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COMMENTARY

MULTIPLE MU OPIATE RECEPTORS: BIOCHEMICAL AND PHARMACOLOGICAL EVIDENCE FOR MULTIPLICITY

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The concept of opiate receptor multiplicity is almost 20 years old. "Receptor Dualism", proposed by Martin in 1967 [1], attempted to explain the complex interactions between morphine and its N-allyl derivative, nalorphine. Since then, investigators have postulated specific receptors for morphine (mu) [2], the benzomorphans (kappa and sigma) [2], the enkephalins (delta) [3] and β-endorphin (epsilon) [4]. Of these sites, the morphine (mu) and enkephalin (delta) sites have been studied most extensively. This Commentary will examine the concept of multiple mu receptors, specifically the biochemical and pharmacological evidence for the mu_1 receptor, a common, very high affinity binding site for opiates and enkephalins [5, 6].

MU AND DELTA RECEPTORS

Soon after the discovery of the enkephalins, Kosterlitz and colleagues [3] noted very interesting differences between the actions of morphine and the enkephalins in the guinea pig ileum and mouse vas deferens bioassays. Although morphine inhibited electrically-induced contractions in the guinea pig ileum more effectively than [Leu^5]enkephalin, their relative potency in the mouse vas deferens was reversed. He concluded that the compounds were acting through morphine, or mu, receptors in the guinea pig ileum and through a distinct enkephalin receptor in the mouse vas deferens, which he termed delta.

Binding studies supported the existence of these two discrete subpopulations of opiate receptors, clearly documenting sites selective for either morphine or the enkephalins [3, 7]. Equally impressive, these two classes of binding sites possessed distinct regional distributions in both homogenate binding assays [8] and detailed autoradiography studies [9]. The existence of regions containing only mu or delta sites provided very strong evidence for selective morphine and enkephalin receptors.

MULTIPLE MU RECEPTORS: MU_1 AND MU_2 SITES

We also find selective binding sites for morphine and the enkephalins and have confirmed their distinct regional distribution in autoradiography studies. However, our studies suggest that, in addition to the classical morphine-selective site, which we termed mu_2, and the enkephalin-preferring delta sites, both morphine and the enkephalins label a common site, termed mu_1, with far greater affinity than their respective selective sites (Fig. 1; Table 1) [5].

Evidence for mu_1 sites rests upon a variety of binding approaches, including competition between the opiates and the enkephalins [5, 10, 11], selective irreversible opiates [5, 10, 11–18], developmental [19, 20] and phylogentic studies [21, 22], and their regional distribution within the brain seen in computerized digital subtraction autoradiograms [23]. However, the most compelling evidence is the correlation of mu_1 sites with specific opiate actions.

Biochemical evidence for mu_1 receptors

Unlike the classical mu and delta receptors, which were originally identified in peripheral bioassays, we initially observed the high affinity, or mu_1, sites in binding studies [24]. Saturation studies using high specific activity antagonist ([^{3}H]naloxone) and mu agonist ([^{3}H]dihydromorphine) radioligands revealed curvilinear Scatchard plots. The lower affinity binding component corresponded to the sites originally reported in 1973 [25–27] while the higher affinity component bound both compounds with far greater affinity (K_D values under 1 nM). The lower capacity (B_{max}) of the high affinity sites, typically

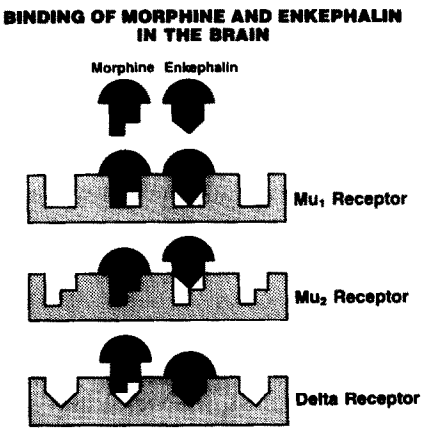

Fig. 1. Schematic representation of mu_1, mu_2 and delta binding sites.

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Table 1. Approximate K_D values of morphine, dihydromorphine and [D-Ala^2,D-Leu^5]enkephalin for mu_1, mu_2 and delta sites

	Approximate K_D values		
	mu_1	mu_2	delta
Morphine	0.4	10	70
Dihydromorphine	0.2	3	ND
[D-Ala^2,D-Leu^5]enkephalin	0.5	50	5

Values are based upon saturation and competition studies. From Wolozin and Pasternak [5]. ND: not determined.

10% that of either the morphine-selective mu_2 or delta sites, coupled with their very high affinity have occasionally presented technical difficulties in documenting their presence. The development of sophisticated computer analysis techniques [28] has been quite helpful. Using similar nonlinear regression analysis programs, a number of laboratories have confirmed the existence of this high affinity binding component for both the opiates and, more recently, the enkephalins [29–34].

