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dissertation entitled

Interactions of Mu and Kappa Opioid Receptor Agonists

presented by

Shannon Laura Briggs

has been accepted towards fulfillment
of the requirements for

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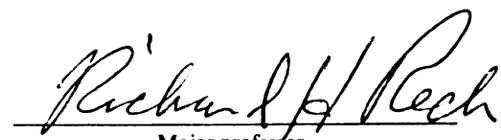

Major professor
Richard H. Rech

Date March 5, 1996

INTERACTIONS OF MU AND KAPPA OPIOID RECEPTOR AGONISTS

by

Shannon Laura Briggs

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1996

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ABSTRACT

INTERACTIONS OF MU AND KAPPA OPIOID RECEPTOR AGONISTS

by

Shannon L. Briggs

Mu and kappa opioid agonists are efficacious analgesics, especially against visceral pain. Interestingly, these opioids also produce various undesirable effects that seem to be in opposition to each other. Thus, the hypothesis that mu and kappa opioids in combination produced at least additive antinociception while reducing total side effects was tested. Regarding one of the side effects, mu-related euphoria, fentanyl and enadoline (mu-kappa opioid combination) demonstrated that a kappa agonist reduced the positive reinforcement (euphoria) of a mu agonist. Also, oxymorphone and butorphanol (mixed mu-kappa opioid combination) in the cat did not affect physiological parameters (respiratory rate, pulse rate, and mean arterial pressure) in analgesic dose levels. Results of antinociceptive studies indicated that mu and kappa opioid combinations in the colorectal distension (visceral model of pain/nociception) in rats and cats produced additive or synergistic interactions. In contrast, mu and kappa opioid combinations tested in the cold-water tail-flick (cutaneous thermal model of pain/nociception) in rats produced antagonistic interactions. These same opioids, tested individually, produced maximal levels of antinociception. Although mechanisms for antinociceptive interactions of mu and kappa opioids in colorectal distension and cold-water tail-flick are not fully understood, data from studies using antagonists (naloxone, beta-funaltrexamine, and nor-binaltorphimine) and methadone-tolerant rats indicated that mu and kappa receptor activity was involved. In conclusion, mu and kappa opioid combinations produced at least additive visceral antinociception with minimal side effects.

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This dissertation and its work are dedicated to those who struggle daily in search for better ways to cope with their pain. This work strongly supports the notion that available pain relievers may be combined to produce less bothersome side effects while also producing superior levels of pain relief. Evidence presented in this thesis should encourage others to continue developing better pain remedies and give pain sufferers more hope and a confident expectation of better things to come.

Acknowledgements

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Sincere thanks

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First, I truly thank God that this dissertation is done!

Sincere thanks go to my mentors; without such a patient, experienced mentor as Dr. Richard Rech and an ambitious, encouraging mentor as Dr. Don Sawyer, the endless work and tedium of this dissertation would have been overwhelming. I also thank the committee members, Drs. Henry Beckmeyer, Greg Fink, Jim Galligan, and Alice Young, for their insightful counsel and guidance that helped create this dissertation.

With much gratitude, I thank Rob Durham for his friendship, encouragement and all his help! Thanks for being a big brother now and then! And without the listening ears and helpful suggestions of Elaine Striler and Kristi Paul, I would have gone nuts. Thank you both for the numerous conversations that were not necessarily related to research! Special thanks go to Dr. Ryhoei Nishimura, Marsha Collins, Jenny Zuvers, Jossette Rousseau, and Stephanie Gollakner for their help in training many rats and assisting in many experiments. I also want to thank Dr. Carsten Neilson for his timely arrival and assistance with experiments, but especially for his cheerful companionship and good "horse sense." Another thank you goes to Jennifer Seguin for being a wonderful friend and encourager! Also, my years spent as a graduate student have been a lot of fun thanks to my fellow graduate students--and those lunchtime conversations at the North-West end of 3rd floor.

With much love, I wish to thank my family and husband for their unconditional love and support. Thank you Mom and Dad for the hours of proofreading and for the much appreciated computer! And thank you Grampa Andy and Grandma for the many dinners that I didn't have to make! Finally, thank you Christopher for "picking me up" so many times and for believing in me more than I believe in myself.

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CHAPTER 1

INTRODUCTION

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Efforts to learn more about pain and analgesia are motivated by one familiar factor; pain can cause suffering. In the past, pharmacological pain relief has been limited to the use of opium derivatives (mu) and only recently have more effective kappa opioid analgesics become available. The discovery of opiate receptors and endogenous analgesic peptides further advanced our understanding of pain. Excitement generated during the discovery of these clues spurred researchers and clinicians on to discover how pain is transmitted and modified. Presently, much is known on how pain is generated, communicated, and perceived. In addition, many therapies are available which can interrupt pain signals at each of those steps. However, even among these "scientific and technological advances there are millions of suffering patients who are not getting the relief they need" (Bonica, 1981). Even after years' of dedicated efforts, the battle for pain control is far from over. Advances in pain management are encouraging, but those accomplishments are not consoling to those who still suffer daily from their pain. Thus the following pages introduce the importance of the work of this thesis.

For as long as humans have existed, so has the quest to control our perception of pain. Pain is terribly unpleasant, but at times it is necessary for our survival. Certain rare individuals are insensitive to pain, which leaves them vulnerable to frequent episodes of bodily damage. For example, deaths have resulted from a ruptured appendix which later became infected because warning signs of pain were not perceived (Sternbach, 1968). Although pain is usually unpleasant, it is a necessary warning to prevent damage or life threatening injury.

If it is necessary to perceive pain, why are we so determined to escape this sensation? If removing painful sensations is not the answer, then the alternative is to find some way to control pain and relieve suffering to a point that is at least tolerable or manageable. Herein lies the essential concept in our ability to control pain: pain detection and tolerance to pain are separate entities. Pain detection is necessary for the preservation

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of self in all species. However, once pain is recognized our perception of pain can then be altered in ways so as to completely erradicate it or at least reduce it to a tolerable level. Unfortunately, the remedy for pain has its own dark side in that while morphine type opioids are commonly used for pain relief, these same drugs can elicit unpleasant or dangerous side effects or make one physically and psychologically dependent upon the drug.

Historical Perspectives

Remedies for the relief of pain have been found dating back as far as 4000 years B.C. (Jaffe and Martin, 1990; Snyder, 1989). Most historical references for the relief of pain include a description for the use of a plant with properties similar to those of opium. Writings of Homer in the Odyessey and the Iliad include references to a plant extract called nepenthe which produced effects similar to those of opium (Levinthal, 1988; Snyder, 1989). The plant that these writings referred to was most likely the opium poppy, Papaver somniferum. Harvesting opium from poppies begins in early evening after the petals of the poppy fall (Levinthal, 1988). The unripened seed pod is slit with shallow cuts which begin to ooze a white, milky substance called opium, a name derived from the Greek word "opius" meaning "little juice" (Jaffe and Martin, 1990). By morning the opium will have turned reddish brown and sticky. This resin is scraped off the pods and made into balls which can be eaten or smoked. The first written reference to the extraction of opium was found in Compositions Medicamenotrum written by Scribonious Largus in 40 A.D (Snyder, 1989). Thirty seven years later, Dioscorides experimented with the whole poppy plant and concluded that only the juice contained the active ingredient (Snyder, 1989). Some of the numerous effects of opium described in ancient writings included intense pleasurable feelings, relief of various pains including headaches, colic, urinary disorders, and labor pain. Ancient Greek and Roman literature include descriptions of the opium poppy as producing blissful somnolence. The Roman god of

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sleep, Somnus, the name from which somnolence is derived, was shown with a filled container of juice from poppy plants (Snyder, 1989). Also, Greek mythology depicts the god of sleep, Hypnos, (from which the word hypnotic is derived) (Snyder, 1989) and the god of dreams, Morpheus (from which the word morphine is derived) as receiving poppy capsules from Nyx, the god of night (Levinthal, 1988). At this point in history, most of what was known of opium appeared in writings during the Roman Empire. After the fall of the Rome Empire, it was the efforts and writings of Arabian physicians that sustained and added to our knowledge of opium. One of the more famous Arabian physicians, Avicenna, used opium extracts for the relief of diarrhea (Snyder, 1989). Throughout history, a few bona fide medicinal uses of opium had been identified, but opium was used more commonly as an ingredient in tonics concocted by physicians. These tonics were prescribed for hundreds of uses. Opium as used before the nineteenth century was described as "God's Own Medicine" (Levinthal, 1988) and considered a panacea. In the 16th century, Paracelsus, an influential physician during the Renaissance era, endorsed the use of opium as the most universal medical treatment. He was quoted as saying that opium "will dissolve disease as fire does snow" (Levinthal, 1988). One of the most popular tonics, called theriaca, was developed by Andromachus, a physician to the Roman Emperor Nero (Snyder, 1989). This tonic was popular into the 18th century. Although there may have been some medical benefits from these tonics, it is more likely that patients felt better from the effects of opium than from any benefit of other ingredients in the tonic.

Finally in 1805, the active ingredient in opium was isolated and named morphine, after the god of dreams Morpheus, by a German chemist Frederich Serturner (Snyder, 1989). He purified morphine and characterized its chemical and pharmacological properties and was awarded the most prestigious award of the Institute of France. Later in 1832, an eminent French chemist, Robiquet, isolated codeine and a few months after another Frenchman, Polletier, isolated thebaine (Snyder, 1989). Codeine and thebain are important compounds used even today; codeine is commonly used as a mild pain reliever

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and cough suppressant and thebain is used as a starting point for synthesizing many opioid compounds used in medicine. The word opioid refers to natural and synthetic derivatives related to any one of 20 alkaloids found in the seed pod of the opium poppy (Jaffe and Martin, 1990). Another important contribution was the discovery of papaverine, a vasodilator, in 1848 by Merck (Jaffe and Martin, 1990).

Although some things were known about morphine and its ability to relieve pain, any speculative notion of its mechanism was crude or rudimentary at best up through the middle ages. Abuse and addiction problems associated with morphine were documented by 16th and 17th century European physicians as in this example from Dr. John Jones:

> "The effects of suddenly leaving off the use of opium after a long and lavish use thereof are great and even intolerable distresses, anxieties, and depressions of spirit, which commonly end in a most miserable death, attended with strange agonies, unless men return to the use of opium; which soon raises them again and certainly restores them." (Jones, 1700)

In 1856 Dr. Alexander Wood developed the hypodermic needle which enabled physicians to induce more reproducible levels of opioid induced analgesia by parenteral injection (Levinthal, 1988) This was an important event since morphine taken orally often produced less predictable and dangerous variations in the onset and level of analgesia to the point of overdose in some cases. Inasmuch as the hypodermic needle contributed to the advancement of pain management, it also ushered in a more intense association between morphine and its euphoric effects, creating a stronger potential for abuse.

Although cases of abuse and addiction existed since the first effects of morphine were noted, it wasn't until the 19th century that abuse and addiction were considered serious problems for society. One example of this "paradoxically bad medicine" is best described using events that occurred during the civil war. Many wounded and dying soldiers were provided immediate and satisfying relief from pain by injected morphine.

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However, after the war, so many veterans were addicted to morphine that the addiction was termed "soldier's disease" (Julien, 1985).

Even though morphine received much attention, the majority of morphine addicts in late 19th century America were addicted to patent medicines containing opium derivatives. In fact in 1890, imports of crude opium in the Unites States used in patent medicines were equivalent to at least 50 doses for every person, including children (Levinthal, 1988). It has been estimated that the total number of opium addicts during 1900 was roughly 250,000 (Musto, 1973). Part of the reason for society's tolerance of opiate addiction was the fact that opium was easily attainable and most commonly taken orally. Opium taken orally lasts longer and was less likely to produce a rush of euphoria than injected morphine.

Regulation of patent medicines started with the Pure Food and Drugs Act in 1906 which required that ingredients be labeled as such and, if opium was an ingredient, that the phrase "habit-forming" also be included (Levinthal, 1988). Within a few years sales of patent medicines dropped by approximately one third (Musto, 1973). Another law was passed in 1909 which prohibited smoking opium (Levinthal, 1988). Opium smoking was especially popular among the Chinese living in America, and patent medicines were popular with the rest of the population. Thus only a tiny segment of Americans was affected. Actual effects of opium restriction were felt in 1914 with the passage of the Harrison Narcotic Act (Levinthal, 1988). This was in part the result of negotiations between the United States, Great Britain and China in regard to ending drug trafficking in China. The Harrison Act did not prohibit opium addiction or restrict physicians from administering it, but interpretation of this Act by Supreme Court decisions brought about certain regulations which gradually restricted availability of opiate containing patent medicines (Levinthal, 1988).

The result was that opium was no longer available for most of the public. Thus oral users of opium were forced to quit their habit overnight. Most of these opium users

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recovered from their habit with minimal side effects. However, addicts with greater dependence on opium continued their habit with illegal opium sources and the underground narcotic industry came into existence (Snyder, 1989). Morphine, previously purchased with only a fraction of a day's wages (in 1897, the Sears Roebuck catalogue advertised laudanum without prescription for about 6 cents an ounce), could now cost an entire month's wages or more (Levinthal, 1988). As the underground narcotic industry grew, it also became wise to the ways of drug characteristics, namely potency. Before the 1920's, morphine was commonly abused but soon lost its popularity to heroin, a drug which was more potent in its ability to produce a "rush" of euphoria. Small amounts of heroin in comparison to morphine produced the desired "high," and drug dealers, realizing the important factor of potency and easy concealment, put the advantages of heroin to use in the illegal drug trade (Snyder, 1989). The cost of maintaining a heroin addiction surpassed most addicts' incomes and most addicts developed criminal behavior to support their addiction (Snyder, 1989).

The infamous legacy of heroin addiction thus began in the 1920's after the Harrison Act and seemed to reach a plateau around 1960 (Snyder, 1989). During these 40 years, heroin addiction attracted minimal attention. However, after 1960 and into the early 1970's, heroin use escalated to epidemic levels (Snyder, 1989). Some reports suggest that heroin use can be correlated to the number of fatal heroin overdoses. Reports of fatal overdoses more than doubled from 1960-1964, almost tripled from 1965-1970, and continued to increase into the early 1970's (Snyder, 1989). Besides epidemic proportions of people from various walks of life pursuing a heroin addiction, United States soldiers stationed in Vietnam were developing addictions to heroin.

Foundations of Opiate Research

In response to public and political pressure, President Nixon officially declared The War on Drugs in 1971. A $100 million dollar budget was allocated to the Special Action

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Office on Drug Abuse Prevention (SAODAP), a new office created to fight drug abuse (Snyder, 1989). The director of this newly created office was Dr. Jerome Jaffe, a pharmacologist and psychiatrist experienced in treating heroin addicts (Snyder, 1989).

