also similar in the two assay systems, with a small increase in the slope of the  $[{}^{3}\text{H}]\text{EKC}$  displacement curve to U69593, and a slight reduction in the maximum inhibition of binding seen with the dynorphin peptides (90-85%). The only difference was in the  $[{}^{3}\text{H}]\text{EKC}$  displacement curve to GLYOL, which was slightly shifted to the right in the presence of DADLE, an effect not seen in the  $[{}^{3}\text{H}]\text{bremazocine}$  assay.

#### **B.HETEROGENEITY OF BINDING:**

Two main features tend to suggest heterogeneity of binding in the HEPES buffer system, the low Hill coefficients seen with the majority of the agonist ligands, and the biphasic displacement curves obtained for the dynorphin peptides. It is not clear to what extent these two observations are related. The Hill coefficients seen with the peptides were markedly increased when the data was recalculated to exclude the second component of the binding. However, all were still significantly less than one, suggesting that the low Hill coefficients may not be entirely due to the presence of this binding-site. These two effects will therefore initially be discussed separately.

#### i. Hill Coefficients.

Binding displacement curves with Hill coefficients significantly less than one, are classically considered to represent binding to more than one binding-site or binding-site conformation. The majority of the antagonists and partial agonists tested in this study displaced [<sup>3</sup>H]bremazocine binding with Hill coefficients close to one. With the exception of etorphine however, all the agonist ligands, whether kappa or mu/delta selective, displayed Hill coefficients significantly less than one.

Robson et al. [116] in their study of [<sup>3</sup>H]bremazocine binding in guinea-pig cerebellum, obtained Hill coefficients close to one for a number of agonist and antagonist displacers. A value of 0.79 was seen with U50488, which was increased to 1.0 in the presence of mu and

delta suppressing agents. This is in agreement with results from this study, which also show an increase in the U50488 Hill coefficient, in the presence of DADLE, and suggests the possible involvement of a mu/delta component in both cases. However, the shallow slopes obtained for EKC and tifluadom in this study were not observed by Robson et al.

Gairin et al. [39] also obtained low Hill coefficients for U50488 and dynorphin A (1-17) against [<sup>3</sup>H]bremazocine binding in guinea-pig cerebellum. Frances et al. [37] however, using [<sup>3</sup>H]diprenorphine to bind to kappa receptors in guinea-pig cerebellum, obtained Hill coefficients greater than 0.8 for all displacers tested, including GLYOL, morphine, EKC and U50488.

The lowest Hill coefficients seen in this study (values of less than 0.7) included those for the selective kappa agonists ICI 197067, U69593, U50488 and ICI 204448, the mu/delta ligands GLYOL, DMPEA, morphine and fentanyl and the dynorphin peptides even after correction for the "dynorphin resistant" site. Slightly higher values were obtained for the kappa agonists EKC and tifluadom. In the case of the selective mu/delta and kappa compounds this pattern does suggest the possible involvement of a mu or delta component, as a greater difference in the affinity of a displacer for separate binding components, would be expected to lead to a shallower slope. However a Hill coefficient of 0.70 was achieved for dynorphin A (1-17) after correction of the binding maximum, although this peptide is only 3 fold kappa selective [25]. This lack of selectivity is also shown by dynorphin B and dynorphin A (1-13), [25, 154] for which low Hill coefficients were obtained in this assay, even after correction of the binding maximum. Similarly, the kappa agonist EKC (Hill coefficient = 0.74) is almost completely non-selective in low ionic strength binding systems, [90], and tifluadom (Hill coefficient=0.80) also does not discriminate between mu and kappa receptors, under these conditions, although it has a lower affinity at the delta receptor [109]. The results obtained with EKC, tifluadom and the dynorphin peptides therefore do not support the suggestion that the low Hill coefficients are entirely due to a mu/delta component, although his may be

involved. The data from the [<sup>3</sup>H]bremazocine assay run in the presence of DADLE also does not fully support this conclusion. Steeper slopes were achieved for U50488 and U69593, but there was no increase in the Hill coefficients obtained for GLYOL, morphine, or the dynorphin peptides following correction for the "dynorphin resistant" subsite.

Heterogeneous agonist displacement of [<sup>3</sup>H]antagonist binding, has been shown in a number of binding systems selective for one receptor type, and is thought to be due to the presence of low and high agonist affinity receptor conformations. Antagonist ligands bind to both conformations with high affinity, and where a [<sup>3</sup>H]antagonist ligand is displaced by an agonist this results in a shallow, extended displacement curve.

The contrast seen, in this study, between the high Hill coefficients obtained with the majority of the antagonist and partial agonist ligands tested, and the low values seen with even non-selective agonists such as tifluadom, EKC and the dynorphin peptides, suggests that under these assay conditions [<sup>3</sup>H]bremazocine may label both high and low agonist affinity conformations of the kappa receptor. The large shifts in agonist affinity seen in the [<sup>3</sup>H]bremazocine binding assay in Krebs /HEPES + GppNHp also clearly show that the kappa receptor is capable of existing in both low and high affinity conformations. The largest shifts in affinity between the HEPES and Krebs assay systems were seen for U69593 and the dynorphin peptides, even after correction for the presence of the dynorphin resistant site. These compounds also showed amongst the lowest Hill coefficients in the HEPES system, supporting the possible involvement of a low affinity kappa conformation.

EKC is generally considered to be a full agonist at the kappa receptor, and in this study showed a 31 fold shift in affinity between the two buffer systems, from an  $IC_{50}$  of 3.87nM in the [<sup>3</sup>H]bremazocine assay in HEPES, to 121nM in the Krebs/HEPES buffer system. A Hill coefficient of 0.83 was obtained in the Krebs assay, suggesting that 120nM represents a reasonable approximation of the affinity of EKC for the low affinity conformation of the kappa receptor. On this basis,

EKC is not likely to bind significantly to a low affinity kappa site at the concentration (0.5nM) used in the  $[^{3}H]$ EKC binding assay.

In this study Hill coefficients significantly lower than one were obtained for all the ligands tested against  $[{}^{3}H]EKC$  binding, both in the presence and absence of 10µM DADLE. These remained low, in the case of the dynorphin peptides, even after correction for the "dynorphin resistant" component of the binding. These results suggest that some other factor may be involved in the low agonist Hill coefficients seen in the  $[{}^{3}H]EKC$  binding assay, and also possibly in the  $[{}^{3}H]$ bremazocine system, although the involvement of a low affinity kappa binding site cannot be excluded.

#### ii. Dynorphin-resistant subsite:

Displacement of  $[{}^{3}H]$  bremazocine binding in HEPES buffer at 25°C, by The dynorphin peptides dynorphin A (1-17), dynorphin A (1-13), dynorphin B and B-endorphin was complex. These ligands all displaced approximately 85% of the specific binding with high affinity, but failed to displace the remaining 15% at concentrations up to 10µM. In the case of dynorphin A(1-17) this effect was most pronounced, with a clear plateau in the displacement curve between 30nM and 1µM dynorphin. Although shallow displacement curves were also seen with many of the other ligands tested, only the dynorphin peptides produced clearly biphasic curves of this particular type, leading to the suggestion of a "dynorphin resistant" binding component. Biphasic displacement of [<sup>3</sup>H]bremazocine binding run at 0<sup>o</sup>C in the presence of peptidase inhibitors was also seen with the kappa selective peptides a and ß neoendorphin, MEAP and MEAGLE. In the case of MEAP and the neoendorphins clear plateaux were formed at 80-85% inhibition of specific binding, with no further displacement at concentrations up to 10µM.