Following the discovery of the enkephalins and the delta receptors, we faced a major question: How did this high affinity binding component fit into the concept of mu and delta sites? If morphine and the enkephalins labeled only two classes of sites, the higher affinity component of each ^{3}H-ligand might represent their own selective site while the lower affinity component would correspond to the other class of sites. Studies with naloxazone and naloxonazine, however, argued against this two-site model [5, 10–18, 35]. Treatment of membranes with either naloxazone or naloxonazine irreversibly eliminated the high affinity binding component of a large series of opiates (morphine, dihydromorphine, ethylketocyclazocine), enkephalins ([D-Ala^2,D-Leu^5]-enkephalin, [Met^5]enkephalinamide, [Met^5]enkephalin, [Leu^5]enkephalin) and antagonists (naloxone and naltrexone). In a two-site model, loss of the high affinity binding component for radiolabeled morphine should be accompanied by the loss of the lower affinity radiolabeled enkephalin site. Similarly, losing the high affinity component of both agonist and antagonist binding implied that the high affinity binding component did not represent agonist/antagonist conformational changes of the receptor. Although other explanations still remained, the above studies raised the possibility that the high affinity binding component might represent a common site for opiates and enkephalins.

Having ruled out the two-site model, we then focused upon the lower affinity binding component remaining following naloxazone or naloxonazine treatment. (Note: although we use the term "low affinity", it is relative. In fact, these "low affinity" sites typically have K_D values under 10 nM.) After treating tissue with naloxazone to eliminate the high affinity binding component, we examined the selectivity of the lower affinity component of [^{3}H]-dihydromorphine (K_D aproximately 3 nM) and [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (K_D approximately 6 nM) [5]. Competition studies indicated that these two sites were highly selective for either morphine or the enkephalins, suggesting that the lower affinity [^{3}H]dihydromorphine binding component represented the classical morphine-preferring mu receptor while the lower affinity [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin component corresponded to the delta site previously reported. However, this still left the question of the high affinity binding component.

The ability of naloxazone and naloxonazine to eliminate the high affinity binding component of both morphine and the enkephalins raised the possibility that they both might be labeling the same site. To examine this possibility more directly, we examined the competitive interactions between morphine and the enkephalins [5, 10, 11]. Early competition studies noted that radiolabeled enkephalin labeled mu sites in addition to delta sites [7]. Chang and Cuatrecasas reported that morphine inhibited [^{125}I]-[D-Ala^2,D-Leu^5]enkephalin in a biphasic manner. Most of the binding represented delta sites and was quite insensitive to morphine (IC_{50} 47 nM). However, morphine did inhibit between 20 and 30% of binding quite potently (IC_{50} 0.3 nM), implying that this portion of binding represented mu (morphine) sites.

Did the mu binding of [^{125}I]-D-Ala^2,D-Leu^5]enkephalin observed in the competition studies correspond to its high (K_D 0.8 nM) or its low (K_D 7 nM) affinity binding components observed in the saturation studies [7]? In the two-state model, the radiolabeled enkephalin should bind to delta sites more potently than mu sites so that addition of a low concentration of morphine should preferentially affect the lower affinity component. On the other hand, if morphine and the enkephalins shared a common high affinity site, low concentrations of morphine should compete with the higher affinity binding component of the radiolabeled enkephalin. When we examined this question, we found that morphine at 1 nM selectively competed with the higher affinity [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin binding component, indicating that radiolabeled enkephalin labeled a mu site with higher affinity than its enkephalin-selective site. The reciprocal experiments using [^{3}H]-dihydromorphine, a mu ligand, and unlabeled [D-Ala^2,D-Leu^5]enkephalin revealed similar results. Low concentrations of [D-Ala^2,D-Leu^5]enkephalin preferentially inhibited the high affinity binding component of [^{3}H]dihydromorphine binding. Together these studies strongly support the concept of a common high affinity site for morphine and the enkephalins.

Naloxazone provided another approach to this question. Previously, we noted that treating tissue

with naloxazone eliminated the high affinity binding component of a variety of radiolabeled opiates and enkephalins, including [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin. Inactivating the high affinity binding component of [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin with naloxazone eliminated the ability of low morphine concentrations to inhibit [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin binding [5, 10, 11]. Thus, two separate experimental approaches both suggest that enkephalins bind with highest affinity to a mu (morphine) site.

Two other major pieces of evidence support the concept of a distinct mu_1 site. The first is the ability of both morphine and [D-Ala^2,D-Leu^5]enkephalin to protect the high affinity binding component of [^{3}H]-dihydromorphine from low concentrations of *N*-ethylmaleimide [10]. The second involves recent digital subtraction autoradiographic studies of mu_1, mu_2 and delta sites in rat brain. In these studies, mu_1 sites labeled with [^{3}H]dihydromorphine and [^{3}H-[D-Ala^2, D-Leu^5]enkephalin had the same regional distribution, which differed from that of either mu_2 or the delta sites [23].