Up to this point in time, narcotic research yielded information on synthetic morphine-type drugs (meperidine and methadone); three mixed action agonists (pentazocine, butorphanol and buprenorphine); a mixed agonist-antagonist (nalorphine), and a relatively pure antagonist (naloxone). The latter two were used predominantly as antidotes for heroin and morphine poisoning (Jaffe and Martin, 1990). However, the mechanisms on how opiates worked remained more or less unknown. From the $100 million dollar budget, a portion of two million dollars was directed for experimental research. Portions of this grant were awarded to six research groups who had competed for the award by proposing experiments that eventually laid the cornerstones of modern opioid research.

In the 1960's, the assumption that an opiate receptor existed was fairly well accepted, especially after the synthesis of etorphine by American Cyanamide (Levinthal, 1988). Etorphine is similar to morphine in its effect and structure, but etorphine is 5,000 to 10,000 times more potent (Snyder, 1977). The unusually high potency of etorphine gave support to the idea that opiates interact with specific receptors to produce their effects, i.e., sedation and analgesia. Further support for the existence of an opiate receptor came from studies which manipulated different parts of the chemical structure of morphine. It has been recognized that analgesic action resides in only one of the enantiomers of a racemic mixture, usually the levorotatory isomer. Drastic changes and even deletions of some parts were without major effect whereas small changes to certain parts of the morphine molecule profoundly affected its pharmacology. "The best studied and most interesting change was the substitution of the methyl group on the tertiary nitrogen by an allyl or cyclopropylmethyl group, which caused the resulting molecule to become a potent specific antagonist against many of the pharmacological effects of

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morphine and related opiates. The recognition of the remarkable stereospecificity and structural constraints placed on analgesic and other actions of opiates led to the receptor hypothesis" (Simon, 1982). Also, important research with opioids and in-vitro experiments had been developed using the guinea pig ileum (Gyand & Kosterlitz, 1966; Kosterlitz & Waterfield, 1975) and the mouse vas deferens (Henderson, Hughes, & Kosterlitz, 1972). In these experiments, opiates were shown to inhibit electrically stimulated contractions in smooth muscle preparations with similar potency profiles as seen in *in-vivo* analgesic assays.

Attempts to produce unequivocal evidence for the existence of an opiate receptor (by actually isolating the receptor) began with results from Van Praag and Simon (1966), which demonstrated opiate binding in brain homegenate, but without distinction between specific and non-specific binding. Later in 1970, using stereospecific enantiomers of methadone injected into lateral ventricles of rats in an attempt to demonstrate that opiate receptors have stereospecific selectivity, Ingoglia and Dole showed that both enantiomers had similar rates of diffusion. Another attempt by Goldstein et. al. (1971), using radioactive levorphanol with either unlabelled dextrorphan or levorphanol, tried to identify opiate receptors in the rat brain by using stereospecificity as an indicator. Their results showed only 2% stereospecific binding. Finally in 1973, convincing evidence for the existence of an opiate receptor was presented by three different laboratories using three different variations of Goldstein's experiment. The three groups were Lars Terenius (1973), Candice Pert & Solomon Snyder (1973), and Eric J. Simon, J.M. Hiller and I. Edelman (1973). Lars Terenius demonstrated stereospecific binding using radioactive dihydromorphine. Fortunately, dihydromorphine, a light-sensitive molecule, was well protected from bright lights in the lab in Sweden since darkness came in mid-afternoon during the winter and only dim incandescent bulbs provided indoor lighting. In contrast, Pert & Snyder tried to use dihydromorphine in their experiments but the same molecule was rapidly destroyed by bright fluorescent lights and sunlight in Snyder's laboratory in

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Baltimore. (Snyder, 1989) Eventually, Pert & Snyder, using a modified technique from Goldstein et al. (1971) and radioactive naloxone, a pure antagonist of morphine, demonstrated that levorphanol displaced 70% of the radioactive naloxone whereas the inactive enantiomer, dextrorphan, did not compete for the binding site. Their results clearly demonstrated stereospecific binding of an opiate receptor. The third group including Simon and a postdoctoral student, Jack Hiller, modified the technique of Goldstein et al. (1971) and used the most potent opiate known, etorphine, as the radiolabeled ligand. Results from this procedure clearly demonstrated stereospecific binding of the opiate receptor. Unbeknownst to each other, the three groups individually demonstrated 50-90% stereospecific binding of an opiate receptor using three different radiolabeled opioid ligands in similar modifications of the technique reported by Goldstein et al. (1971).

In addition to the mounting evidence for the existance of an opiate receptor, Martin and colleagues (Gilbert & Martin, 1976; Martin et al., 1976) added that they had pharmacological evidence for the existence of not just one opiate receptor but multiple opioid receptors. They showed that morphine and several analogs differed in their pharmacological profiles in chronic spinal dogs. In addition to the different profiles, these drugs were unable to substitute for each other in the prevention of withdrawal symptoms in animals made dependent on one of them. From these studies, three types of opioid receptors were characterized according to the drugs that were active: mu for morphine, kappa for ketocyclazocine, and sigma for SKF 10047 (N-allylnormetazocine).

The idea of multiple opioid receptor types was supported by Lord et al. (1977) who reported that a receptor in the guinea pig ileum was sensitive to morphine and antagonized by naloxone, indicating that this receptor was the mu receptor. In addition, they reported that a different receptor, found in the mouse vas deferens, was more sensitive to enkephalin than morphine and was not as easily antagonized by naloxone as

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compared to morphine. The new binding site was called the delta receptor after the deferens tissue in which it was discovered.

Evidence for the existence of a kappa receptor gained further support by *in vivo* expermental results which showed that monkeys responded differently to benzomorphans such as ethylketocyclazocine or bremaocine when compared to morphine (Woods et al., 1979). Additional evidence for kappa receptors was shown in morphine-dependent monkeys. Results showed that benzomorphans could not suppress withdrawal symptoms in the morphine-dependent monkeys. In addition, drug discrimination studies showed that rats trained to recognize bremazocine generalized to other benzomorphans but not to fentanyl or morphine (Shearman and Herz, 1981). Benzomorphans also showed inhibitory effects in the guinea pig ileum that were antagonized by naloxone concentrations which were 3 to 7 times greater than required to antagonize morphine (Hutchinson et al., 1975). Furthermore, dynorphin had been suggested as the endogenous ligand for kappa receptors (Chavkin and Goldstein, 1981a,b; Chavkin et al., 1982), having 10-fold higher affinity for kappa sites compared to mu and delta sites (Corbett et al., 1982). Dynorphin was also shown to be about 700 fold more potent than [Leu5] enkephalin in the guinea pig ileum and far less sensitive to naloxone antagonism (Goldstein et al., 1979). Successful identification of kappa receptor binding was finally accomplished by Kosterlitz and coworkers (Kosterlitz and Paterson, 1980; Kosterlitz et al., 1981; Magnan et al., 1982). Using guinea pig brain, this group showed that mu (DAMPGO) and delta (DADL) enkephalins inhibited [3H]ethylketocyclazocine binding in a biphasic manner, suggesting that a portion of the binding sites which were bound by [3H]ethylketocyclazocine were resistant to the two peptides.

Further evidence for multiple receptor types was presented by LeMaire et. al. (1978) and Schulz et. al. (1979). The two groups showed that "the isolated rat vas deferens (RVD) was highly sensitive to inhibition by beta-endorphin but not enkephalins, morphine and other related opiate alkyloids." Wuster et. al., (1978) suggested that the

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opiate receptors in the RVD were selective for beta-endorphin and named the binding site epsilon. Tolerance studies were used again to show that beta-endorphine was still active after the RVD were made highly tolerant to etorphine, (Schulz et al 1981).

Current knowledge of opiate receptors includes the identification of at least five different opiate receptors: mu, delta, kappa, sigma, and epsilon (Gilbert and Martin, 1976; Martin, 1976; Lord et. al., 1977; LeMaire et. al., 1978; Wuster et. al., 1978; Chang & Cuatrecasas, 1979; Schulz et. al., 1979; Kosterlitz and Paterson, 1980; Chavkin and Goldstein, 1981a,b; Kosterlitz et. al., 1981; Schulz et.al., 1981; Chavkin et. al., 1982; Magnan et. al., 1982; Chang et.al., 1984; Goldstein and James, 1984; Wood and Iyengar, 1986; Nock et.al., 1990). There is further evidence, albeit inconclusive, for the existence of three more opioid receptors: iota (intestinal), lambda, and zeta (Oka, 1980; Grevel & Sadee, 1983; Zagon et. al., 1989). Besides these families of receptor types, Pasternak and Wood (1986) introduced data that indicated multiple subtypes among the individual receptor types. Of the five opiate receptors first mentioned, studies have shown evidence for subtypes of receptors such as mu1, mu2, (Rothman et. al., 1987; Toll et. al., 1984; Lutz et.al., 1985; Wolozin & Pasternak, 1981; Pasternak & Wood, 1986) and kappa 1,kappa 2, and kappa 3 (Attali et.al., 1982; Su 1988; Zukin et.al., 1988; k3 references Price et. al., 1989; Clark et.al., 1989; Gistrak et. al., 1989; Paul et. al., 1990; Paul et.al., 1991).

Physiological effects associated with mu receptor subtypes include supraspinal antinociception, catalepsy, hypothermia, and prolactin release for the mu1 receptor and spinal antinociception, inhibition of gastrointestinal propulsion, respiratory depression, sedation, straub tail (stiffened tail), bradycardia, and growth hormone release for the mu2 receptor (Pasternak and Wood, 1986; Ling and Pasternak, 1983; Ling et. al., 1985; Nath et. al., 1994). Additional studies using [^{3}H]dihydromorphine and [^{3}H]naloxone demonstrated high and low affinity binding components (Pasternak and Snyder, 1975). Wolozin and Pasternak (1981) reported evidence suggesting the lower affinity binding

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sites corresponded to mu2 receptors and higher affinity binding sites represented mu1 receptors. These findings were confirmed from studies of Fischel and Medzihradsky (1981) and Lutz et. al. (1984).

Current information on kappa receptor subtypes indicates that subtypes are not yet distinguishable by their physiological effects but rather they derive their identity from differences in binding affinties. Experimental results show that at least four arylacetamide kappa agonists (U-69,593, U-50,488, ICI 197,067, and PD 117,302) demonstrate more selective binding for the kappa1 site than the kappa2 site (Nock et. al., 1988; 1989; Zukin et. al., 1988; de Costa et. al., 1989; Paul et. al., 1990; Horan et. al., 1991; Unterwald et. al., 1991; Horan et. al., 1993). Clear evidence for kappa2 receptor binding remains controversial. Originally, ethylketocyclazocine demonstrated affinity for the kappa2 site (Iyengar et. al., 1986), but other studies indicated that [^{3}H]ethylketocyclazocine binds to epsilon rather than kappa receptors (Nock et. al., 1990). More recently, Horan et. al. (1993) showed that ethylketocyclazocine mediated antinociception was antagonized by (-)-UPHIT, a kappa1 antagonist. Thus further investigation is required before any conclusions can be made about the kappa2 receptor. In contrast to the confusion surrounding the kappa2 receptor, there has been considerable convincing evidence reported for the existence of the kappa3 receptor. Naloxone benzoylhydrazone (NalBzoH) is a novel opiate proposed to be a kappa3 agonist (Clark et. al., 1989; Gistrak et. al., 1989; Price et. al., 1989). This agonist has been shown to act in producing antinociception in the tail flick test, hot plate, and acetic acid writhing test, as well as acting as an antagonist against morphine induced antinociception (Gistrak et. al., 1989; Paul et. al., 1990). Also, antinociceptive effects of NalBzoH demonstated no cross-tolerance to morphine or U-50,488. In addition, NalBzoH was antagonized by naloxone, but not at the same degree as was morphine. In other studies, NalBzoH was not antagonized by b-FNA (mu antagonist), naltrindole (delta-antagonist), or nor-BNI (Paul

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Endogenous Opioids

Even before identification of the opiate receptor, Reynolds (1969) demonstrated profound, long-lasting analgesia induced by electrically stimulating the central gray region of a rat brain, thus suggesting that the brain itself has the capacity to produce analgesia. This finding has been confirmed by others (Mayer et al., 1971). Electrical stimulation has even been effectively used for relieving intractable pain in humans (Richardson and Akil, 1977). Electrically-induced analgesia was thought to be mediated by an opiate (morphine) type of substance and results showed that naloxone partially antagonized electrically-induced analgesia (Akil et al.,1976; Hosobuchi et al., 1977). In another experiment, results demonstrated cross tolerance between the analgesic effects from exogenous opioids and those of electrical stimulation (Mayer and Hayes, 1975).

The first attempts to identify an endogenous ligand responsible for producing analgesia occurred after the isolation of the opiate receptor. Experimental approaches to identify the ligand included screening all known neurotransmitters and hormones. This approach proved fruitless, and "thus began the search for a new substance with high affinity for the receptor and opiatelike actions" (Simon, 1982). Hughes and Kosterlitz in Aberdeen, Scotland (Hughes, 1975) and Terenius and Wahlstrom (Wahlstrom and Terenius, 1974) in Uppsala, Sweden were the first two groups to report that a substance in animal brain could produce opioid activity *in-vitro*. Hughes and Kosterlitz demonstrated that their aqueous extract from the brain inhibited electrically induced contractions in the guinea pig ileum and mouse vas deferens which was naloxone reversible. Terenius and Wahlstrom reported that their animal brain extract demonstrated competitive binding for the opiate receptor. Later that year, Goldstein reported on work done with two substances he had extracted from pituitary tissues, Pituitary opioid peptide

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(Pop)-one and Pop-two. Pop-one turned out to be beta-endorphin, and Pop-two was dynorphin. An interesting similarity was noted between dynorphin and leu-enkephalin, which was that the first 5 amino acids (aa) were identically matched. The appreciable potency of dynorphin (its name derived from the Greek, dynamis, meaning power) was best demonstrated by comparing its effect to other opioids in a tissue bath assay. Dynorphin was shown to be 730 times more potent than leu-enkephalin, 190 times that of morphine, and 54 times more potent than beta-endorphin (Goldstein et al., 1979). Goldstein's 17 aa peptide was termed Dynorphin A to distinguish it from a 13 aa version called Dynorphin B. (Goldstein et al., 1981). An even shorter version of Dynorphin A, only 8 aa in length, was called simply dynorphin (Weber et al., 1982).