In the  $[{}^{3}H]$  bremazocine assay run at 25°C, the suppression of any possible mu or delta component by high concentrations of DADLE, did not abolish or in any way reduce the proportion of the binding

resistant to dynorphin A (1-13) or (1-17). The position of the binding plateau seen with these ligands was in fact slightly lowered in the presence of DADLE. This was probably due to the displacement of some kappa receptor binding by this concentration of DADLE, leading to a relative increase in the proportion of "dynorphin resistant" binding.

Similar results were also obtained in the [<sup>3</sup>H]EKC binding assay run in HEPES buffer. Displacement curves to dynorphin A (1-17) and (1-13) were clearly biphasic, with a "dynorphin resistant" component comprising approximately 10% of the specific binding. This component was again slightly increased in the presence of 10µM DADLE.

The inability of DADLE to suppress or even reduce the proportion of "dynorphin resistant" binding in either of these two assays clearly confirms that this component cannot be defined as either a high affinity mu or delta binding-site.

Two reports are available in the literature providing clear evidence for a similar binding-site to that described here. In the first of these, Morre et al., [97] using a suppressed [<sup>3</sup>H]EKC binding assay in guinea-pig whole brain membranes, obtained a classic kappa receptor profile with a range of standard ligands. Dynorphin A (1-17) however displaced 80% of the specific binding with an  $IC_{50}$  value of 1.6nM, whereas the remaining 20% was resistant to displacement at concentrations up to 1µM. This "dynorphin resistant" component was then studied in the presence of 100nM dynorphin A (1-17) plus standard mu/delta suppressors. The affinities obtained for standard kappa ligands such as bremazocine, diprenorphine, naloxone and U50488, were similar to those seen against "total" kappa binding, and no displacement was seen with either DADLE or GLYOL. A range of peptides, including dynorphin A (1-17), B-neoendorphin and MEAP, with high affinity for total kappa binding, were however completely inactive against this "dynorphin resistant" site, at concentrations up to 100µM. Any possible PCP or sigma binding component was excluded by the addition of 10µM PCP as a suppressing agent, and confirmed by the high affinity of naloxone. The absence of any displacement of [<sup>3</sup>H]EKC

from the dynorphin resistant subsite by either GLYOL or DADLE, clearly shows that this component cannot be defined as a high affinity mu or delta binding-site.

In the second report, [110] human amygdala membranes were used to study the binding of [ ${}^{3}$ H]diprenorphine to the kappa receptor, in the presence of 100nM GLYOL and DADLE to suppress mu and delta binding. Dynorphin A (1-17) and dynorphin A (1-13) displaced only 50% of the specific binding with high affinity, whereas affinities for the remainder of the binding were approximately 200 fold lower, with only 70-80% displacement at 10 $\mu$ M. The profile obtained was otherwise kappa-like.

In addition to these two studies, Robson et al. [115] have reported that dynorphin A (1-9) readily displaced only 75% of the kappa selective binding of  $[{}^{3}\text{H}]$ bremazocine in guinea-pig whole brain membranes, at  $0^{\circ}\text{C}$ .

The "dynorphin resistant" binding site defined by these groups under kappa selective conditions, shows clear similarities with the "dynorphin resistant" binding component seen in this study. Low affinities were obtained by Morre et al., at this site, only for the kappa selective peptides, thus confirming our results with MEAP, MEAGLE and the neoendorphins. In all three reports mu and delta binding was adequately suppressed, and the possibility of a PCP or sigma component excluded. This data therefore also supports our conclusion that this site cannot be explained in terms of high affinity mu or delta receptor binding.

The exact nature of the "dynorphin resistant" binding-site is therefore, unclear. The relatively steep displacement curves obtained for such ligands as ICI 204448 and ICI 204879, in the HEPES assay excludes the possibility of a low affinity mu receptor binding-site. These compounds have negligible affinity for this binding site, and would be expected to yield clearly biphasic displacement curves, similar to those seen in the [<sup>3</sup>H]bremazocine binding assay in Krebs buffer, if such a receptor conformation were involved. Little information is available as to the properties of a low affinity delta binding site. However, in the absence of any measurable high affinity delta binding in this study, the presence of a low affinity binding-site for this receptor is extremely unlikely.

The possibility remains, that the "dynorphin resistant" subsite represents a low affinity conformation of the kappa receptor. However, the  $IC_{50}$ s obtained for the standards in the [<sup>3</sup>H]bremazocine assay run in Krebs/HEPES buffer do not correlate well with their ability to interact with the "dynorphin resistant" site in HEPES. Dynorphin A (1-17) and dynorphin A (1-13), for instance, had  $IC_{50}$ values of 59.7nM and 12.6nM respectively in Krebs buffer, following correction for the low affinity mu site, but did not interact with the "dynorphin resistant" site in HEPES at concentrations below 100nM. In addition the continued presence of the "dynorphin resistant" site in the [<sup>3</sup>H]EKC binding assay also does not lend support to this conclusion.

Further work would therefore be needed to establish the nature of the "dynorphin resistant" binding site, possibly involving the use of a dynorphin suppressed assay system, to block binding to the classical kappa receptor. This might also help to clarify the extent to which the low agonist Hill coefficients were due to the same cause, another point which remains unclear. Numerous reports are available in the literature, suggesting the presence of kappa receptor subtypes, in a number of different tissues (see appendix). In the absence of any other explanation, the possibility of a novel binding-site cannot be excluded.

## 2. KAPPA RECEPTOR BINDING TO GUINEA-PIG CEREBELLAR MEMBRANES: KREBS/HEPES BUFFER +GPPNHP

A. ASSAY VALIDATION:

The specific binding of 0.2nM [<sup>3</sup>H]bremazocine to guinea-pig cerebellar membranes was virtually identical under these altered conditions, with

no apparent loss in affinity, as shown by the displacement curve to  $(\pm)$  bremazocine. The affinities of the agonist displacing agents were however markedly reduced in the Krebs/HEPES buffer system. The largest changes were seen with the dynorphin peptides and U69593, followed by U50488, tifluadom, EKC and ICI 197067. Large rightward shifts were also seen with the mu agonists morphine, GLYOL and DMPEA, and the delta ligands DADLE and ICI 174864 were virtually inactive under these conditions, with IC<sub>50</sub>s greater than 50µM. Smaller reductions in affinity were seen with the kappa agonists ICI 204448 and ICI 204879, and also with the partial agonists levallorphan, pentazocine and nalorphine. No alterations were seen in the IC<sub>50</sub>s of the antagonists naloxone, naltrexone and diprenorphine.

The majority of the literature evidence on the effects of Na<sup>+</sup> and GTP on kappa receptor binding is difficult to interpret, due mainly to the lack of selective ligands, and the consequent use of suppressing agents, mostly agonists, under conditions in which their selectivity would be likely to be lost, because of lowered affinity. Two studies are however available, in which guinea-pig cerebellar membranes have been used, and these problems therefore do not arise.