Correlation of mu_1 sites with pharmacological actions

Correlating a binding site with pharmacological effects is essential in establishing the relevancy of the site. Sites lacking this association have little meaning. Thus, establishing a relationship between mu_1 sites and opiate actions was a major goal. Although a number of opiate actions have been studied (Table 2) [6, 14–16, 19, 20, 33, 35–42], this review will focus upon analgesia, respiratory depression and dependence. The availability of naloxazone and naloxonazine has greatly facilitated these studies. Under specific conditions *in vivo*, both compounds show the same long-lasting mu_1-selective blockade they possess *in vitro*, permitting studies of the role of mu_1 sites in a number of opiate actions. Unlike all the other opiate receptor subtypes, the mu_1 sites have not been identified in any peripheral bioassay systems. Therefore, only central actions have been studied.

Analgesia. On the basis of his classic studies of the chronic spinal dog, Martin *et al.* [2] concluded that mu sites mediated morphine analgesia. However, our binding studies indicated that morphine labeled both mu_1 and mu_2 sites. Which mu subtype was responsible for this very important opiate action? Attempts to answer this question relied heavily upon naloxonazine and naloxazone. Treating rats or mice *in vivo* with either drug under specific conditions markedly lowers the levels of mu_1, but not mu_2, sites in the central nervous system for greater than 24 hr [12, 13]. We examined the role of mu_1 sites in opiate analgesia by determining full analgesic dose–response curves for a variety of opiates and opioid peptides 24 hr following naloxazone. Naloxazone treatment markedly diminished the analgesic potency of morphine, shifting the dose–response curve over 12-fold to the right in mice [12, 13]. Similar shifts were observed for other opiates, the enkephalins and even β-endorphin [15, 16, 33, 35, 40]. Together, these results indicated a significant role of mu_1 sites in opiate analgesia.

In these experiments, we did notice an interesting trend. Although mu_1 blockade diminished the analgesic responses of the various compounds, their shifts varied from approximately 3- to 12-fold. Martin's original hypothesis of Receptor Dualism proposed two classes of opiate receptors capable of mediating analgesia. Could the difference in the shifts of the various compounds result from their interactions with other receptor subtypes? When we compared the shifts of the various compounds with their affinities for a number of binding subtypes, we found an excellent correlation between analgesic shifts and affinity for delta sites [40]. Naloxazone shifted those compounds active at delta sites far less than morphine, supporting previous suggestions that delta sites might be important in opiate analgesia [43–46]. Additional experiments in mice suggest that mu_1 sites are involved with supraspinal analgesia while delta sites are important at the spinal cord level [40].

Respiratory depression. A major goal of opiate research over the past half-century has been the development of analgesics lacking side-effects, particularly respiratory depression. Clearly, an important question is whether different receptor classes mediate these two opiate actions. We examined the role of mu_1 sites in morphine analgesia and respiratory depression in the rat [41, 42]. Naloxonazine shifted morphine's analgesic dose–response curve,

Table 2. Tentative classification of opiate actions

mu_1 Mediated	Not mu_1 mediated
Supraspinal analgesia	Spinal analgesia (delta and/or kappa)
Prolactin release	Growth hormone release (mu_2 or delta)
Acetylcholine turnover	Dopamine turnover (mu_2 and delta)
Catalepsy	Sedation (kappa)
Hypothermia	Respiratory depression (mu_2)
Feeding (free and deprivation-induced)	Inhibition of guinea pig ileum contractions (mu_2)
	Bradycardia (mu_2)
	Reversal of endotoxic shock (delta)
	Feeding (deoxyglucose-induced)

determined with the tailflick assay, 4-fold to the right. Using serial arterial blood samples from unrestrained, free-moving rats, we then studied the effects of naloxonazine on the actions of morphine on pO_2, pCO_2 and pH. Unlike analgesia, naloxonazine had no detectable effect on morphine respiratory depression, leading us to conclude that mu_2, not mu_1, sites mediate the respiratory depressant actions observed under our experimental conditions.

Physical dependence. A major problem with the continued use of opiates is the development of physical dependence. Having determined that mu_1 sites are important in analgesia, we questioned the role of mu_1 sites in the various aspects of morphine physical dependence [38]. Physical dependence was produced by administering morphine intravenously for 24 hr and precipitating withdrawal with naloxone. Under these conditions, mu_1 blockade had little effect on most withdrawal signs monitored. Despite its antagonism of morphine analgesia, naloxonazine antagonized only two of the sixteen signs measured. These findings suggest that withdrawal is a summation of signs resulting from a variety of receptor subtypes. Analgesics active at a limited number of receptor subtypes might well produce less physical dependence.

IMPLICATION OF MU_1 SITES

This concept of a common high affinity, or mu_1, site for both opiates and opioid peptides is quite unusual. The relationship of most transmitters to their receptors is divergent: one transmitter labeling a number of receptor subtypes. However, the suggestion of mu_1 sites is an example of convergence: multiple transmitters binding to a single site. Similar examples of receptor convergence in other neurotransmitter systems might prove to be an interesting approach to the modulation of the central nervous system.

The concept of multiple mu receptors is a useful one. It offers a unified explanation for both biochemical and pharmacological evidence from a variety of experimental approaches. Equally important, it is easily testable. However, it remains an hypothesis. Regardless of how compelling the current evidence appears, alternative explanations still remain.

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