Later in December 1975, Hughes et al. (1975) presented data of two pentapeptides from extracts of the pig brain that produced opioid activity. The pentapeptides were named according to their end amino acid, met-enkephalin (Tyr-Gly-Gly-Phe-Met) and leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). The following year, Simantov and Snyder (1976) characterized peptides with the same structure in the bovine brain except they noted that the peptides were present in a different ratio.

Identifying endogenous opioid peptides was a major breakthrough, but the peptide sequence had actually been determined 10 years previously in 1964. C. H. Li (1964) isolated what he called beta-lipoprotein (bLPH), a 91 amino acid peptide hormone from pituitary glands. Within the 91 amino acid sequence was the identical sequence for met-enkephalin at residues 61-65. In 1976, Roger Guillemin (Ling et. al., 1976), isolated two peptides from sheep hypothalami and pituitaries which were left over from the "Nobel prize-winning studies on hypothalamic releasing factors" (Simon, 1982). These peptides when sequenced were similar to the bLPH sequence at 61-76 and 61-77 and represented the first 16 or 17 amino acids in the 31 amino acid sequence of beta endorphin (Ling et. al., 1976). Two other investigators (Cox et al., 1976; Bradbury, 1976) working independently demonstrated opiate activity using the C-terminal fragment (61-91) of

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bLPH. The peptides discussed have now been named alpha (61-76)-, beta (61-91)-, and gamma (61-77)-endorphin (Simon, 1982).

By 1982, research efforts had revealed a distinguishable endorphin system with at least three opioid families (enkephalin, beta-endorphin, and dynorphin) originating from 3 large precursor molecules and distributed in 3 separate anatomical pathways (Akil et al., 1984). The "main site of expression for the precursor of endogenous opioids is the pituitary gland, in particular the corticotrophic cells of the anterior lobe and the melanotrophic cells of the intermediate lobe." (Buzzetti et al., 1992). The opioid peptides are synthesized from a precursor called pro-opiomelanocortin (POMC) the gene for which is located on the short arm of chromosome 2 in man (Owerbach et al., 1981).

In summary, research efforts uncovered the identity and anatomical distribution of three types of receptors which produced analgesia (mu, kappa, delta). In addition, three separate families of endogenous ligands (endorphins) were indentified and characterized according to their synthesis, site of action, and possible functions.

> It would have been convenient if it were the case that a single receptor type were linked up with only one family of endorphins, but it seems that there is considerable overlap. The met-enkephalin and leu-enkephalin peptides are primarily attracted to the delta receptors, while one of the variations of the enkephalin peptides seems equally attracted to the mu receptor. Beta-endorphin is strongly attracted to both mu and delta receptors, with a slight bias toward delta. All dynorphin peptides show a preference for kappa, but even here there are tendencies toward the other receptors (Akil, 1984).

In addition to the overlapping binding patterns, "multiple forms of endorphins were evidently released simultaneously in the brain." Thus it is possible that "slight differences

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Opioids and Pain Pathways

Since the body has endogenous chemicals which modulate the perception of pain, there also must be target tissues where these chemicals produce their effect. Our perception of pain arises from neural tracts that transmit information regarding nociceptive stimuli. A painful stimulus is usually an intense level of heat, cold, pressure, cutting, or some variation of trauma which produces actual or potential tissue damage which activates a nociceptor. Nociceptors are peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root and trigeminal ganglia (Jessell and Kelly, 1991). Activating nociceptors involves depolarizing the membrane of the sensory ending and generating an action potential. Although the transduction mechanism is not fully understood, it has been proposed that each type of noxious stimulus produces a distinct change since the threshold for one stimulus can be changed without altering the threshold of another. Nociceptors are basically located in two groups of structures or tissues: soma and viscera. The soma includes skin, cutaneous tissues and bone, whereas viscera include hollow organs such as the stomach, bladder, and colon and solid organs such as the liver, pancreas, and spleen. Nociceptors have been characterized by their response to stimuli. Thermal and mechanical nociceptors have small diameter and lightly myelinated a-delta fibers that are fast-conducting (5-30 m/s) and have high thresholds. They enter the spinal cord through the dorsal horn, terminating mostly in laminae I and V. Because of the high threshold, a strong mechanical stimulus is required to stimulate somatic nociceptors served by A-delta fibers, thus pain from these neurons is perceived as sharp, intense, and brief.

This type of pain is referred to as the "first pain." Another type of nociceptor is the polymodal nociceptors which are mostly unmyelinated, slower-conducting (0.5-2 m/s), and mediate mechanical, chemical and thermal stimuli (greater than 45°C and less than 0°C). C fibers enter the spinal cord through the dorsal horn and terminate mostly in lamina II and V. This pain is referred to as the "second pain."

The proportion of A-delta versus C fibers vary depending on the location. This difference can have functional significance. For example, the ratio between A-delta and C fibers in visceral primary afferents is 1:8 or 1:10, whereas the ratio at the dorsal root is 2:1 (Jänig and Morrison, 1986). Visceral afferent fibers have been estimated to comprise only 2-15% of all afferent fibers to various levels of the spinal cord (Ness and Gebhart, 1990). Since the visceral sensory field has such a low number of visceral afferents, there is extensive branching and convergence. Some receptive fields of adjacent roots have up to 100% overlap (Bonica, 1990). Also, Sugiura et al. (1989) described individual visceral afferent fiber terminals as having a much wider rostrocaudal distribution in the spinal cord than individual cutaneous afferent fiber terminals. Since visceral afferent fiber terminals converge onto the same second-order spinal neurons as the more numerous, less widely distributed cutaneous afferent terminals, nociceptive information is processed with other inputs. That is, virtually all second-order spinal cord neurons in the cervical lumbar spinal cord that receive visceral input also receive a cutaneous input; which are viscerocutaneous in character (Gebhart & Ness, 1990). Neurons with input from joints and muscles that converge with visceral inputs onto second-order neurons are termed viscerosomatic. It has been said, however, that input to the sacral spinal cord (L6-S2) may be only visceral,

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although convergence of input from tail muscle and joint has been noted (Ness & Gebhart, 1987a). Finally, there also exists viscerovisceral convergence onto the same second-order neurons. There is convergence of inputs from vagina, cervix, urinary bladder, and descending colon as well as from cutaneous structures (see Ness & Gebhart, 1990, for citations). In humans, convergence is observed when colorectal distension produces referred pain to the lower back and abdominal wall (Bonica, 1990). It seems that almost every visceral afferent input shares a second-order neuron, which produces convergence and a lesser ability to identify specific areas of noxious stimuli. Thus, complaints of visceral type pain are characterized as diffuse, dull, aching, or burning (Wingard et al., 1991).

Nociceptive signals from the periphery are carried by A-delta and C-fibers to the dorsal roots of the spinal cord where they bifurcate and synapse with other neurons. Some axons ascend and descend one to three segments as part of the Lissauer tract and some axons terminate in laminae I, II, and V in the dorsal horn. At the synapse, an A-delta terminal has small electron translucent synaptic vesicles that are thought to contain excitatory amino acids and a C-fiber terminal that contains large core dense vesicles that store peptides in addition to the smaller clear vesicles (Jessell & Kelly, 1991). Both A-delta and C-fibers release excitatory neurotransmitters which evoke fast and slow synaptic potentials in dorsal horn neurons. Glutamate seems to be the neurotransmitter evoking fast synaptic potentials and peptides such as substance P seem to be the neurotransmitters evoking slow synaptic potentials. Evidence for glutamate and substance P as neurotransmitters of fast and slow synaptic potentials comes from studies which showed

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that antagonists of excitatory amino acids (glutamate) blocked transmission and application of substance P produced slow synaptic potentials that mimicked those evoked from high intensity stimulation of primary afferents (Jessell and Kelly, 1991).

As mentioned earlier, nociceptive primary afferents synapse with projection neurons mostly in laminae I, II, and V of the dorsal root (spinal cord gray matter), which is divided into laminae based on structural organization of neurons (Rexed, 1954). Lamina I is called the marginal zone, Lamina II is called the substantia gelatinosa, Laminae III-V, the nucleus proprius, and Lamina VI the base of the dorsal horn (Bonica, 1990). Within these laminae are a variety of neurons which modify the nociceptive signal relayed from A-delta and C-fibers to projection neurons. Myelinated afferents (non-nociceptive), local excitatory interneurons (send sensory input to projection neurons), inhibitory interneurons, and even A-delta and C-fibers contribute to modifying the nociceptive signal that will be transmitted by projection neurons to higher centers. These various neurons are found throughout the laminae of the spinal cord, each lamina having distinct types of neurons.

A large portion of cells in lamina I are projection cells which form long ascending pathways and shorter intersegmental connections involved with sensory transmission (Basbaum, 1984; Yaksh and Hammond, 1982), while some cells in lamina I respond only to noxious stimuli and thus are known as nociceptive specific neurons (NS) (Cervero et al., 1976). The substantia gelatinosa (lamina II) contains stalk cells (excitatory influence) and islet cells (inhibitory influence) which function as dorsal horn nociceptive local-circuit neurons (Gobel, 1978). Dendrites of stalk cells receive signals from primary afferents and

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possibly descending serotonergic axons (Dubner and Bennett, 1983). Islet cells also receive descending serotonergic input, but in contrast to stalk cells, islet cells produce inhibitory influences which are GABAergic and enkephalinergic. (Dubner and Bennett, 1983; Gobel, 1978). As well as the local circuitry, the substantia gelatinosa has some interneurons projecting into surrounding laminae and into long ascending tracts (Willis, 1985). Cells in lamina V are called wide dynamic range (WDR) neurons. These neurons are also called polymodal since they are sensitive to various input: low to high mechanical, chemical, and thermal stimuli which are relayed by A-delta and C-fiber primary afferents (Wall, 1989). Axons of lamina V cells also contribute to ascending nociceptive pathways (Cervero and Morrison, 1986).

It is not just one of these neurons in a particular lamina that modifies a nociceptive signal relayed by a projection neuron, but the contribution of all the neurons and the balance of their activity that modifies a signal. This theory is called the Gate Theory, proposed by Melzack and Wall (1965). The theory is that three types of local input (*Aa/Ab*-reg primary afferent, C fibers, and inhibitory neurons) synapse onto a projection neuron.

> "Both *Aa/Ab* and C fibers have excitatory input on the projection neuron, whereas the inhibitory neurons provide a tonic inhibition of projection neurons. Thus, *Aa/Ab* and C fibers can increase and inhibitory neurons can decrease the intensity of the nociceptive stimulus. The crucial modulatory action is that the *Aa/Ab* myelinated fibers also

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activate inhibitory neurons while unmyelinated fibers suppress inhibitory neurons. In other words, activated myelinated fibers reduce the activity of projection neurons, thus pain perception is reduced. On the other hand, unmyelinated fibers increase activity of projection neurons, thus pain perception is increased" (Jessell & Kelly 1991).

These neuronal interactions are the result of many neurochemicals which have been found in the dorsal horn. Neurochemicals such as substance P, choleocystokinin (CCK), vasoactive intestinal peptide (VIP), somatostatin, and neurotensin have an excitatory influence on neurons, whereas 5- hydroxytryptamine (5-HT), gamma-butyric acid (GABA), enkephalin, and endorphin have inhibitory influences (Otsuka et al., 1982; Seybold and Elde, 1982; Stanzione and Zieglgansberger, 1983; Leah et al., 1985).

Cellular Actions

Inhibitory neurons such as islet cells of lamina II utilize neurotransmitters such as GABA and enkephalin and descending neurons release 5HT onto stalk cells of lamina II which attenuates their activity. Ruda et al. (1982) and Miletic et al. (1984) showed that electrical stimulation of the nucleus raphe magnus (NRM) would inhibit nociceptor specific (NS) input, WDR lamina I neurons, and stalk cells of lamina II. Also Guilbaud (1973) showed that LSD-25 (5-HT antagonist) inhibited electrically induced analgesia (analgesia via electrical stimulation of dorsal raphe nuclei) as measured by recordings from interneurons of lamina V. This action was most likely attributed to 5-HT (Willcockson et al., 1984). In addition, GABAa and GABAb receptors have been found presynaptically on

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A-delta and C-fibers of primary afferents (Barber et al., 1978; Patrick et al., 1983). GABAa receptors have been shown to be associated with Cl- channels and GABAb receptors have been shown to decrease Ca^{++} conductance (Desarmenien et al., 1980). Thus, GABAergic mechanisms (increased Cl- conductance and/or decreased Ca^{++} conductance) have been associated with inhibition of synaptic transmission. Besides these type of neurons and neurochemicals, there are endogenous opioid (enkephalin and dynorphin) containing interneurons. Enkephalinergic and dynorphinergic interneurons are located close to terminals of nociceptive afferents and dendrites of dorsal horn neurons that receive nociceptive afferent input (Jessell & Kelly, 1991). In addition, opioid binding sites have been found on primary afferents (Fields et al., 1980) and presynaptic opioid receptors (μ, δ, κ) have been detected in the superficial layers of the dorsal horn (Gamse et al., 1979; Fields et al., 1980; Tam and Liu-Chen, 1982) where nociceptive primary afferents containing substance P are known to terminate (Hunt et al., 1981). Furthermore, substance P-containing primary afferents have been located in close proximity to cell bodies and dendrites containing enkephalin and dynorphin (Standaert et al., 1986). Regulation of nociceptive transmission by opioids is done in part by activating opioid receptors which decrease Ca^{++} entry into the terminal of nociceptive afferents during a depolarizing stimulus and by hyperpolarizing dorsal horn neurons by activating K^{+} conductance, thus making depolarization more difficult and decreasing action potential duration (Werz and MacDonald, 1982a; Weinreich and Wonderlin, 1987). Reduction of Ca^{++} in sensory nerve terminals decreases the release of glutamate, substance P, and other excitatory neurotransmitters (Mudge et al., 1979; Yaksh et al., 1980). Specific opiate

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receptor functions have also been determined. Mu receptors, which bind some enkephalins, hyperpolarize dorsal horn neurons via an increase in K^+ conductance (which reduces transmitter release) (Werz and MacDonald, 1982a,b; Yoshimura and North, 1983; Williams et al., 1982; Pepper and Henderson, 1980; Morita and North, 1981). Also, enkephalins have been shown to block a Ca^{++} current which produces broad action potentials of C-type DRG neurons in culture (Mudge et al., 1979). Kappa receptors have been shown to decrease Ca^{++} action potentials in somata of cultured DRG cells without affecting K^+ conductance (Werz and MacDonald, 1982a; Werz and MacDonald, 1983). The endogenous kappa ligand, dynorphin, has been shown to inhibit populations of DRG neurons (MacDonald and Werz, 1986) by blocking the Ca^{++} current in C-type DRG neurons in culture (Chavkin et al., 1982). Besides endogenous opioids, exogenous mu opioids such as morphine, normorphine, and D-Ala2, MePhe4, Met5 (o)enkephalin-ol, and kappa opioids such as U50488H and tifluadom inhibited neuronal activity similarly to their endogenous counterparts (Cherubini and North, 1985). In summary, a decrease in neurotransmitter release has been shown to be brought about by different mechanisms for the mu and kappa receptor, which is most interesting to note in light of the fact that both mu and kappa receptors have been shown to coexist on the same nerve fibers. Thus, the possibility exists that mu and kappa receptor activation may act synergistically to inhibit neurotransmitter release (Cherubini et al., 1985). Taken together, all of these neural and neurochemical interactions function in the dorsal horn of the spinal cord to accomplish the intricate balance of nociceptive signal integration. The final output from the spinal cord is

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then passed to supraspinal regions which results in complex somatic and autonomic responses to noxious stimuli.