Kosterlitz et al. [78] have followed the effect of increasing Na<sup>+</sup> concentrations on the binding of a range of ligands to guinea-pig cerebellar membranes, in a TRIS buffer system. [ ${}^{3}$ H]bremazocine binding was reduced by approximately 30% at 100mM Na<sup>+</sup>. This compares with 80% inhibition of [ ${}^{3}$ H]dynorphin A (1-9) binding at the same concentration, and approximately 45% for [ ${}^{3}$ H]tifluadom. A similar effect on [ ${}^{3}$ H]bremazocine and [ ${}^{3}$ H]diprenorphine binding was also seen in rabbit cerebellar membranes, which contain a preponderance of mu receptors (75-83%), with binding levels reduced 15-20% by 100mM Na<sup>+</sup>.

Frances et al. [37], in a more detailed study, obtained a linear Scatchard line for [ ${}^{3}$ H]bremazocine in guinea-pig cerebellar membranes, with an affinity of 0.06nM. The addition of 120mM Na+ and 50µM GppNHp produced a decrease in affinity, but had no effect on the receptor density. In rabbit cerebellar membranes however, [ ${}^{3}$ H]bremazocine binding was relatively insensitive to Na<sup>+</sup> and GppNHp, with only a

small reduction in affinity. Bremazocine displacement of [<sup>3</sup>H]diprenorphine binding in guinea-pig cerebellar membranes was also shifted 7 fold in the presence of Na<sup>+</sup> and GppNHp, compared with only 1.8 fold in rabbit cerebellar membranes, under the same conditions.

The results obtained in these two papers therefore do suggest some reduction in the kappa receptor affinity of bremazocine in the presence of Na<sup>+</sup> and GppNHp, although the effects on the mu receptor are less clear. This is not in line with our results, in which the altered buffer conditions had no apparent effect on bremazocine binding. However the Krebs/HEPES buffer systems used in this study also contained other ions, notably  $Mg^{2+}$ , which may exert opposing effects, that have not been separately investigated.

Using  $[{}^{3}H]$ diprenorphine as the labelled ligand in guinea-pig cerebellar membranes, Frances et al. also obtained displacement curves to a number of agents in the presence and absence of Na<sup>+</sup> and GppNHp. The affinities achieved in the presence of Na<sup>+</sup> and GppNHp were reduced, compared to control, for all the agonist and partial agonists tested, whereas antagonist displacement curves were unaffected. The presence of Na<sup>+</sup> and GppNHp however had no effect on either the displacement curve to diprenorphine, or its specific binding.

These results are in good general agreement with this study, although the shifts in affinity do not correlate exactly. One notable difference between the two studies however was in the slopes of the displacement curves. In the Frances et al. study, all the Hill coefficients quoted were greater than 0.8, both in the presence and absence of Na<sup>+</sup> and GppNHp. This was not the case in this study, where very low Hill coefficients were obtained for the majority of the compounds tested, suggesting substantial heterogeneity of binding. This is likely to have affected the calculation of the IC<sub>50</sub> values, and hence of the size of the affinity shift, making exact comparison difficult.

It would appear therefore that the reductions in agonist affinity widely reported in the literature, for numerous receptor systems in

ionic media containing Na<sup>+</sup> and GTP or one of its stable analogs, have also been seen in the [<sup>3</sup>H]bremazocine binding assay studied here, suggesting that the kappa receptor is capable of existing in a low agonist affinity conformation similar to that reported for the mu receptor.

#### **B.HETEROGENEITY OF BINDING:**

Displacement of [<sup>3</sup>H]bremazocine binding in Krebs/HEPES + GppNHp was clearly complex, and low Hill coefficients were obtained for the majority of the compounds tested. There was no indication of any steepening of the displacement curves compared with the HEPES assay, with lower Hill coefficients for most of the kappa agonists, and a value significantly less than one for naloxone. In addition biphasic lines were obtained for both the dynorphin peptides and ICI 204448, 204879 and 197067, with plateaux in the binding curves at 70% inhibition of specific binding. Although biphasic binding curves were also seen with the dynorphin peptides in HEPES buffer, the displacement curves to ICI 204448 and ICI 204879 were fairly steep, with no indication of biphasic binding. A very low Hill coefficient was however achieved for ICI 197067 in both buffer systems.

These initial results therefore suggested that the "dynorphin resistant" component identified in the HEPES assay was still present in Krebs/HEPES + GppNHp, and had in fact increased from 15 to 30% of the specific binding of  $[{}^{3}$ H]bremazocine. Recalculation of the data for the dynorphin peptides and the ICI kappa agonists assuming a maximum of 70% inhibition of binding, increased the Hill coefficients to values not significantly different from one for all agents except dynorphin A (1-17). This also led to a decrease in IC<sub>50</sub> values, thus considerably reducing the affinity shift between the HEPES and Krebs/HEPES assays, from 608 to 111 in the case of dynorphin A (1-17). In particular, no remaining shift was seen with ICI 204879 and 204448, despite the fact that these agents behave as full agonists at the kappa receptor [28]. Although it was not possible to assess the exact extent to which the apparent Hill coefficients seen with the other displacers were affected by their affinity for the "dynorphin-

-resistant" component, the recalculated data for the dynorphin peptides, and particularly for ICI 204448 and 204879, suggests that in the absence of this component, the Hill coefficients might have been closer to one for all the agonists tested, possibly leading to smaller changes in affinity at the kappa receptor, under the altered buffer conditions. "Na<sup>+</sup> shifts" at the kappa receptor may therefore be lower than those reported at the mu receptor for compounds of equivalent intrinsic activity, or may vary for different structural groupings, irrespective of agonist activity.

The nature of the "dynorphin resistant" component in Krebs/HEPES buffer was however unclear, based on these initial results. Current receptor theory would suggest that under these conditions it would not be likely to be a high affinity mu or delta binding site. This is borne out both by the displacement curves to GLYOL and morphine, which show no significant inhibition of binding at concentrations below 1 $\mu$ M, and by the lack of activity seen with DADLE and ICI 174864. It is also unlikely to be a high affinity kappa site, due to the low affinities of the dynorphin peptides and ICI kappa agonists, all of which have high affinity for the majority of the specific binding of [<sup>3</sup>H]bremazocine in HEPES buffer. The possibility of a low affinity mu or delta binding site therefore remains.

# C. "KAPPA SUPPRESSED" [<sup>3</sup>H]BREMAZOCINE BINDING:

A [<sup>3</sup>H]bremazocine binding assay in Krebs/HEPES buffer +10µM GppNHp and 1µM ICI 204879, was set up in order to further investigate the "dynorphin resistant" component seen in the unsuppressed Krebs/HEPES buffer system.

The binding profile obtained under these conditions was completely different to that from either of the unsuppressed [<sup>3</sup>H]bremazocine assays. Compared with the unsuppressed assay in Krebs/HEPES buffer, the most marked changes were those seen with the dynorphin peptides and the kappa agonists, all of which predictably showed large decreases in affinity. In addition, the affinities of U50488 and U69593 were decreased approximately 40 fold. The affinity of EKC was

#### however unaffected.

By contrast, the affinities of the mu/delta agents were increased in the kappa suppressed assay, with significant decreases in the  $IC_{50}$ values for GLYOL, DADLE and DMPEA. Slightly higher affinities were also seen with morphine, ICI 174864, fentanyl, nalorphine and Q.nalorphine, although the increase was not so marked. A similar effect was observed with the slightly mu selective antagonists naloxone and naltrexone, with  $IC_{50}$ s of 5.76 and 1.27nM respectively in the kappa suppressed assay, compared with values of 25.4 and 10.7nM in the unsuppressed binding system.