Spinal Organization of Pain Pathways

In order for supraspinal regions to receive nociceptive signals from the dorsal horn, they must be linked. In primates, nociceptive information is carried by projection neurons that make up the 5 major ascending pathways that originate in different laminae of the dorsal horn (Katz and Ferrante, 1993). The first three of the five tracts make up the anterolateral fasciculus (ALF) and are the primary tracts for the transmission of nociceptive signals from the dorsal horn to supraspinal centers. The five tracts are 1) spinothalamic tract, 2) spinoreticular tract, 3) spinomesencephalic tract, 4) spinocervical tract (or propriospinal multisynaptic ascending system), and 5) dorsal column postsynaptic spinomedullary system.

The spinothalamic tract (STT) consists of axons of NS and WDR neurons from laminae I and V-VII (Giesler et al., 1981a) that cross the midline, ascend in the anterolateral white matter on the contralateral side (Willis et al., 1979; Jones et al., 1985), and terminate in the ventrobasal complex of the thalamus (Peschanske et al., 1983). A small group of cells in laminae I and V project directly to the ventroposteriolateral nucleus and medial part of the posterior thalamus where they synapse and continue to project to somatosensory cortex. These neurons are more specifically called the neospinothalamic tract (Kerr and Lippman, 1974; Brown, 1981; Yaksh and Hammond, 1982). Remaining cells of the STT branch out and join with SRT and SMT axons to form the paleospinothalamic tract. These axons project to regions where further integration of the

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nociceptive stimuli will take place. These regions include the nuclei of the medullary reticular formation, to synapse and project onto periaqueductal gray (PAG), hypothalamus, medial intralaminar thalamic nuclei, and limbic structures. Evidence that these areas are involved in pain transmission is clear from studies that showed direct stimulation or implantation of drugs in these areas produced profound analgesia without general behavioral depression (Basbaum and Fields, 1978; Fields and Basbaum, 1978; Kerr, 1975). Evidence that the STT is involved with pain sensations is supported by data from human patients who report that pain sensation is absent below the segmental level and on the contralateral side of a lesion in the anterolateral tracts (White and Sweet, 1969). In addition, lesions in the STT diminished responses to noxious stimuli in rats (Peschanski et al., 1986). On the contrary, in an individual case history all but the anterolateral quadrant of the spinal cord was severed and pain sensations remained (Noordenbos and Wall, 1976).

The spinoreticular tract consists of axons of nociceptive neurons (Willis, 1985) from laminae VII and VIII that may or may not cross the midline and ascend in the anterolateral quadrant of the spinal cord. Axons in this tract branch out and terminate mostly in the pontomedullary reticular formation (Mehler, 1969) and some in the thalamus (Giesler et al., 1981; Keveter and Willis, 1982). This evidence supports the theory that SRT neurons trigger arousal and contribute to motivational-affective responses in response to nociceptive stimuli in addition to autonomic, motor, and somatic reflexes (Bowsher, 1976).

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The spinomesencephalic tract consists of neurons responding exclusively to noxious stimuli originating in laminae I and V which eventually terminate primarily in the PAG and cuneiform nucleus, and with a smaller contribution in mesencephalic reticular formation and other midbrain sites (Swett et al., 1985; Hylden et al., 1986a). (The periaqueductal gray region has reciprocal connections with the limbic system through the hypothalamus.) Stimulation of PAG has resulted in analgesia as well as aversive effects (Willis, 1982), including sensations of diffuse pain referred to the central part of the body or a feeling of fear (Nashold et al., 1969). From these studies and others, it has been proposed that midbrain projections influence affective aspects of pain, autonomic responses, and pain modulation (Hylden et al., 1985, 1986a,b).

The spinocervical tract consists of neurons originating in laminae III or IV which mostly respond to only tactile stimuli, but some respond to noxious stimuli even in the presence of anesthesia (Giesler et al., 1979b). These neurons ascend in the dorsolateral spinal cord to the lateral cervical nucleus (a small group of neurons lateral to the dorsal horn in the upper cervical segments of the spinal cord). Neurons from this nucleus cross the midline and ascend in the medial lemniscus in the brainstem to midbrain nuclei and thalamus (ventroposterior lateral and posterior medial nuclei). These neurons seem to be adaptive since it has been shown in humans that chronic pain returns after a period of months or years after successful cordotomy of the anterolateral quadrant of the spinal cord (White and Sweet, 1969). It has been postulated that remaining spinocervical and postsynaptic dorsal column tracts conduct nociceptive signals in the absence of a functional anterolateral quadrant.

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The dorsal column polysynaptic system consists of nociceptive neurons in laminae III & IV that project axons in the dorsal column of the spinal cord (Bennett et al., 1984) along with axon collaterals of large diameter myelinated primary afferents to the cuneate and gracile nuclei in medulla (Jessell and Kelly, 1991). Projections from the medulla ascend in the medial lemniscus to the lateral hypothalamus (Dennis and Melzack, 1977). The function of this system in relation to human sensation is unknown, but it may modulate signals transmitted in the STT.

Neural communication between spinal cord and supraspinal areas proved to be more complex than originally thought. Experiments showed that when certain areas in the brain stem were stimulated that nociceptive neurons in the spinal cord were inhibited. In other experiments, pain responses evoked by brain stem stimulation were abolished by making lesions in the dorsal lateral funiculus. These results indicated that another form of a pain modulating system existed. Further experimentation has produced a current theory which proposes that the periaqueductal-periventricular gray (PAG) is the integration center for this system. Input from the hypothalamus, limibic system (especially the amygdala), insular cortex, pontine and medullary reticular formation, and other brain stem regions converge into the PAG. Thus the PAG integrates signals related to emotion, stress, affect, general somatic sensation, and pain. In response to this input, the PAG region may release endorphins which bind to postsynaptic receptors. In addition, the PAG provides substantial excitatory input to the serotonergic nucleus raphe magnus of the medulla. Raphe nuclei axons descend to the spinal cord in the dorsal lateral funiculus terminating in the outer laminae (I, II, and V) of the dorsal horn. At this point raphe

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nuclei axons synapse with local circuits in the dorsal horn (inhibitory interneurons) where they release serotonin. In conjunction, noradrenergic neurons from the medulla and pons participate with other descending fibers to dampen nociceptive activity. Descending serotonergic and noradrenergic neurons exert inhibitory influence in at least three ways. First, when descending fibers are active, they suppress activity of inhibitory interneurons, such as GABA neurons which tonically inhibit neural activity of descending axons. Second, descending fibers synapse on dendrites of STT and thus produce direct inhibition of projection neurons. Third, descending neurons synapse and excite enkephalinergic neurons which in turn synapse and inhibit STT activity. Thus STT neurons receive direct and indirect inhibitory influences which dampens their nociceptive activity.

Pain Issues

Even though the body has many diverse mechanisms to alleviate and moderate the perception of pain, still at times, the perception of pain can be unbearable and intolerable. From an ethical philosophy, our society has determined that no subject should endure unnecessary suffering (Halsburry, 1973; Iggo,1979), thus it is our responsibility to relieve this suffering by either curing the disease producing pain or alleviating the painful symptoms. Opium derived remedies for the relief of painful suffering have been used commonly since ancient times (4000 years B.C.). It has only been during the last 30 years that new insights and methods for pain relief have been proposed and utilized by researchers and clinicians. The previous pages described the current understanding of how nociception originates and can be perceived as painful, at least by human standards. To continue, the next few pages will focus on treatments available for pain relief and the

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ability to minimize side effects. Minimizing side effects is crucial for the success of any pain relieving treatment. For example, during the 1950's, frontal lobotomies were performed to provide relief from severe pain due to malignancy (Barber, 1959). This surgical technique severed projection neurons of the medial spinoreticulothalamic pathway at the point where they synapsed in the frontal lobe. "In these patients, pain intensity and threshold were unaffected but the emotional aspects (suffering anguish) were abolished. Unfortunately, severe personality changes accompanied the procedure." (Kleinman et al., 1987).

Usually pain remedies are not nearly as invasive as the one just mentioned. Most pain relief is provided from non-steriodal anti-inflammatory drugs, alpha2 agonists, and opioids. However, opioids are used more frequently in circumstances of moderate to severe pain. Opioids as a group can be characterized by their site of action, efficacy, and potency, among other effects. For mild to moderate pain, less efficacious and less potent opioids are used, but as pain increases to moderate to severe, then more efficacious and more potent opioids are used. Besides describing pain in terms of intensity, it is also described in terms of duration. Moderate to severe acute pain is usually accompanied by sympathetic activation (Erian and Shih, 1987) and is described as having a short duration and is self-limiting, eg. postoperative incisional pain, postburn pain, severe pain associated with visceral diseases, or labor pain (Erian and Shih, 1987). These types of pain are usually manageable with a variety of drugs, especially opioid narcotics. But in cases where moderate to severe pain is present for extended periods (6 to 9 months or more), beneficial effects of opioid treatment are less appealing since opioids also produce

undesirable effects including development of tolerance, risk of addiction, and other side effects (Pasternak, 1982). Thus for severe chronic pain, clinicians have successfully treated patients using multiple techniques including longterm use of an opioid-containing pain cocktail, transcutaneous nerve stimulation, and repeated trigger point injections. When an opioid-cocktail is used long term, the dose of active drugs must be carefully monitored and varied with the fluctuation of the pain. In this manner, it is possible to treat patients for many years with low doses of opioids and prevent the development of significant tolerance. This form of therapy can only work if patients are closely followed by the physician and the pain re-evaluated frequently (Bonica, 1990).

Unfortunately, a large percentage of patients in pain (estimated to be 65 million) are not adequately treated due to insufficient administration of opioids (Bonica, 1981). Also, inadequate relief of chronic pain costs approximately $60 billion annually in health care services and loss of work productivity (Bonica, 1981). Bonica wrote that these shortcomings were due to common beliefs held by physicians and nurses that lead to "underestimation of the dosage, overestimation of the duration of action, and fear of addiction and respiratory depression" (Bonica, 1982). It was generally thought that the risks of physical and psychological drug dependence, drug abuse, increased psychological distress, and impaired cognition were too great to warrant the extended use of narcotic analgesics for sever chronic pain (see, for example Maruta et al., 1979; Maruta and Swanson, 1981: Medina and Diamond, 1977) In response to problems of physical and psychological addictions, Himmelsbach (1943) showed that for physical dependence to become a concern required regular administration of optimal therapeutic dosage of

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narcotics (demerol) four to six times daily for six weeks. In addition, the incidence of addiction has been found to be 1:4,000 in hospitalized patients who received narcotics (Porter and Jick, 1980). Furthermore, more recent reports indicate that long term therapy with opioids can be successful. Portenoy and Foley (1986) found that 24 out of 38 patients administered opioid analgesics for at least 4 years for nonmalignant chronic pain achieved "acceptable or fully adequate relief of pain." Thus for patients suffering from pain, physical dependence is not so much a problem. However, respiratory depression and nausea and vomiting seem to be the most troublesome side effects of potent analgesics (Dundee, 1977). Opioid dosage is a primary factor which influences these side effects (Grzesiak and Perrine, 1987), thus proper selection of drug in the management of acute pain depends on the intensity and cause of pain (Erian and Shih, 1987). In attempts to alleviate pain as well as undesirable side effects, alternate routes of administration such as intrathecal or epidural injections have been used with equivocal results. Opioids administered into spinal cord spaces produced long lasting analgesia, but also nausea, vomiting, urinary retention, pruritis (an annoying, less serious side effect), and respiratory depression (a major drawback in the use of intrathecal opioids) (Bromage et al., 1980; Lanz et al., 1982; Bromage et al., 1982; .Rawal and Wattwil, 1984). Thus despite innovative treatments, the problem of pain management still looms as well as the various side effects that accompany treatments. Although much is known about pain systems, there is still much more to learn. The hope for finding better methods for controlling pain lies in the specific neuroanatomical systems related to pain. Understanding these systems

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Nociceptive Models

Although all painful conditions are worthy of study, it seems that "most of what is known about the anatomy and physiology of pain is from studies of experimentally induced cutaneous (skin) pain, while most clinical pain arises from deep tissues" (Kleinman et al., 1987; Gebhart, 1992). Pain remedies available for visceral pain relief are far from optimal since most potent analgesics capable of providing pain relief also produce bothersome side effects such as euphoria, constipation, sedation, etc. To study antinociceptive effects of mu and kappa agonists on visceral pain, the colorectal distension assay was chosen as a visceral nociceptive model. As previously mentioned, there is more available information on cutaneous or somatic nociception than visceral nociception. Thus, the cold-water tail-flick was chosen as a cutaneous nociceptive model to use as a comparison to the visceral nociceptive model, CRD. Before the nociceptive models are used, it is important to have a clear understanding of the physiology and anatomy of the tissues or organs affected by the nociceptive stimuli.

Colorectal Distension

Since the visceral nociceptive model, CRD, stimulates nociceptors in the colon, the following discussion will introduce sensory and nociceptive systems of the lower gut. Sensory input from the intestines to the central nervous system is continuous and complex. A number of receptor types have been identified, but it is not known as to how different receptors interact within portions of the central nervous system or which types of gut

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stimuli provoke a central nervous system response. The enteric nervous system exhibits three levels of reflex activity. One is entirely intrinsic, with afferent and efferent fibers probably relaying in the enteric ganglia. Another level of reflex activity involves connections between afferent and efferent pathways in the prevertebral ganglia. The third level involves connections in the spinal cord and the medulla (Bonica, 1990). Ascending colonic and rectal primary afferents pass through the pelvic nerves and enter the spinal cord via ventral and some dorsal roots of S2, S3, and S4 (Bonica, 1990). Upon entrance into the spinal cord, there are three pathways (spinothalamic, spinoreticular, spinomesencephalic) which transmit nociceptive information from the dorsal horn to higher centers (Jessell and Kelly, 1991). Recordings from single fibers in the vagus show that fibers from small bowel receptors fire continuously at low frequency and the frequency increases in direct proportion to increased distension brought about by inflating a balloon within the lumen of the colon. These receptors also fire at a higher frequency during muscular contraction, which is an indication that these mechanoreceptors are connected "in series" rather than "in parallel" with the contractile units. (Bonica, 1990).

Furthermore, when studying visceral antinociception it is desirable to identify which receptors modulate these effects. Schmauss and Yaksh (1984) and Quirion (1984) have concluded that populations of spinal opioid receptors modulating the cutaneous thermal response possess distinguishable pharmacological characteristics which resemble those described as mu and delta, whereas the visceral responses are modulated by spinal receptors with profiles having characteristics resembling those of mu and kappa receptors. Thus, it has been suggested that visceral noxious stimuli are modulated primarily by mu

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and kappa receptors, in comparison to other receptors. An important difference to remember with the thermal data described above is that it came from studies using either radiant heat or warm (45° to 50° C) or hot water (55° C). Therefore, available information on tail-flick assays can only serve as an analagous model for the cold-water tail-flick due to the temperature differences.

Cold Water Tail Flick

The tail-flick utilizes a stimulus which elicits tail withdrawal from the stimulus in approximately 3 seconds in naive animals (D'Amour and Smith, 1941). Studies have shown that the tail-flick latency is a measure of response threshold (Light et al., 1979). This indicates that the response is "all or none." However, the response latency can be modulated in a graded and quantal manner (D'Amour and Smith, 1941; Levine et al.,1980; Chapman et al., 1965; Yoburn et al.,1985).

Additional studies have shown that the tail-flick latency is a spinal reflex (Willis, 1982; Lewis et al., 1980) which is normally subject to tonic descending inhibition. Studies showed that the tail-flick latency was reduced in spinally transected or lightly anesthetized rats compared to awake rats (Willis, 1982; Sandkühler and Gebhart, 1984). The reflex arc of the tail-flick consists of thermal nociceptors with unmyelinated (C fibers) or thinly myelinated (A-δ) afferent fibers. Nociceptive fibers in the rat tail give increasing responses to thermal stimuli from 37° to 45° C (threshold) to greater than 50° C (Fleischer et al.,1983; Neckar and Hellon, 1978). Nociceptive neurons project to spinal segments S3 to Co3 and terminate in the superficial dorsal horn (Grossman et al., 1982). Neural mechanisms related to tail-flick suppression are not clearly understood, but the proposed

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hypothesis is that interneurons are activated at rates sufficient to excite motor neurons located in the ventral horn of L4 to Co3 (Grossman et al., 1982; Gebhart, 1992). These motor neurons make up the efferent limb of the reflex arc that ennervates the extensor caudae medialis and lateralis, and abductor caudae dorsalis, three back muscles responsible for tail movements (Gebhart, 1992). Since the tail-flick is a spinal reflex, neuromuscular blockers could abolish responses (block motor response) without affecting pain sensation. Thus increased latencies in the tail-flick should be carefully tested (Thurston et al., 1988).

Although the neuroanatomical details of the tail-flick response are not clearly defined, pharmacological manipulations and electrical stimulation of brain nuclei have added to our understanding of how the tail-flick response can be modulated. Ness and Gebhart (1986) showed that electrical stimulation of the lateral periaqueductal gray (PAG), medial PAG, and ventromedial medulla increased threshold responses. The ventromedial medullary raphe nuclei (particularly NRM) and the dorso-lateral funiculi have been shown to mediate tail-flick antinociception (Besson and Chaouch, 1987; Willis, 1982; Fields and Basbaum, 1989). Descending spinal projections from the rostral ventromedial medulla include two types of reflex-modulating neurons: off-cells and on-cells. Studies showed that just prior to a tail flick, off-cells ceased firing whereas on-cells started firing (Fields et al., 1983). During opioid-induced antinociception, off-cells fired continuously, suggesting that tail-flick suppression was related to activity of off-cells (Fields et al., 1983). Hentall et al. (1984) later showed that less than 30 active off-cells could suppress the tail-flick response.

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The previous information has been generated from studies using a different stimulus temperature than the one in the CWTF. As helpful as the information from the warm/hot water models may be, more precise information related specifically to the CWTF should be considered. First, the CWTF has been shown to be responsive to mu and kappa opioid agonists, including morphine, dynorphin A, U-50488, and pentazocine (Tiseo et al., 1988; Pizziketti et al., 1985). One note to mention about the CWTF is that temperature is a key factor. At 0° C, kappa agonists produced poor antinociceptive effects in the CWTF (Tyers, 1980; Clark et al., 1988). However, kappa agonists (dynorphin A, U-50488, and pentazocine) were efficacious in producing antinociception in the CWTF at -10° C (Tiseo et al., 1988; Pizziketti et al., 1985). In addition to pharmacological studies, single fiber recordings of the saphaneous nerve in anesthetized rats (Kajander et al., 1994) and monkeys (Simone et al., 1994) have shown that A-delta and C "mechanoreceptors" were excited by noxious cold, and that C nociceptors were active at temperatures at or above 0° C, whereas both A-delta and C nociceptors were active at temperatures below 0° C. As results from studies using 0° and -10° C demonstrated the unique differences in nociceptive systems and their modulation, it is evident that the previous discussion of nociceptive systems related to radiant heat and warm or hot water tail flick assays can only serve as an analogous model for nociceptive systems related to the CWTF.

Criteria of Nociceptive Models

When studying a nociceptive stimulus, there are at least six criteria that have been proposed by the International Society of Pain that should be considered before using a

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nociceptive model. First, a stimulus should simulate naturally occurring conditions to activate only those receptors normally involved in eliciting a painful sensation. Second, the stimulus should be repeatable so that effects can be measured over time and for a number of presentations. Third, the stimulus should elicit reproducible responses. Fourth, if presentation of a stimulus requires restraint, then training or conditioning to the restraint before testing is an effective method of minimizing stress-related release of endogenous endorphins which might confound the study. Fifth, only one stimulus type should be presented per study due to complications of stimulus-induced analgesia, ie., cold water swim test. Finally, a nociceptive stimulus should be presented at the most minimal level which elicits a behavioral or physiological response that is reproducible and can be used as an experimental endpoint.

The CRD and CWTF meet all of these criteria. In addition, the focus of this project is the interaction of mu and kappa opioids in their ability to relieve visceral pain in comparison to cutaneous (thermal) pain. Another advantage of CRD and CWTF is that they are sensitive to clinically relevant doses of analgesics (Sawyer et al., 1991b; Gebhart, 1992). This observation gives more credence to the proposal that CRD (Ness and Gebhart, 1990) and CWTF (Kreh et al., 1984) simulate naturally occurring conditions (which is one of the criteria just mentioned). (In humans, hollow organ distension has already been used extensively for characterizing nociception in the gastrointestinal tract, urinary tract, vagina, uterus, biliary system, and gall bladder; Ness and Gebhart, 1990). The importance of using a naturally occurring nociceptive stimulus has been discussed by Shaw et al. (1988). They pointed out that the use of 55° C thermal stimulus or 9% acetic

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Another advantage of using CRD and CWTF is that they are non-invasive and applicable to many species. CRD has been adapted for use in ponies, cats, dogs, rats, and rabbitts (Pippi & Lumb, 1979; Muir, 1982; Jensen et al., 1988; Sawyer et al., 1985; Sawyer and Rech, 1987; Houghton et al., 1991; Sawyer et al., 1991a,b; Rech et al., 1987; Sawyer et al., 1990; Danzebrink and Gebhart, 1990; Gebhart, 1992; Diop et al., 1994). The CWTF has been studied in at least two species, the rat and monkey (Pizziketti et al., 1985; Simone et al., 1994). Moreover, frequent testing over long periods yield stable, reproducible nociceptive thresholds for both CRD and CWTF. The application of a model to many species has the advantage that comparisons across a number of species can be made, including humans. Application in human studies is helpful because human subjects can verbalize what distension feels like and how drugs are working to alter their perception.

Mu and Kappa Opioid Agonists

Since the focus of this thesis is on the ability of mu and kappa opioids to produce analgesia, it is necessary to be familiar with not just analgesic effects, but also their effects on other physiological systems. Mu and kappa receptors are similar in that they both

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provide pain relief but they differ dramatically in other effects. Mu receptor activation produces analgesia in numerous conditions (Martin, 1983) as well as positive reinforcement (Woods 1979), self administration (Young et al., 1984), significant place conditioning in rats (Carr et al., 1989; Mucha & Herz, 1985), discriminating effects (Herling & Woods, 1981a, b; Holtzman, 1985, Tang & Collins, 1985), withdrawal effects such as irritability, restlessness, nausea, depression, excitability (Jasinski, 1985), physical dependence (Martin, 1983), prominent tolerance (Martin, 1983), respiratory depression (Keats, 1985), constipation (Martin, 1983), and urinary retention (Dykstra, 1987a; Leander, 1983a; Richards & Sadée, 1985). Mu agonists are also antagonized by comparably lesser amounts of common opioid antagonists than are kappa agonists (Schmauss & Yaksh, 1984; Dykstra, et. al., 1987b; Negus, 1993a; France et al, 1994). More specific mu antagonists, such as β-FNA, antagonized the effects of morphine and alfentinal (mu agonists) in Rhesus monkeys in the tail withdrawal assay, in urine output, and in drug discrimination studies (Dykstra et. al., 1987b), but are not effective against kappa agonists.

Kappa receptors also produce analgesic responses (Martin, 1983) and are responsible for producing negative reinforcement (Woods et al, 1979; Pfeiffer et al, 1986) and significant place and taste aversion (VonVoightlander, 1983; Mucha & Herz, 1985; Iwamoto, 1986; Shippenberg & Herz, 1986; Bechara & van der Kooy, 1987; Shippenberg & Herz, 1991). Further evidence for this "aversive" trend includes results indicating that non-human subjects seldom administer kappa opioids for reinforcing effects (Tang & Collins, 1985; Young et al, 1984). Other studies showed that a kappa agonist did not

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produce cross-tolerance with morphine in regard to conditioned placement (Shippenberg & Herz, 1988). Also, in monkeys, others have concluded that there is convincing evidence that mu and kappa agonists possess distinct stimulus characteristics (Herling & Woods, 1981a,b; Holtzman, 1985; Tang & Collins, 1985). Kappa opioids also produce dependence and withdrawal symptoms that are distinct from and less severe than those of mu opioids (Woods & Gmerek, 1985; Gmerek et al., 1987). Respiratory functions are minimally affected by kappa opioids at analgesic doses (Dykstra, 1987a, Butelman 1993; France & Woods, 1990, Howell, 1988). However, recent evidence indicates that some kappa agonists produce changes in respiratory function: U-69,593 decreased frequency of respiration and volume of respiration to less than 40% of control and CI-977, DUP 747, PD 117302, and spiradoline had limited effects (France, et al 1994). Kappa agonists produce diuresis, in contrast to mu agonists which produce urinary retention. Although mu and kappa receptors seem to be opposing each other in this function, data suggest that this opposing interaction is distinctly related to their individual effects on urination via their own receptors (Leander, 1983a; Richards & Sadée, 1985). Also, doses of kappa agonists that produce diuresis and discriminative effects are well below those of analgesic doses, muscle relaxation, and stupor (Dykstra, 1987a). In antagonist studies, higher doses of naloxone (Schmauss & Yaksh, 1984), nalorphine (France et al, 1994), and quadazocine (France et al, 1994; Dykstra et al, 1987b; Negus et al 1993a) were required to antagonize effects of kappa agonists (ie: bremazocine, CI-977, DUP 747, PD117302, Ethylketazocine, spiradoline, U-50488H, and U-69593) in comparison to mu agonists (alfentanil, morphine). The specific mu antagonist β-FNA did not change the profile of

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kappa agonist effects (U-50, ethylketazocine, bremazocine) in tail withdrawal, urine output, and drug discrimination studies (Dykstra, 1987b). In contrast, nor-BNI, a specific kappa antagonist, attenuated anitnociception of a kappa agonist, spiradoline, in the hot plate test without affecting morphine antinociception (Jones & Holtzman, 1992).

The idea of using lesser amounts of mu and kappa agonists in combination to produce superior pain relief with less side effects of each drug is an attractive hypothesis. France et al. (1994) commented that despite some of the adverse effects of kappa agonists, they might be clinically useful in pain management, especially when mu agonists would be contra-indicated. The advantage of kappa agonists is that they are efficacious in producing analgesia and they have a potentially wide margin of safety. Furthermore, kappa agonists are unique from other pain relievers in that they have opposing side effects to mu agonists (see earlier discussion in this section). Thus, not only are kappa agonists appealing as pain relievers, but also as adjuncts to mu agonists. To study these effects of mu and kappa agonists in combination, agents listed below were chosen based on information available that these agonists are either mu or kappa selective.

Agonists used in Nociceptive Testing

There are two important factors, efficacy and potency, used to characterize agonist activity. Efficacy is a term used to describe the intrinsic ability of a compound to elicit an effect at its receptor. Some compounds are highly efficacious (full agonists), minimally efficacious (partial agonists), and some have no efficacy (antagonists).
Potency describes the quantity of compound required to elicit a response. A highly potent compound, such as etorphine, can produce dramatic effects with extremely smaller

quantities than morphine. These two terms, efficacy and potency will be used at times to describe and compare the following agonists used in this work. The following agonists were used in this study on the basis of either their selectivity or clinical usefulness.

Mu Agonists

Fentanyl is a congener of meperidine and a member of the phenylpiperdine group (Ferrante, 1993). Although fentanyl is a mu agonist, it is structurally different from morphine and its derivatives. As an analgesic, fentanyl was estimated to be 80 times more potent than morphine (Jaffe and Martin, 1990). Pharmacological profiles characterized fentanyl as a highly selective mu1 and mu2 agonist. Its effects at these receptors include antinociception, discrimination, respiratory changes, emesis (urinary retention), and motivational effects (Jang and Yoburn, 1991). (In analgesic studies the ED_{50} dose of fentanyl is approximately 0.003 mg/kg SC [Hayes, et al., 1987]). As previously mentioned in the section *Mu and Kappa Agonists*, the effects of fentanyl have also been shown to be antagonized by doses of naloxone and naltrexone appropriate for antagonizing mu activity and by the selective mu antagonist β-FNA in pigeons, monkeys, and rats .