The profile obtained here clearly does not conform to that of either a high or low affinity kappa binding site. It is also not likely to represent binding to a combination of sites, as the Hill coefficients obtained were very considerably improved, compared with the unsuppressed [<sup>3</sup>H]bremazocine assay in Krebs/HEPES buffer. Values significantly less than one were seen only with ICI 200940, and interestingly, with the ligands Q.nalorphine, etorphine, GLYOL, DADLE, DMPEA and ICI 174864. These values were in all cases higher than those achieved in the unsuppressed assay system.

The use of the Krebs/HEPES buffer system + GppNHp, makes it difficult to compare this assay with any of the standard opioid binding profiles achieved in low ionic strength buffer systems. However antagonist affinities should not be affected by the altered conditions, and the high affinities obtained with naloxone and naltrexone in the kappa suppressed assay do suggest that the "dynorphin resistant" binding site might represent a mu receptor binding component. The higher affinities obtained with the mu/delta agonists also support this conclusion.

3. [<sup>3</sup>H]NALOXONE BINDING IN RAT BRAIN MEMBRANES:

In order to further investigate the hypothesis that the binding profile obtained for the "dynorphin resistant component" in the kappa suppressed assay system might represent a mu receptor binding

component, data from this assay was compared with the affinity profile obtained in a "low affinity" mu receptor binding assay, run in the same Krebs/HEPES buffer system.

The standard mu receptor agonists such as GLYOL and DHM could not be used as labelled ligands in this assay, due to their reduced affinity in the Krebs/HEPES buffer system, and the antagonist ligand  $[{}^{3}$ H]naloxone was therefore chosen. This compound is only slightly selective for the mu receptor, with affinities of 2.65nM, 14.4nM and 27nM respectively [43] for the mu, kappa and delta sites. It was however hoped to overcome this problem by using  $[{}^{3}$ H]naloxone at a very low concentration (0.2nM) compared with its kappa receptor affinity, such that only a small number of kappa receptors would be occupied, and also by running the assay in rat brain membranes, where the proportion of kappa receptors is very low [44].

The  $IC_{50}$  values obtained in this assay for the antagonists, and also for the partial agonists bremazocine and levallorphan, were in good agreement with their Ki values against [<sup>3</sup>H]GLYOL binding in TRIS buffer, [90] and therefore consistent with binding to a mu receptor. The profile obtained with the agonists and the majority of the partial agonists was however markedly different to that expected for a high affinity mu binding site. The mu agonists in particular had much lower affinities in this assay than would be expected from a classical mu receptor interaction, with the  $IC_{50}$ s of morphine and GLYOL reduced 2-300 fold compared with their Ki values against [<sup>3</sup>H]GLYOL binding [10]. The affinities of DADLE and DMPEA were also shifted by a factor of X350 and X1000 respectively in the [<sup>3</sup>H]naloxone assay, with respect to their reported mu receptor affinities [26, 10].

Similar large decreases in affinity were also seen with the kappa agonists, with the biggest shifts apparently occurring with the compounds with lowest kappa receptor selectivity, such as the dynorphin peptides [25, 109, 44], whereas U69593 and U50488, with lower reported mu affinities [26] were less markedly affected, and showed smaller rightward shifts. Small decreases in affinity were also seen with the partial agonists nalorphine, nalbuphine and

pentazocine, and the non-selective agonist etorphine.

The Hill coefficients obtained in the  $[{}^{3}H]$ naloxone binding assay were close to one for all the antagonist and partial agonist ligands, except for Q.Nalorphine and Nalbuphine. Markedly shallow slopes were however seen for dynorphin A (1-17), the ICI kappa agonists, and a number of the mu/delta ligands, including in particular DADLE, DMPEA and ICI 174864. It is interesting to note, in this context, that the only compounds for which the IC<sub>50</sub>s obtained in the  $[{}^{3}H]$ naloxone assay were lower (by approximately 10 fold) than the reported mu receptor affinities were the delta selective antagonists ICI 154129 and ICI 174864 [26]. Since ICI 174864 is an antagonist ligand, with a delta receptor affinity of approximately 150nM in standard binding assays [30], this data suggests that there might be a delta component in the  $[{}^{3}H]$ naloxone assay. This would be quite consistent with the high proportion of delta receptors in rat brain, and the low selectivity of naloxone.

The binding profile obtained in the [<sup>3</sup>H]naloxone binding assay described here, although clearly different from that of a high affinity mu receptor binding site, does show some correlation with affinity data obtained at the mu receptor in a number of pharmacological preparations. In particular, Carroll et al. [10] have compared the Ki values obtained both in a [<sup>3</sup>H]GLYOL binding assay run in HEPES buffer, and a [<sup>3</sup>H]naloxone system identical to that described here, with affinity measurements obtained in two isolated tissue preparations. Values were determined both in RVD, by antagonism of GLYOL, and also in GPI, using the receptor occlusion technique of Furchgott et al. [38]. Good correlations were found in this study, between the affinities obtained in the [<sup>3</sup>H]naloxone binding assay and those from the isolated tissue preparations, both in the order of activity and the absolute values, with correlation coefficients of 0.98 and 0.96 for GPI and RVD respectively. No such correlation was seen between the [<sup>3</sup>H]GLYOL binding data, and any of the other assays, with low correlation coefficients in all cases. This agreement is unlikely to be fortuitous, and suggests that the low affinity mu receptor binding profile obtained in the [<sup>3</sup>H]naloxone binding assay

described both here and in the literature is not simply an artefact but is likely to represent a relevant physiological state of the mu receptor.

A very close correlation was seen in this study, between the binding profiles obtained in the kappa suppressed [<sup>3</sup>H]bremazocine assay in Krebs/HEPES buffer, and the [<sup>3</sup>H]naloxone system in rat brain membranes, confirming that the suppressed [<sup>3</sup>H]bremazocine assay does represent binding to a low affinity mu receptor site. There were however some remaining discrepancies between the the two assays.

Although similar affinities and Hill coefficients were obtained for almost all the compounds tested in these two assays, the  $IC_{50}s$ achieved in the kappa suppressed [<sup>3</sup>H]bremazocine assay were in general slightly higher than those from the [<sup>3</sup>H]naloxone system. In most cases the difference was only 2-4 fold, and may simply reflect differing receptor occupancies by the two labelled ligands, at the concentrations used. However, the slightly larger discrepancies, in the region of 3-4 fold, appeared to occur mainly in the mu/delta agonist group, with morphine and fentanyl in particular, showing lower affinities in the suppressed [<sup>3</sup>H]bremazocine assay. The majority of the low Hill coefficients were also obtained with this group, in both assays.

In addition to these small shifts, markedly different results were obtained, between the two assay systems, for ICI 174864, dynorphin A (1-17) and ICI 204879. Clearly biphasic displacement curves were seen with dynorphin A (1-17) and ICI 204879 in the unsuppressed  $[^{3}H]$ bremazocine assay in Krebs/HEPES buffer, with no significant inhibition of the binding of  $[^{3}H]$ bremazocine to the "dynorphin--resistant" site, at concentrations up to 10µM. These agents were also virtually inactive in the kappa suppressed  $[^{3}H]$ bremazocine assay, with IC<sub>50</sub> values of greater than 50µM. However, in the  $[^{3}H]$ naloxone assay, IC<sub>50</sub> values of 6990nM and 6410nM respectively were obtained for dynorphin A (1-17) and ICI 204879, with full displacement of specific  $[^{3}H]$ naloxone binding, although shallow slopes were seen in both cases. The reason for these discrepancies is unclear, although it clearly indicates some remaining differences between the receptor populations labelled in the [<sup>3</sup>H]naloxone and kappa suppressed [<sup>3</sup>H]bremazocine binding systems.