In contrast to fentanyl and other phenylpiperidines, oxymorphone is similar in structure to morphine and is a member of the semisynthetic group (Ferrante, 1993). Oxymorphone is at least 10 times more potent as an analgesic than morphine (Beaver et al., 1977; Sinatra and Harrison, 1989), but less potent than fentanyl. Since oxymorphone produces similar effects to morphine, it was suggested that oxymorphone binds to mu1 and mu2 receptors (Ferrante, 1993).

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Kappa Agonists

Spiradoline (U-62066), a racemic mixture, has been characterized as a selective kappa receptor agonist in antinociceptive, discriminative, and binding studies (Lahti et al., 1985; Clark et al., 1988; Von Voightlander & Lewis, 1988; Piercey & Einspahr, 1989; Holtzman et al., 1980; Holtzman et al., 1991; Balster & Willetts, 1988; Meecham et al., 1989). The (+) enantiomer has been characterized as having a micromolar affinity for kappa receptors and a nanomolar affinity for mu receptors. Although the (+) enantiomer has affinity for the mu receptor, it also has low potency at this site (results thus far do not indicate if it is an agonist or antagonist) (Meecham et al., 1989). In contrast, the (-) enantiomer has been shown to have a high affinity (more than twice the mu/kappa affinity ratio than the racemic mixture) for the kappa receptor (Von Voightlander & Lewis, 1988; Meecham et al., 1989). In antinociceptive studies, intraperitoneal and intraspinal injections of spiradoline in mice have been shown to be 260 times more potent in the tail flick and 120 times more potent in the hot plate assay than the selective kappa agonist U-50488 (Piercey & Einsphar, 1989). Also, in comparison to the effects of U-50488, it has been suggested that spiradoline can rapidly penetrate the blood brain barrier (Piercey & Einspahr, 1989). Unlike U-50488, spiradoline has been shown to be only marginally antagonized by serotonin depleting agents (reserpine and p-chlorophenylalanine), whereas these agents profoundly antagonized the antinociceptive effects of U-50488 (Von Voightlander & Lewis, 1988). The effects of spiradoline on smooth muscle bioassays have also been shown to be antagonized by a selective kappa antagonist, nor-binaltorphimine (nor-BNI) (Meecham et al., 1989). The effects of spiradoline have also been shown to be antagonized by doses of naloxone and naltrexone that are approximately

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ten times more than those effective against mu agonists (Von Voightlander & Lewis, 1988; Holtzman 1983, 1985, 1991; Dykstra, 1988; Meecham et al., 1989). There is a growing body of evidence showing that spiradoline (racemate) produces its effects predominately via kappa receptors, whether the experiments are designed to test analgesia or discriminitive effects.

Enadoline (CI-977 or PD129290) has also been characterized in various assays as a selective kappa agonist (Lahti et al., 1985; Clark et al., 1988; Leighton et al., 1987; Von Voightlander & Lewis, 1988; Hunter et al., 1990) and has been shown to have the highest potency (more potent than U50488H and spiradoline) and efficacy at kappa receptors in comparison to other arylacetamides (Hunter et al., 1990). Results of binding studies demonstrated a mu/kappa affinity ratio of almost 1000 (Meecham et al., 1989). Enadoline also has (+) and (-) enantiomers, but in contrast to spiradoline, results showed that the (+) enantiomer displayed very low affinities for both kappa and mu receptor sites and that the (-) enantiomer showed an even greater affinity for kappa over mu sites than the racemic mixture (Meecham et al., 1989). Included in the effects of enadoline are its antinociceptive efficacy against a paw pressure test in the rat and hot plate and acetylcholine-induced abdominal constriction in the rat (Hunter et al, 1990). The effects of enadoline have also been antagonized by naloxone at doses indicative of kappa activity (Dykstra et al., 1987b) and by the kappa selective antagonist, nor-BNI (Hunter et al., 1990).

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Statement of Purpose

This thesis will present research that tests combinations of various specific opioids for potential to be used in a manner which (1) decreases addiction liability, (2) produces minimal changes in physiological parameters (respiratory rates, pulse rates, and mean arterial pressure) and (3) most importantly, provides superior pain relief relative to either single agent alone. In addition, the selective use of opioid combinations for specific types of pain will be discussed using the CRD and CWTF models as examples.

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CHAPTER 2

OXYMORPHONE-INDUCED ANALGESIA AND COLONIC MOTILITY MEASURED IN COLORECTAL DISTENSION

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Summary

Changes in colonic motility in rats following intravenous (I.V.) administration of oxymorphone (0.1 mg/kg), atropine (0.1 mg/kg), or saline were monitored to determine if opioid-induced changes in colonic motility affect antinociceptive measurements when using colorectal distension (CRD) as a nociceptive assay. Polygraph recordings of colonic pressures, contraction frequencies, and the pressure-volume relationship of the stimulus showed that oxymorphone produced a transient increase in contraction frequencies when compared to atropine- and saline-treated rats. The transient increase in contraction frequency caused by oxymorphone declined to baseline levels 30 minutes after administration, the time point at which the nociceptive threshold for CRD was tested. Neither oxymorphone nor atropine changed baseline pressures or the pressure-volume curve for the balloon stimulus. Antinociceptive results from CRD 30 minutes post treatment showed that only oxymorphone produced significant antinociception. We conclude that oxymorphone does not produce changes in colonic motility that complicate antinociceptive measurements in CRD and that CRD is an effective means of testing opioid-induced visceral antinociception.

Introduction

Distending hollow organs, such as the colon, is a means of studying visceral nociception in a variety of species (Diop et al., 1994; Gebhart and Ness, 1990; Jensen et al., 1988; Nakayama et al., 1990; Ness and Gebhart, 1988; Sawyer and Rech, 1987; Sawyer et al., 1990; Sawyer et al., 1991). Studies using CRD in testing opioids or other antinociceptive agents generally have not included data regarding how colonic motility may affect measurements of antinociception. Mu opioids are known to produce effective

antinociception as well as powerful effects on gastrointestinal motility (Galligan and Burks, 1983; Nakayam et al., 1990; Porreca et al.,1983; Tavani et al.,1990). Thus, when using CRD as a nociceptive assay in testing opioids, especially mu opioids, it is important to know how these agents affect distensibility of the colon, i.e., elastic and contractile properties of the colon. If opioids relax the colon and increase distensibility at the time that antinociceptive measurements are taken, then those measurements may be misleading. In addition, an increase in the threshold pressure stimulus following opioid treatments could be due to the increased distensibility of the colon and not a true antinociceptive effect. If the colon is affected by mu opioids in this manner, then CRD may not be appropriate as a nociceptive assay due to the fact that opioids may be creating an apparent antinociceptive effect by increasing colonic diameter. On the other hand, if colonic motility is not changed by opioids, then CRD would be useful as a nociceptive assay. These studies were done to assess changes in colonic motility caused by oxymorphone to determine if oxymorphone would change colonic motility at a time when antinociceptive measurements were made.

Materials and Methods

Subjects: Five Harlan Sprague Dawley rats (480 to 590 g), trained for the CRD protocol, randomly received one I.V. dose of each of the following treatments: atropine sulfate (0.1 mg/kg) (The Butler Company, Columbus, OH), oxymorphone hydrochloride (0.1 mg/kg), (Numorphan) (Dupont Pharmaceuticals, Manati, Puerto Rico), and saline (control). All drugs were administered in a blinded manner. The dose of atropine was selected for its gastrointestinal effects (Galligan and Burks, 1986, Yokotani et al., 1983) and the dose of oxymorphone was selected for its analgesic effects (Durham, 1992). Oxymorphone at 0.1 mg/kg produced sedation, but not to such a level that prevented ambulation. Rats were trained to lie quietly while wrapped in a towel with a balloon catheter inserted into the colon per rectum. To obviate stress during the study, I.V.

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catheters were implanted into tail veins at least 15 minutes prior to each study. Nociceptive thresholds were established using an air-filled colonic balloon catheter. Air was used to distend the balloon to designated pressures and then released into a volume displacement system where the volume of liquid displaced by the pressurized air was measured. When the pressure stimulus distended the balloon sufficiently to reach the minimum nociceptive threshold, the rat responded with an increased tone of abdominal muscles, also referred to as a guarding response. These contractions activated an abdominal belt equipped with a strain gauge (Omega Engineering, Inc.), which caused significant deflections of an oscillograph trace. An oscillograph trace deflection denoting a nociceptive response is at least 6 times larger than that of background deflections in the absence of a stimulus (Durham, 1992). Nociceptive threshold data were represented graphically as a percent of maximum possible effect (MPE) by subtracting the pre-drug control (C) from the post-drug pressure at time = n (PDn), and dividing that value by the difference of the maximum pressure (M) and the control pressure (C), and multiplying by one hundred (Harris and Pierson, 1964).

$$MPE = \frac{(PDn - C)}{(M - C)} \times 100$$

Next, the air-filled balloon catheter was removed and replaced with a water-filled balloon catheter to measure pressures and contraction frequency of the colon. Colonic pressures and contractions were recorded for fifteen minutes using a pressure transducer connected to a polygraph (Grass Model 7D), Grass Instruments Co., Quincy, MA. Only contractions producing at least a 5 mm Hg change in pressure were considered significant; these recorded deflections were later counted manually. After fifteen minutes, I.V. catheters were flushed with 0.2 ml saline, the coded drug administered, and catheters again flushed with 0.2 ml saline. Pressures and colonic contractions were recorded for 30 minutes after

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drug administration. The water-filled balloon was then removed and replaced with the air-filled balloon and the CRD threshold was again measured.

A one-way repeated-measures analysis of variance was performed on data from colonic pressures and the pressure-volume relationship of the stimuli. A two-way repeated measures ANOVA on two factors was used to test for differences between groups for frequency of contractions, and Student-Newman-Keuls test was used to determine differences between pairs. The criterion for a significant difference was $p < 0.05$.

Results

Oxymorphone, (0.1 mg/kg) I.V., produced a significant antinociceptive effect at 30 minutes post-injection (MPE mean ± S.E. = 100 % ± 0, $p < 0.05$), while neither atropine nor saline injections produced antinociception (MPE mean ± S.E. = 0 % ± 0, $p < 0.05$). Atropine or saline did not alter colonic motility following I.V. administration. Oxymorphone, however, did cause a transient increase in the frequency of phasic contractions. The increase in contraction frequency reached a peak at 5 minutes and declined toward baseline levels over the next 25 minutes (Figure 1). Colonic pressures from oxymorphone-, atropine-, and saline-treated rats were compared and there were no significant differences between groups. Also, colonic pressures for each treatment recorded for 30 minutes after drug injection were not different from pressures recorded for 15 minutes before drug injections. The stimulus pressure producing a guarding response during pre-drug trials (control pressure) was presented again 30 minutes after drug administration. Volumes of water displaced by the control pressure at 30 minutes did not differ from that displaced by those same pressures during pre-drug trials. Furthermore, volumes of water displacement were not changed by oxymorphone, atropine, or saline (Table 1).

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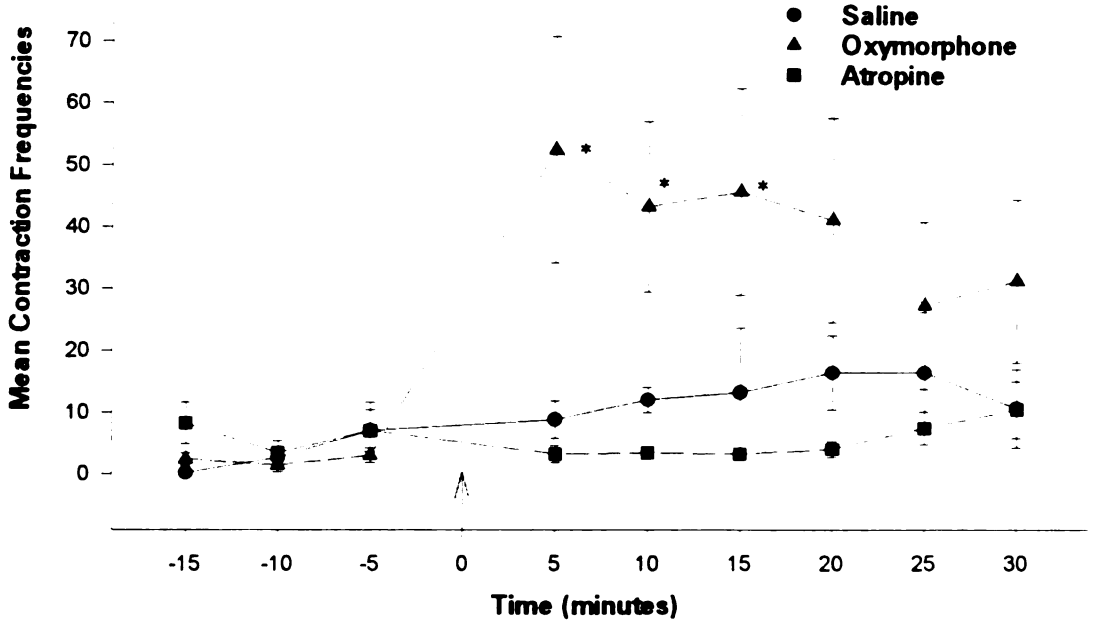

Figure 1. Mean contractions per 5 minute periods as measured for 15 minutes before drug injection and 30 minutes after drug injection.
Each point represents the mean ± S.E. of data from 5 rats. Only oxymorphone-treated rats showed a significant (* = $p < 0.05$) difference from control.

Tab

Pre

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Table 1. Volume (ml) displacement from control pressure (mmHg) stimuli, mean ± S.E.

	Before drug administration		After drug administration	
Treatment	Volume (ml)	Control Pressure (mmHg)	Volume (ml)	Control Pressure (mmHg)
Saline	7.8 ± 0.2	242 ± 6.6	6.8 ± 0.6	244 ± 6.7
	7.7 ± 0.4	242 ± 6.6		
Oxymorphone	8.1 ± 0.4	256 ± 13.6	7.7 ± 0.5	256 ± 13.6
	7.7 ± 0.5	256 ± 13.6		
Atropine	8.0 ± 0.2	244 ± 8.1	7.3 ± 0.2	244 ± 8.1
	7.5 ± 0.2	244 ± 8.1		

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Discussion

Mu agonists have potent analgesic and gastrointestinal effects, including profound changes in gastrointestinal motility. When using CRD to study opioid induced antinociception, it is necessary to establish that the "analgesic effect" is pharmacodynamic influence and not an artifact resulting from an alteration in colonic distensibility. Since mu agonists can affect phasic and tonic contractions of the colon, measurements were made of the frequency of phasic contractions and of the baseline pressure or tone of the colon in oxymorphone-, atropine- and saline-treated rats. Results showed that oxymorphone did not alter the tone of the colon compared to effects in saline and atropine treated rats. This is indicated by data in Table 1 which shows that drug treatment did not alter the pressure-volume relationship of the colon. Oxymorphone produced an increase in contraction frequency, as has been previously demonstrated (Galligan and Burks, 1983; 1986; Gillan and Pollock, 1980; Nakayama et al., 1990). However, contraction frequency returned to levels similar to that of saline-treated rats at 30 minutes, at which time a maximum antinociceptive effect was observed. Furthermore, the pressure-volume relationship of the stimulus observed in all three treated groups did not change in this study, indicating that colonic distensibility was not affected by any treatment, including oxymorphone. A similar observation was reported by Diop et. al., who found that morphine did not affect the tone of the colon. Thus, the observed "antinociception" of this study appeared to be due to pharmacological alteration of nociceptive mechanisms rather than physiological changes in colonic motility. From this study, we conclude that CRD is a reliable nociceptive assay and oxymorphone-induced changes in colonic motility do not confound measurements of antinociceptive drug effects.

CHAPTER 3

KAPPA ANTINOCICEPTIVE ACTIVITY OF SPIRADOLINE IN THE COLD-WATER TAIL-FLICK IN RATS.

Summary

Spiradoline (U62066E) is a racemic mixture of the two enantiomers U63639(+) and U63640(-). As a racemic mixture, spiradoline appears to have kappa opioid receptor activity, but the contribution of each enantiomer toward this activity is unclear. To determine the activity of the enantiomers in comparison to spiradoline, the racemic mixture was tested in the cold-water tail-flick (CWTF) assay in male Sprague-Dawley rats. Antinociception by spiradoline was antagonized completely by naloxone 0.05 mg/kg, a dose 5 times that required to antagonize antinociception by fentanyl in this same assay. In a second procedure, rats were made tolerant to chronic methadone and then tested for altered antinociceptive effects in CWTF of fentanyl and spiradoline. Fentanyl-induced antinociception was markedly reduced, while spiradoline-induced antinociception was essentially unchanged in the methadone-tolerant animals relative to non-tolerant control subjects. U63640 (levo-enantiomer of spiradoline) produced antinociceptive levels not significantly different from that of the racemic mixture, whereas U63639 (dextro-enantiomer) failed to affect the nociceptive response in the effective dose-range of the racemate. Additionally, in animals pretreated with nor-binaltorphimine (nor-BNI), spiradoline failed to produce antinociception while fentanyl produced the usual response. Furthermore, in animals pretreated with β-funaltrexamine (β-FNA), fentanyl failed to show antinociception but spiradoline induced antinociception remained unchanged. These results show that spiradoline is a full antinociceptive agonist in the CWTF assay and that the effects of the drug are mediated through kappa opioid receptors.

Introduction

Spiradoline, is a racemic mixture of two enantiomers U63639(+) and U63640(-). The racemic mixture, U62066E, appears to have kappa opioid recepter activity in rodents (VonVoigtlander and Lewis, 1988; Meecham et. al., 1989) and primates (France et. al, 1994). However, U63639, the levo-enantiomer, has been shown to have slight affinity for mu receptors, although studies have not clearly shown whether this activity is agonistic or antagonistic (VonVoigtlander and Lewis, 1988). Pitts and Dykstra (1994), using monkeys, showed that antinociceptive dose-effect curves of the racemic mixture of spiradoline were not altered by a dose of β-funaltrexamine (β-FNA) (8.0 mg/kg, s.c.) that produced marked shifts in dose-effect curves of morphine. This evidence suggests that spiradoline acts as a selective kappa agonist in attenuating a nociceptive response in monkeys. However, the selectivity of spiradoline at the kappa receptor and its ability to produce antinociception in rodents has not been tested.

This study was done to test the hypothesis that spiradoline produces its antinociceptive effect in rats via kappa opioid receptors. The nociceptive model used in this experiment was the cold-water tail-flick. Cold stimuli have been used to study pain in human subjects (Kreh, et al., 1984), as well as animals (Pizziketti et al., 1985). The CWTF in rats is a nociceptive assay with the advantage that many subjects can be easily tested repeatedly over short intervals with reproducible results. Furthermore, Pizziketti et al. (1985) and Tiseo et al. (1988) have shown that the CWTF is specific for opioid agonists and that it is sensitive to both mu (morphine) and kappa opioid receptor agonists (dynorphin A, U-50488H, and pentazocine). Spiradoline has not been tested in this assay.

Thus, these experiments were conducted for two purposes: (1) to determine that spiradoline produces antinociception in the CWTF assay and (2) that this effect is specifically mediated via kappa receptors.

Methods

Subjects

Male Sprague Dawley rats weighing 300 to 500 grams were approved for use in the following experiments by the All-University Committee on Animal Use and Care of Michigan State University. All rats were trained over a two-month period to lie quietly in a towel which was snugly fitted around them. Training started for rats between ages of 60 to 80 days. At approximately 6 weeks, rats accomodated to being restrained in towels without struggling. The subjects were reinforced after training sessions by access to Cheerios® cereal "treats" and time to "play and socialize" on a large table-top among towels and plastic boxes and tunnels.

Drugs

Spiradoline racemic (U62066E), U63639 (levo-enantiomer), and U63640 (dextro-enantiomer) were generously provided by P.F. VonVoigtlander from The Upjohn Company, Kalamazoo, MI. Fentanyl citrate was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Methadone was purchased from Mallinckrodt (Mundelein, IL). All agonists were dissolved in saline. Naloxone was purchased from Mallinckrodt (Mundelein, IL) and diluted in saline. The selective antagonists, β–FNA and nor-BNI, which were generously provided by the National Institute on Drug Abuse, were dissolved in sterile water.

Procedure for Log Dose-Response Patterns of Agonists in CWTF

Trained rats were restrained in towels as described earlier while their tails were dipped into tap water at 27 to 30° Celsius (dummy stimulus) or a solution of ethylene glycol and water (1:1 volumes) maintained at -10° Celsius (nociceptive stimulus).

Nociceptive thresholds were determined by dipping tails into the cold solution with a timer used to measure the latency until the rat flicked its tail from the cold solution. Tail dips using tap water were also used to extinguish any conditioned pattern of tail flicking with the cold solution. After four tail dips, latencies were averaged and the mean latency was used as baseline response. Most rats removed or "flicked" their tails in less than 3 seconds. After determining thresholds, rats were released from towel restraint and given an injection of a coded drug (experimenter blinded). Rats were again restrained and responses to the cold solution were recorded at 15, 30, 45, and 60 minutes after injection. Tail dips in tap water were interspersed between tail dips in the -10° Celsius for each time point. The subjects' tails were never left in the cold solution for more than 60 seconds.

Procedure for Naloxone Antagonism

Nociceptive thresholds (controls) were determined as previously described. Trained rats were then given an injection (SC) of saline or various doses of naloxone (0.005 - 0.5 mg/kg) immediately followed by either fentanyl, 0.03 mg/kg, or spiradoline, 1.0 mg/kg. Doses of the agonists were previously determined to be ED_{50}'s in attenuating the nociceptive response. Nociceptive thresholds were again determined at 15, 30, 45 and 60 minutes post-injection.

Procedure for Methadone Tolerance

Two groups of trained rats (n=15 each) were treated every 12 hours with either methadone or saline. Doses of methadone were gradually increased to 7.6 mg/kg, at which time experiments were conducted. Thus, rats became tolerant to 7.6 mg/kg, i.p. methadone during this study. Experiments were scheduled such that residual methadone contributed no antinociceptive effect (Figure 4). On test days, both methadone-tolerant rats and saline-treated rats were randomly put into two groups each. One methadone-tolerant group and one saline group were randomly administered fentanyl (0.018 mg/kg SC) or spiradoline (0.6 mg/kg SC) and then tested. After 3 hours (time at which agonists

were no longer active), those rats that received fentanyl were injected with spiradoline and rats that first received spiradoline were injected with fentanyl. Thereafter, rats were again tested. A blinded experimenter observed and recorded latency of responses.

Procedure for Enantiomers

Eight trained rats were administered once per week an injection of a different coded drug for four weeks. Thus, all rats randomly received one dose each of: 1.0 mg/kg s.c. U62066E, 1.0 mg/kg s.c., U63639, 1.0 mg/kg s.c., U63640, and saline (vehicle). Nociceptive thresholds were determined as described earlier, followed by s.c. injection of one of the coded drugs and then followed by testing for nociceptive thresholds at 15, 30, 45, and 60 minutes post-injection.

Procedure for Selective Antagonism

Seven trained rats were pretreated s.c. with 2.5 mg/kg β–FNA. Twenty-four hours later, three of these rats received fentanyl (0.018 mg/kg) and four rats received spiradoline (1.0 mg/kg). Eight trained rats were pretreated s.c. with 10.0 mg/kg nor-BNI. After 48 hours, four of those rats received fentanyl (0.018 mg/kg) and four received spiradoline (1.0 mg/kg). A blinded experimenter observed and recorded latency of responses 15 minutes post-injection.

Data Analysis

The ED_{50} dose of fentanyl and of spiradoline was determined by using the linear regression function of Sigma Plot® for Windows. All drug comparisons were tested using a random ANOVA, except data from the enantiomers, which were tested using a repeated measures ANOVA. Student-Neuman-Keuls method was used to determine significant group differences. Significance was set at $p < 0.05$. For graphical representation, antinociceptive data were standardized as a maximum percent effect (MPE) (Harris and Pierson, 1964):

$$MPE = \frac{PDn - C}{Max - C} \times 100$$

where PDn is the stimulus level that a subject responds at n minutes post-injection. C is the stimulus level to which a naive subject normally responds. Max is the maximum stimulus level presented to a subject.

Results:

Dose-Response Patterns in CWTF

Results of the log dose-response effects in the CWTF demonstrated that spiradoline acted as a full agonist in producing antinociception with an ED_{50} of 0.56 mg/kg s.c. (Figure 2). This result is in good agreement with results of spiradoline tested in other nociceptive assays in the rat (VonVoigtlander and Lewis, 1988).

Naloxone Antagonism

To determine specificity of spiradoline at the kappa receptor, incremental doses of naloxone were used to antagonize antinociceptive effects of fentanyl and spiradoline. Results showed that naloxone (0.0025 mg/kg) did not alter the effect of fentanyl, while 0.005 mg/kg of naloxone reduced fentanyl's effect by 50%. Naloxone at 0.05 mg/kg fully antagonized fentanyl, but had no effect on spiradoline. A naloxone dose of 0.10 mg/kg also fully antagonized fentanyl without affecting spiradoline, and 0.5 mg/kg of naloxone fully antagonized both fentanyl and spiradoline, to an equivalent effect of saline (Figure 3).

Methadone Tolerance

Control responses of rats made tolerant to methadone were not different from those of control rats. Results showed that the antinociceptive effect of fentanyl in methadone-tolerant rats was markedly lower ($p < 0.05$) than that observed in non-tolerant rats. Antinociception induced by spiradoline in methadone-tolerant rats was not significantly altered from antinociception induced in non-tolerant rats (Figure 4).

These results indicate that methadone-tolerant rats were also tolerant to the mu agonist fentanyl but not to the kappa agonist, spiradoline, indicating that the antinociceptive effects of spiradoline were mediated by kappa receptors.

Antinociceptive Activity of Enantiomers of Spiradoline

U63639(+) was without effect as was saline for the 60-minute period that testing was conducted ($p > 0.05$) (Figure 5). However, U62066E and U63640(-) produced significant antinociception during this same period ($p < 0.05$) (Figure 5). Also, U62066E and U63640(+) produced similar levels of antinociception at all time points with the exception of 30 minutes. These results indicate that the antinociceptive effects of the kappa enantiomer were similar to those of spiradoline.

Selective Antagonism

β-Funaltreximine was found to be most potent and selective as an antagonist 24 hours after administration and nor-BNI was most selective and potent as an antagonist 48 hours after administration. Also, results showed that both β-FNA and nor-BNI produced agonistic activity in the CWTF during the first 12 hours, but not at any time thereafter (24, 48, and 72 hours). After the 24 hour pretreatment, β–FNA antagonized antinociceptive effects of a fentanyl dose of 0.018 mg/kg s.c. by 80%, whereas the antinociceptive effect of a spiradoline dose of 1.0 mg/kg s.c. in β-FNA pre-treated rats was similar to that in untreated rats, ($p < 0.05$) (Figure 6). After 48 hours, nor-BNI did not affect fentanyl-induced antinociception but antagonized spiradoline-induced antinociception. (The latency decreased from 40 seconds to less than 10 seconds; controls were at 4 seconds). These results also indicate that spiradoline-induced antinociception in the CWTF is mediated by kappa receptors.

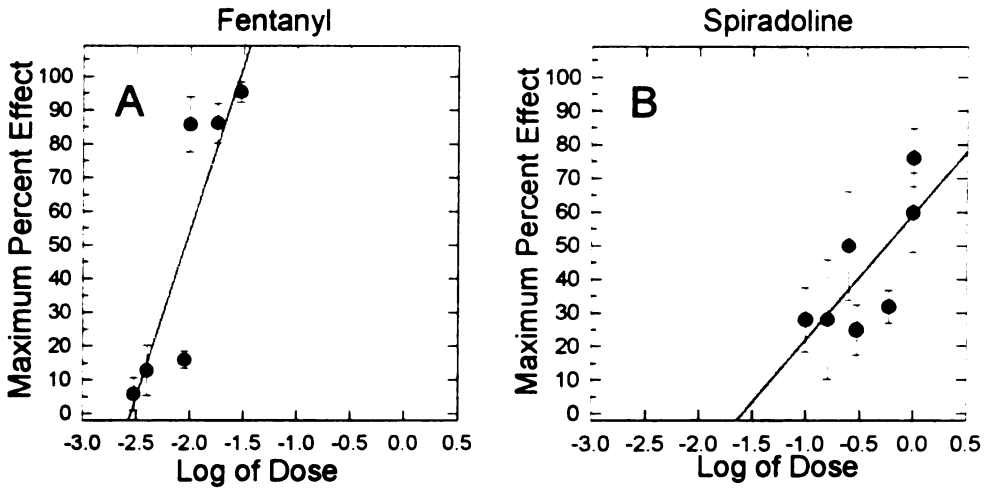

Figure 2. Antinociceptive dose-responses for fentanyl and spiradoline.
Graph A: Mean (± SEM) for fentanyl dose response in the CWTF assay 15 minutes post-injection; ED_{50} = 0.004 mg/kg SC; n = 3 to16 subjects per dose. Graph B: Mean (± SEM) for spiradoline dose response in CWTF 15 minutes post-injection; ED_{50} = 0.56 mg/kg SC; n = 3 to 12 subjects per dose.