An  $IC_{50}$  of 33800nM was also obtained for ICI 174864 in the kappa suppressed [<sup>3</sup>H]bremazocine assay, compared with a value of 4570nM in the [<sup>3</sup>H]naloxone system. This is in fact in good agreement with the reported mu receptor affinity of ICI 174864, in contrast to the [<sup>3</sup>H]naloxone binding value, although shallow slopes were seen in both cases, and again suggests that there may be a delta component in the [<sup>3</sup>H]naloxone assay. This would not however explain the low Hill coefficients obtained in both assays, mainly for the same compounds, unless a delta component were postulated in both the [<sup>3</sup>H]naloxone and suppressed [<sup>3</sup>H]bremazocine binding systems. The possibility of a kappa component, particularly in the [<sup>3</sup>H]naloxone assay, can also not be completely excluded.

#### 4. SUCCESSIVE SUPPRESSION EXPERIMENTS:

In order to investigate the possibility that the low Hill coefficients seen in the [ ${}^{3}$ H]naloxone or kappa suppressed [ ${}^{3}$ H]bremazocine assays might be due to either a delta component or to some remaining kappa binding, displacement curves were generated for DMPEA against [ ${}^{3}$ H]bremazocine binding in the presence and absence of high concentrations of ICI 174864 and ICI 204879.

DMPEA is reported to be a mu selective agonist ligand with a Ki of 0.27nM against [ ${}^{3}$ H]GLYOL binding to the high affinity mu receptor site [10]. In this study the IC<sub>50</sub> of DMPEA against [ ${}^{3}$ H]naloxone binding was 276nM, indicative of a large "Na<sup>+</sup> shift" at the mu receptor, with a Hill coefficient of 0.633. In the kappa suppressed [ ${}^{3}$ H]bremazocine assay the IC<sub>50</sub> of DMPEA was shifted approximately 3 fold to the right, to a value of 787nM. There was however no change in the Hill coefficient. It was hoped therefore that by blocking any possible delta or kappa binding components in both the [ ${}^{3}$ H]naloxone and suppressed [ ${}^{3}$ H]bremazocine assays, steeper slopes might be obtained for DMPEA, with possibly a closer correlation between the IC<sub>50</sub> values.

A concentration of 1µM ICI 174864 was chosen in these experiments, to block any delta receptor binding in either the [<sup>3</sup>H]naloxone or [<sup>3</sup>H]bremazocine assay systems. This compound has a reported delta receptor Ki of 190nM, [30] compared with a value of only 24700nM at the mu binding-site, and negligible kappa receptor affinity [26]. This concentration should therefore be sufficient to block approximately 90% of any delta binding, without significantly affecting interactions at the mu receptor.

As a result of the discrepancy between the  $IC_{50}$  values obtained for ICI 204879 in the [<sup>3</sup>H]naloxone and kappa suppressed [<sup>3</sup>H]bremazocine systems, different concentrations were used to suppress kappa receptor binding in these two assays. The minimal affinity of ICI 204879 for the low affinity mu site as defined in the kappa suppressed [<sup>3</sup>H]bremazocine system allowed a concentration of 10µM to be used in this assay, clearly sufficient to block any remaining kappa binding. In the [<sup>3</sup>H]naloxone binding system however a kappa suppressing concentration of only 1µM ICI 204879 was selected, because of the lower IC<sub>50</sub> value of 6.41µM obtained for ICI 204879 in this assay.

The addition of 10 $\mu$ M ICI 204879 to the suppressed [<sup>3</sup>H]bremazocine assay had no effect on the specific binding beyond that produced by 1 $\mu$ M ICI 204879 alone. This confirms that all kappa receptor binding in this system was adequately blocked at this concentration, and that 1 $\mu$ M ICI 204879 should also be sufficient to prevent any kappa receptor binding in the [<sup>3</sup>H]naloxone assay. Kappa suppressed [<sup>3</sup>H]bremazocine binding was also unchanged by 1 $\mu$ M 174864, either in presence or absence of 10 $\mu$ M 204879. This lack of effect is in line with the very low affinities of these compounds in the suppressed [<sup>3</sup>H]bremazocine assay and does not support the suggestion of either a delta or kappa binding component under these assay conditions.

In the  $[{}^{3}\text{H}]$ naloxone assay the specific binding was reduced 16.4%, and 21.2% respectively by 1µM ICI 204879 and 1µM ICI 174864, and 32.9% by these agents added together. This effect is very nearly additive and given the IC<sub>50</sub>s obtained for ICI 174864 and ICI 204879 in this assay,

would again be quite consistent with displacement from a low affinity mu site, although other binding components could be involved.

The results obtained with DMPEA using these suppression conditions were confusing. Displacement of [<sup>3</sup>H]naloxone binding by DMPEA appeared to be almost entirely unaffected by the presence of ICI 174864 and/or 204879, with no change in either the IC<sub>50</sub> values or Hill coefficients, despite the 30% reduction in specific binding in the presence of both these ligands. In the suppressed [<sup>3</sup>H]bremazocine assay however, where the addition of 1µM ICI 174864 and 10µM ICI 204879 had no effect on the specific binding, small rightward shifts were seen in the displacement curve to DMPEA, in the presence of 10µM ICI 204879, with or without ICI 174864, and a slightly higher Hill coefficient was also obtained in the presence of these two agents together, although the change was small and possibly not significant. In the case of the [<sup>3</sup>H]naloxone binding assay this data can be most easily interpreted by suggesting that all displacement by ICI 174864 and ICI 204879 was from the mu receptor and that any heterogeneity of binding is not due to either a delta or kappa binding component. The reason for the effect of 10µM ICI 204879 on the DMPEA displacement curve in the kappa suppressed [<sup>3</sup>H]bremazocine assay is however unclear, but may represent a non-specific effect on [<sup>3</sup>H]bremazocine binding at this high concentration.

Overall these results, although not particularly helpful, do not support the suggestion of either a kappa or delta component in either the [<sup>3</sup>H]naloxone or the suppressed [<sup>3</sup>H]bremazocine assays. The problem of the remaining low Hill coefficients in these assays however remains unsolved and would require further work.

The very close correlation achieved in this study between the binding profiles from the kappa suppressed [<sup>3</sup>H]bremazocine and [<sup>3</sup>H]naloxone binding assays therefore suggests that the "dynorphin resistant" component obtained in the unsuppressed [<sup>3</sup>H]bremazocine assay in Krebs/HEPES buffer + GppNHp is also likely to be a low affinity mu receptor binding site. The "dynorphin resistant" component in the HEPES assays cannot however be explained on this basis, mainly due to

the data obtained with the ICI kappa agonists 204448 and 204879. These compounds fully displaced the binding of  $[{}^{3}$ H]bremazocine and  $[{}^{3}$ H]EKC in HEPES buffer at low concentrations, but had negligible affinity for the low affinity mu site as defined by the  $[{}^{3}$ H]naloxone or the suppressed  $[{}^{3}$ H]bremazocine assays in Krebs. If a low affinity mu component were present in the HEPES binding systems, these compounds would be expected to produce clearly biphasic displacement curves under these conditions. No such effect was seen in the HEPES buffer assays and therefore although the dynorphin resistant binding components obtained in HEPES and Krebs/HEPES + GppNHp appear superficially similar, it must be concluded that they may have quite separate causes.

It follows from this that the binding profiles of  $[{}^{3}H]$  bremazocine must be different in the two buffer systems, with in particular, a mu receptor component of 30% in Krebs/HEPES, compared with only 5-10% in HEPES alone. Since the specific binding of  $[{}^{3}H]$  bremazocine was almost identical in the two unsuppressed assays, it is possible that the kappa affinity of  $[{}^{3}H]$  bremazocine is reduced in Krebs/HEPES buffer, as suggested in the studies discussed earlier [78, 37], whereas the mu receptor affinity may be unaffected or possibly slightly increased under these conditions. This would be consistent with reported antagonist action of bremazocine at the mu receptor [95] and might explain the fact that the displacement curve to ( $\pm$ ) bremazocine was also unchanged in Krebs/HEPES buffer + GppNHp.

#### 5.SUMMARY AND CONCLUSIONS:

The [ ${}^{3}$ H]bremazocine binding systems developed in this study, in guinea-pig cerebellum membranes, were intended to provide assays selective for the kappa receptor, both in HEPES buffer, and Krebs/HEPES +GppNHp. However, detailed investigation of the binding profiles obtained has shown that this aim was not achieved, and that [ ${}^{3}$ H]bremazocine did not bind to a single receptor or receptor conformation, in either buffer system.

In the HEPES buffer system, the heterogeneity of  $[{}^{3}H]$  bremazocine binding was apparent both in the low Hill coefficients obtained for the agonist displacing ligands, and in a 15% component of the specific binding that could not be displaced by dynorphin A (1-13) or (1-17) at concentrations up to 1µM. Recalculation of the data so as to exclude the "dynorphin resistant" site did not result in any improvement in the Hill coefficients, suggesting that these two effects might have different explanations.

A small mu receptor component was identified in this assay, in line with the findings of other groups working with guinea-pig cerebellar membranes. However the evidence did not suggest that this was responsible for either the low Hill coefficients or the "dynorphin resistant" binding-site. The possibility that [<sup>3</sup>H]bremazocine might be binding to a low affinity conformation of the kappa receptor was therefore investigated, initially using the full kappa agonist [<sup>3</sup>H]EKC. A very similar binding profile was however also obtained with this ligand in the HEPES buffer system, with low Hill coefficients for all the agonist displacers, and a "dynorphin resistant" specific binding component. There was no suggestion that the binding of  $[^{3}H]EKC$ , was in any way less complex than that of  $[^{3}H]$  bremazocine, and it was therefore not possible to resolve the heterogeneity of binding seen with these two ligands, in the HEPES buffer system. The [<sup>3</sup>H]bremazocine binding assay in Krebs/HEPES buffer + GppNHp was set up, in the hope of converting all the receptors to the low agonist affinity conformation, and thus possibly obtaining a simpler binding profile.

[<sup>3</sup>H]bremazocine binding in Krebs/HEPES buffer + GppNHp was however still complex, and showed an apparently similar profile to that seen in HEPES buffer, with low agonist Hill coefficients and a "dynorphin-resistant" component, comprising in this case 30% of the specific binding, although the affinities obtained for the agonist ligands were markedly reduced, compared with those obtained in HEPES.

There was however, one important distinction between the profiles obtained in the two buffer systems, in that the highly selective kappa

agonists ICI 204879 and ICI 204448, were able to displace all of the specific binding of  $[{}^{3}$ H]bremazocine in HEPES, but had very little affinity for the "dynorphin resistant" component as defined in Krebs/HEPES buffer + GppNHp. Based on the use of ICI 204879 as a suppressing agent for the kappa receptor, it was therefore possible to identify the "dynorphin resistant" component in the  $[{}^{3}$ H]bremazocine assay in Krebs/HEPES buffer, but not that in HEPES, as a low affinity mu receptor binding component. In addition, recalculation of the data for the dynorphin peptides and ICI 204448 and ICI 204879 in the Krebs buffer system, using a 70% binding maximum, led to Hill coefficients not significantly different from one, suggesting that  $[{}^{3}$ H]bremazocine binding to guinea-pig cerebellum membranes, under these conditions, could be resolved into two components, a low affinity kappa receptor binding site, and a low affinity mu receptor conformation, comprising 70% and 30% of the binding respectively.

These findings did not however shed much light on the nature of the "dynorphin resistant" site in the  $[{}^{3}H]$ bremazocine assay in HEPES. The possibility of a low affinity mu site can be excluded here, by the high affinities and relatively steep displacement curves obtained for ICI 204879 and ICI 204448. Similarly, the likelihood of a low affinity kappa site is reduced by the very low affinities achieved for dynorphin A (1-13) and (1-17), and the fact that the "dynorphin resistant" component was retained in the  $[{}^{3}H]$ EKC binding assay, although the possibility that some agonists may bind to the low affinity receptor conformation cannot be excluded.

Therefore, although it can be concluded that  $[{}^{3}H]$  bremazocine binding to guinea-pig cerebellar membranes in HEPES buffer is made up mainly of binding to the high affinity conformation of the kappa receptor, and also includes a small high affinity mu receptor component, comprising approximately 10% of the specific binding, it is not clear whether a low affinity kappa component is also involved, either in the low Hill coefficients or the "dynorphin resistant" binding component. This problem could possibly be resolved by suppression of  $[{}^{3}H]$  bremazocine binding to the classical kappa receptor, in the HEPES buffer system, using high concentrations of dynorphin A (1-17). This

would allow the "dynorphin resistant" subsite to be characterised, and its binding profile could then be compared with other assays. The possibility remains that the dynorphin resistant site defined in HEPES buffer may represent a novel receptor or binding-site.

One clear conclusion from this study, however, is that [<sup>3</sup>H]bremazocine has a different binding profile in the two buffer systems, with a much larger mu receptor component in Krebs/HEPES buffer + GppNHp, comprising 30% of the specific binding, compared with only 10% in HEPES alone. This is likely to be due to the differing intrinsic activity of bremazocine at the mu and kappa receptors, such that its kappa, but not its mu receptor affinity, is reduced in the Krebs/HEPES buffer system.

The complexity of the binding profiles obtained with  $[{}^{3}H]$  bremazocine under both sets of assay conditions illustrate the difficulties of using  $[{}^{3}H]$  ligands that are either non-selective, or not full antagonists at all three opioid receptors, in this type of study. It is also clear that where the possibility of novel receptors or binding-sites is being investigated, binding data obtained with  $[{}^{3}H]$  antagonists or partial agonists must be interpreted with the greatest caution.

#### 6. APPENDIX: KAPPA RECEPTOR SUBTYPES:

Numerous reports are available in the literature, suggesting that the binding of certain opioid ligands, in particular that of the kappa agonists [<sup>3</sup>H]EKC and [<sup>3</sup>H]etorphine, is heterogeneous under apparently kappa selective conditions. These effects have been shown in a number of different tissues, including rat and guinea-pig whole brain and spinal cord, and bovine adrenal medulla, and have led to the proposal of multiple kappa receptor sub-types.

The proposal of a "kappa 2" binding site was first made by Attali et al. in 1982, based on [<sup>3</sup>H]EKC and [<sup>3</sup>H]etorphine binding data in rat and guinea-pig lumbo-sacral spinal cord [49]. The binding profile of these ligands was found not to be entirely kappa-like, despite the apparent absence of mu or delta receptors in these tissues . In a subsequent paper [2] the displacement of [<sup>3</sup>H]EKC binding in guinea-pig lumbo-sacral spinal cord by DADLE, etorphine and the mu ligand FK33824 was shown to be biphasic, with a proportion of the binding not displaced by DADLE at concentrations up to 10µM. This component was designated as " DADLE insensitive" and thought to represent the classical kappa receptor (K1). The displacement of [<sup>3</sup>H]etorphine binding in this tissue, was however monophasic in all cases, with no specific binding remaining in the presence of 5µM DADLE, suggesting that this ligand was not binding to the classical kappa site, for which DADLE has a very low affinity. The [<sup>3</sup>H]etorphine binding sites were therefore identified as "DADLE sensitive" (K2).

This proposed sub-division was based on the assumption that no mu or delta receptors are present in the lumbo-sacral spinal cord. This is however not the case. The total opioid receptor population in rat lumbo-sacral spinal cord, has been shown by Traynor et al. [143], using [ $^{3}$ H]bremazocine as the tritiated ligand, to consist of 30% mu, 14% delta, and 56% kappa. Similarly Gouarderes et al. [51] in an autoradiographic study, have identified all three opioid receptor types in the cervical, thoracic and lumbo-sacral regions of rat and guinea-pig spinal cord. Given the lack of selectivity of [ $^{3}$ H]EKC and [ $^{3}$ H]etorphine, the assays described above are therefore likely to

contain a significant mu /delta component, and the DADLE sensitive  $[{}^{3}\text{H}]$ etorphine site (K2) can be explained on this basis. However, the loss of  $[{}^{3}\text{H}]$ etorphine specific binding in the presence of 5µM DADLE, implying the absence of kappa receptor binding, is difficult to reconcile with the non-selective binding profile normally seen with this ligand [90], and remains an anomaly.

This work was followed by two more interesting papers from this group. In the first of these, [50] [<sup>3</sup>H]etorphine was used to study the binding to both K1 and K2 sites at different levels of rat spinal cord, taking account of the likely mu and delta contamination. The K1 classical kappa receptor was identified as the binding remaining in the presence of 10µM DADLE, suggesting that [<sup>3</sup>H]etorphine did bind to the total kappa receptor population in this study. The K2 sites were defined as [<sup>3</sup>H]etorphine binding in the presence of mu/delta suppressors and U50488 to prevent binding to the K1 site. A high concentration of apparent K2 sites was found in lumbo-sacral cord, with a profile that was not in line with high affinity mu/delta or kappa receptor binding. Similar results were also obtained by this group in guinea-pig striatal membranes, [3] using [<sup>3</sup>H]etorphine in the presence of 1µM morphiceptin and 0.1µM DSLET to identify the K2 binding sites. In this study there was again no residual  $[^{3}H]$  etorphine binding in the presence of 5µM DADLE.

The K2 binding profiles obtained in these two studies were not consistent with a classical kappa receptor interaction, as defined in both guinea-pig whole-brain and guinea-pig cerebellum tissues by Kosterlitz et al. [116, 77]. [<sup>3</sup>H]etorphine binding was displaced with high affinity by EKC, etorphine and bremazocine, but a much lower affinity was obtained for U50488 (1.11 $\mu$ M). Intermediate Ki values (0.3-1.2 $\mu$ M) were seen with the mu/delta peptides DADLE, DSLET and DTLET. This profile was compared by the authors with that of the "benzomorphan receptor" characterised by Chang and Cuatracasas [15] in rat brain membranes using a suppressed [<sup>3</sup>H]diprenorphine assay system. This was also not entirely kappa-like, with higher than expected affinities for the delta peptides met and leu enkephalin, and DADLE. The proportion of kappa receptors in rat whole brain tissue is very low, representing only 12% of the total opioid receptor population, compared with 40% in guinea-pig whole brain [44]. Earlier studies, largely based on unsuppressed assay systems, using non-selective benzomorphan ligands, have led to the suggestion that the kappa receptor in rat brain differed from that characterised in guinea-pig neural tissues [49]. It is possible however, that this idea may have arisen from the different ligands used in these two tissues. Weyhemeyer and Mack [151] have compared the binding of [<sup>3</sup>H]EKC and [<sup>3</sup>H]diprenorphine in rat whole brain membranes, in the presence of mu and delta suppressing agents. Under these conditions [<sup>3</sup>H]EKC binding was displaced with high affinity by dynorphin A (1-17), U50488 and EKC itself. Against [<sup>3</sup>H]diprenorphine binding however, Ki values of 275nM, 224nM and 13.4nM respectively were obtained for these compounds. Very low Hill coefficients were seen for these displacing agents in both assays, whereas bremazocine displaced both [<sup>3</sup>H] ligands with high affinity and Hill coefficients close to one.

It is clear from this study, that two different kappa receptor profiles can be obtained in the same tissue, by using different  $[{}^{3}H]$ ligands. Of these the  $[{}^{3}H]$ EKC binding profile was more closely in line with that of the kappa receptor as originally defined by Kosterlitz et al. in guinea-pig whole brain membranes [77], also using a suppressed  $[{}^{3}H]$ EKC binding system, and subsequently confirmed in guinea-pig cerebellum using  $[{}^{3}H]$ bremazocine [116]. Equally the  $[{}^{3}H]$ diprenorphine assay could be said to show some similarities with both the K2 profile defined by Gouarderes et al., using  $[{}^{3}H]$ etorphine in rat spinal cord, and the "benzomorphan" site proposed by Chang and Cuatracasas.
that diprenorphine will also bind all available low affinity receptor conformations, but that EKC may not, the possibility cannot be excluded that both the K2 binding site, defined as mu/delta suppressed [<sup>3</sup>H]etorphine binding in spinal cord, and the "benzomorphan" binding-sites (suppressed [<sup>3</sup>H]diprenorphine binding in rat brain), may represent a mixture of the low agonist affinity conformations of one or more opioid receptor types, particularly as no Hill coefficients are given in either of the two studies.

A third kappa receptor sub-type, the kappa 3 receptor (K3) has also been proposed in recent years, by Castanas et al. [11] based on binding studies in the bovine adrenal medulla.

The adrenal medulla is an important source of endogenous opioid peptides. A number of agents, including met and leu enkephalin, MEAP, MEAGLE and dynorphin A (1-13) have been isolated from both adrenal chromaffin cells, and the axons of the splanchic nerves [125]. Opioid receptor agonists have also been shown to inhibit the nicotine or acetylcholine induced release of catecholamines from cultured chromaffin cells [27, 125]. A neuromodulatory role has therefore been proposed for the opioid peptides in this tissue.

Stereospecific opioid receptor binding in bovine adrenal medullary membranes was first demonstrated by Chavkin et al. in 1978 [16], using [<sup>3</sup>H]naltrexone as the labelled ligand. More detailed studies have confirmed the presence of all three opioid receptor types in this tissue. However some controversy remains over the nature of the receptor involved in the effects of opiates on catecholamine release from adrenal chromaffin cells.

Dean and Lemaire published a study in 1982 [16], suggesting that the inhibition of nicotine induced [<sup>3</sup>H]noradrenaline release from cultured chromaffin cells produced by morphine, levorphanol, dextrorphan and dynorphin A (1-13) was not due to an interaction with any classical opioid receptor type. This conclusion was based on a number of unusual findings, including the absence of naloxone reversibility, and the similar potencies of levorphanol and dextrorphan.

In a second study from the same year however, Saiani and Guidotti [125] obtained a good correlation between the Ki values of a range of ligands as displacers of  $[{}^{3}H]$ etorphine binding, and their ability to inhibit the nicotine induced release of catecholamines from isolated adrenal chromaffin cells. An unusual profile was also seen in these two assays, with much higher potency shown by  $\beta$ -endorphin and MEAP than EKC, morphine and DADLE. The opiate inhibition of noradrenaline release was however naloxone reversible in all cases. Costa and Guidotti [27] also obtained very high receptor densities for  $[{}^{3}H]$ etorphine and  $[{}^{3}H]$ diprenorphine in bovine adrenal medullary membranes, compared with other non-selective ligands such as  $[{}^{3}H]$ EKC,  $[{}^{3}H]$ naloxone and  $[{}^{3}H]$ SKF10047.

Dumont and Lemaire [35] in a later study comparing [<sup>3</sup>H]EKC binding with opiate inhibition of acetylcholine evoked catecholamine secretion from chromaffin cells obtained another very different profile, with dynorphin A (1-13) and U50488 showing the highest activity out of a range of opiates, including ß-endorphin and EKC. The effects of U50488 on catecholamine release were significantly reversed by diprenorphine and Mr2266, but not by naltrexone. MEAP was shown to be virtually inactive in both assays.

The presence of kappa receptor subtypes in bovine adrenal medullary membranes was first proposed by Castanas et al. in 1985 [12], based on a suppressed [ ${}^{3}$ H]etorphine binding system. The proportion of [ ${}^{3}$ H]etorphine binding resistant to high concentrations of DADLE (16% of specific binding) was found to yield a binding profile that was different in some respects from that of [ ${}^{3}$ H]EKC under the same suppressing conditions. In particular, very high affinities were obtained for MEAP and MEAGLE ( 1.2 and 19nM respectively), whereas dynorphin A (1-13) was inactive. Lower affinities were also seen in the [ ${}^{3}$ H]etorphine assay for morphine, fentanyl, EKC and naloxone. The kappa 3 receptor was therefore proposed as a novel subtype of the kappa opioid site in bovine adrenal medulla, highly selective for MEAP, and based on the reported inability of [ ${}^{3}$ H]etorphine to bind to the classical kappa receptor.

The high affinity obtained for MEAP in the [<sup>3</sup>H]etorphine assay does show some correlation with both the binding and release profiles seen by Saianni and Guidotti, in which MEAP was one of the more active of a range of opiate agonists. Dumont and Lemaire however, in marked contrast to Castanas et al., achieved good activity with dynorphin A (1-13) but none with MEAP.

Apart from the high affinity obtained for MEAP in the Castanas et al. study, the picture here appears very similar to that seen in the literature both for the K2 site and for kappa receptor binding in rat brain membranes, with a classical kappa receptor binding profile when the agonist ligand [ ${}^{3}$ H]EKC is used, but a slightly different pattern with [ ${}^{3}$ H]etorphine. Assuming that [ ${}^{3}$ H]etorphine may also bind to low affinity opioid receptor conformations, it is therefore possible that the K3 site could also be explained as a mixture of low affinity opioid receptor binding sites, and no new site need therefore be proposed.

It is not clear however, whether or not the low agonist Hill coefficients, and the " dynorphin resistant" component obtained in this study can be explained on the same basis, although the possibility cannot be excluded, especially as some agonist ligands, such as [<sup>3</sup>H]etorphine may well bind to the low affinity receptor conformations, even when used as labelled ligands.

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cAMP - Cyclic adenosine triphosphate. DADLE - [D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin. DPDPE - [D-Pen<sup>2</sup>, D-Pen<sup>5</sup>]enkephalin. DHM - Dihydromorphine.  $DMPEA - [D-Met^2, Pro^5] enkephalinamide.$ DSLET - [D-Ser<sup>2</sup>, Leu<sup>5</sup>, Thr<sup>6</sup>]enkephalin. DTLET - {D-Thr<sup>2</sup>, Leu<sup>5</sup>, Thr<sup>6</sup>]enkephalin. EKC - Ethylketocyclazocine. G-protein - GTP binding protein. GLYOL - [D-Ala<sup>2</sup>, (Me)Phe<sup>4</sup>, Gly(ol)<sup>5</sup>]enkephalin. GPI - Electrically stimulated longitudinal muscle of the guinea-pig ileum. GppNHp - Guanylylimidodiphosphate. GTP - Guanosine triphosphate. HEPES - N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid. ICI 154129 - (N,N-Diallyl Tyr-Gly-Gly-(CH<sub>2</sub>S)-Phe-Leu-OH).

ICI 174864 - (N,N-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH).

ICI 200940 = 2-(4-nitrophenyl)-N-methyl-N-[(IRS)-1-(4-hydroxyphenyl)

-2-(1-pyrrolidinyl)ethyl]acetamide .

- ICI 197067 2-(3,4-dichlorophenyl)-N-methyl-N-[(IS)-1-(1methylethyl)- 2-(1-pyrrolidinyl)ethyl]acetamide.
- ICI 204448 2-(3,4-dichlorophenyl)-N-methyl-N-[(IRS)-1-(3-(carboxymethoxy)phenyl)-2-(1-pyrrolidinyl)ethyl]acetamide.
- ICI 204879 2-(3,4-dichlorophenyl)-N-methyl-N-[(IRS)-1-(3,4dimethoxyphenyl)- 2-(1-pyrrolidinyl)ethyl]acetamide.

IC<sub>50</sub> - Concentration producing a half-maximal effect.

Ke - antagonist affinity value, obtained in vitro or in vivo.

Ki - Affinity value obtained from a binding displacement curve.

MEAGLE - [Met<sup>5</sup>]enkephalin (Arg-Gly-Leu).

MEAP - [Met<sup>5</sup>]enkephalin (Arg-Phe).

Mr2034 - (-)-(1R,5R,9R,2"S)-5,9-dimethyl-2'-hydroxy-2tetrahydrofurfuryl-6,7-benzomorphan.

MVD - Stimulated mouse vas deferens preparation.

N - Hill coefficient.

NEM - N-Ethylmaleimide.

NG108-15 - Mouse neuroblastoma / rat glioma hybrid cell line.

PCP - Phencyclidine.

Q.Nalorphine - N-Methyl-Nalorphine Chloride Salt.

Q.Tifluadom - N-Methyl-Tifluadom Chloride Salt.

SKF-10,047 - N-allylnormetazocine.

U50488 - Trans-3,4-dichloro-N-Methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]benzeneacetamide Methane Sulphonate.

U69593 - (-)-(5a,7a,8b)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro (4,5)dec-8-yl] benzeneacetamide. . 1