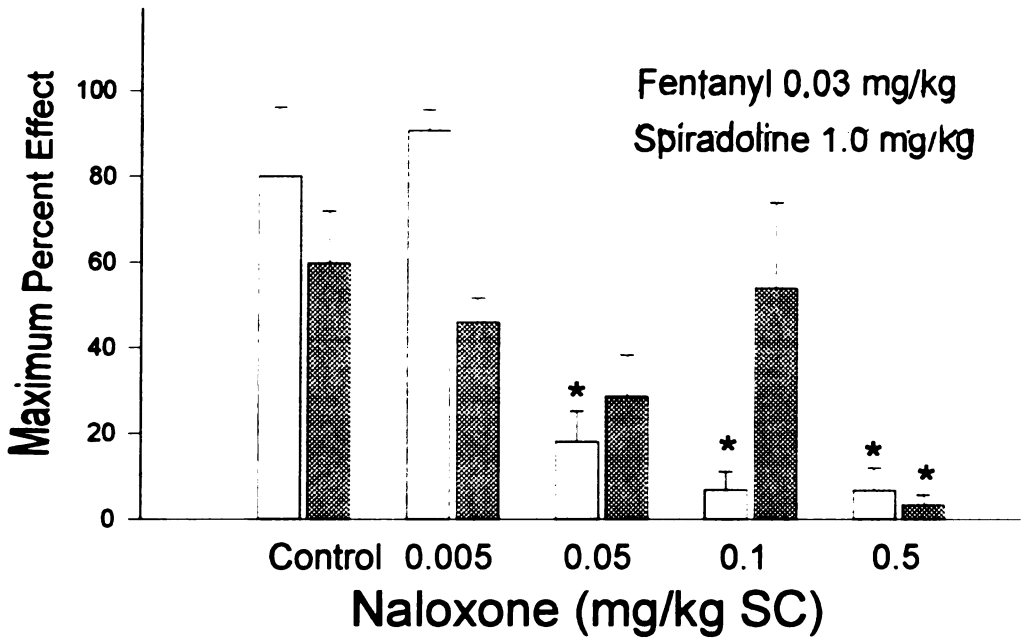

Figure 3. Naloxone antagonism of fentanyl- and spiradoline-induced antinociception. **Mean** (± SEM) of the level of antinociception (MPE) 15 minutes post-injection in subjects **administered** naloxone (N) in incremental doses (0.005, 0.05, 0.1, 0.5 mg/kg) and either **fentanyl** 0.03 mg/kg SC or spiradoline 1.0 mg/kg SC. Asterisk indicates significant **difference** from control ($p < 0.05$).

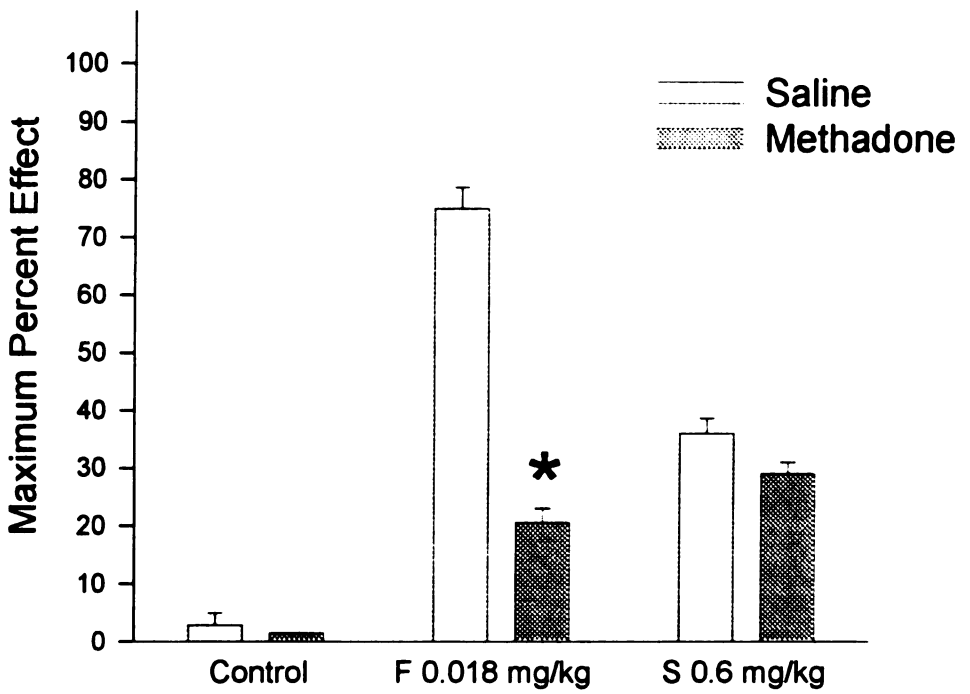

Figure 4. Antinociception of fentanyl (F) and spiradoline (S) in methadone-tolerant rats. **Mean** (± SEM) of controls and of the level of antinociception (MPE) 15 minutes post-**injection** in non-tolerant (saline) vs. methadone-tolerant subjects. Asterisk indicates **significant** difference from non-tolerant (saline-treated) subjects ($p < 0.05$).

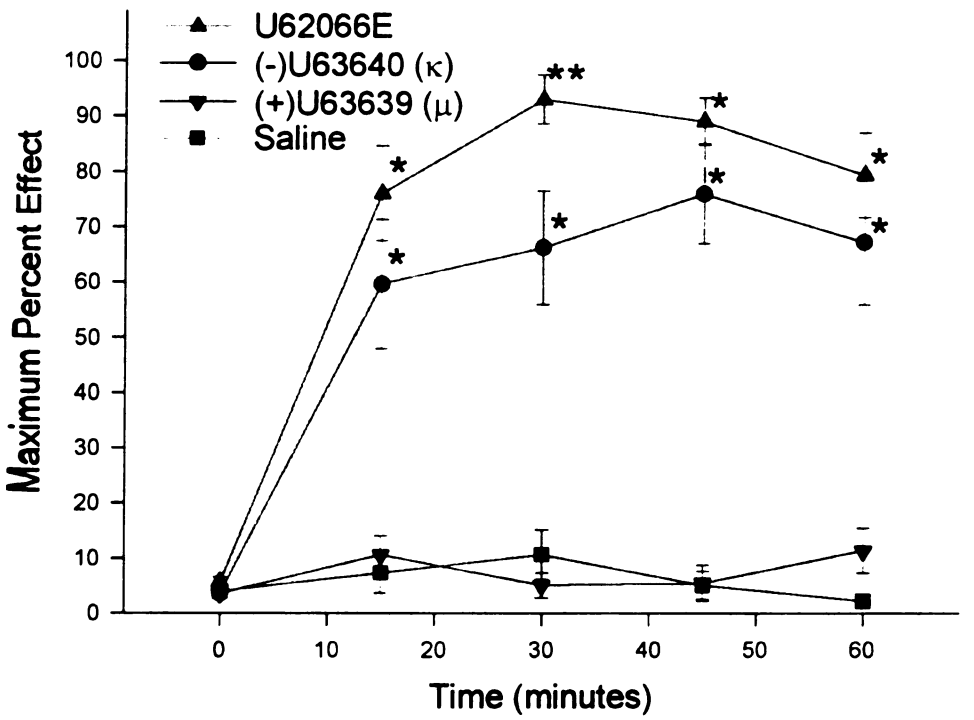

Figure 5. Antinociception of spiradoline and its enantiomers.

Mean (± SEM) of MPE 15, 30, 45, and 60 minutes post-injection in subjects administered **with** either saline (control), U62066E, U63640(-), U63639(+). Asterisk indicates **signi**ficant difference from control ($p < 0.05$). Double asterisk indicates significant **difference** from U63640(-) ($p < 0.05$).

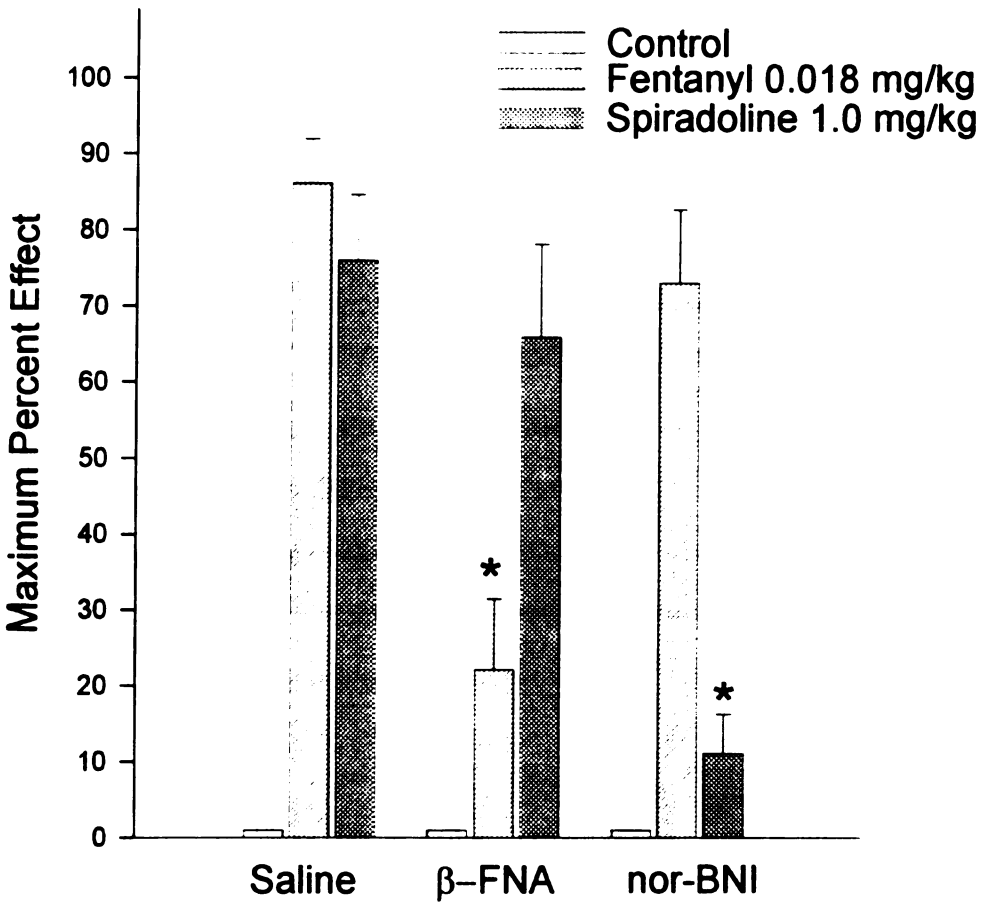

Figure 6. Antinociception of fentanyl and spiradoline in saline-, b-FNA-, and nor-BNI-pretreated rats.

Mean (± SEM) of the MPE 15 minutes post-injection in subjects administered either saline (C), fentanyl (F), or spiradoline (S). Asterisk indicates significant difference from corresponding saline pretreatment ($p < 0.05$).

Discussion

These results indicate that spiradoline acts as a full agonist in the CWTF at -10° Celsius and that spiradoline produces its antinociceptive effect by selective action at kappa receptors. Our dose-response curves of fentanyl and spiradoline indicate that mu and kappa agonists can be equally effective in the CWTF at -10° C. The present studies with naloxone antagonism are in agreement with the literature in that the spiradoline-induced antinociception was antagonized by a dose which was 5 times the dose required to antagonize fentanyl-induced antinociception (Ward and Takemori, 1983; Dykstra et al., 1987; Dykstra and Massie, 1988). Results of testing spiradoline in methadone-tolerant rats also demonstrated kappa selectivity in that spiradoline-induced antinociception remained unchanged whereas fentanyl- induced antinociception in methadone-tolerant rats was significantly reduced. In addition, results of testing the enantiomers and racemic mixture of spiradoline in the CWTF revealed that the proposed mu enantiomer (+ U63639) produced no measureable antinociceptive effect, whereas the proposed kappa enantiomer (-U63640) produced an antinociceptive effect very similar to the racemic mixture for a period of at least one hour. The significant difference between U62066E and U63640 at the 30-minute time-point could indicate that U63639 contributed to the antinociceptive effect in the racemic compound, but the antinociceptive effect of this compound at 30 minutes was equivalent to the effect of saline. Thus, U63639 would have contributed no measureable effect. Another explanation for the observed difference at 30 minutes could be that the enantiomers when combined produced some dynamic interaction which produced higher levels of antinociception than the additive sum of their effect-- which seems unlikely since U63639 by itself produced no antinociceptive effect. In any case, antinociception of U62066E and U63640 was remarkably similar for 60 minutes, indicating that U63640 contributes predominantly to the antinociceptive effect of the racemic compound, U62066E. Finally, the most convincing evidence that spiradoline is a

selective kappa agonist in the CWTF came from studies using selective antagonists, β-funaltrexamine and nor-binaltorphimine. Results showed that β–FNA antagonized fentanyl-induced antinociception without affecting spiradoline-induced antinociception. Results also showed that norBNI was without affect on fentanyl-, but completely antagonized spiradoline-induced antinociception. Thus, the results of these experiments demonstrate 1) that a kappa agonist can be equally efficacious to a mu agonist in a nociceptive assay, and 2) that spiradoline-induced antinociception in the CWTF assay is selectively mediated by activation of kappa receptors.

The conclusion that a kappa agonist can be equally efficacious to a mu agonist may only to be applicable at a specific temperature of -10° C of nociceptive challenge. It is interesting to note that kappa agonists have been shown to produce poor antinociceptive effects in the CWTF (Tyers, 1980; Clark et. al., 1988). However, these investigators used a temperature of 0° Celcius as a cold noxious stimulus. Others have shown that by decreasing the temperature to -10° C, that a variety of kappa agonists, e.g., dynorphin A, U-50488H, and pentazocine are efficacious in producing antinociception (Pizziketti et. al., 1985; Tiseo et. al., 1988). In an analogous manner to the difference seen between 0° and -10° C, opioid agonists in nociceptive assays using warm (45 to 50° C) vs. hot (55° C) also show differences in efficacy depending on the type of opioid. For instance, Davis et al., (1992) showed that a kappa agonist (enadoline, CI-977) was 1000 times more potent than morphine as an antinociceptive agent at 50° C, but enadoline was less effective than morphine when tested in water at 55° C. Others have shown this trend as well, i.e., that kappa agonists seem to lack antinociceptive efficacy to stimuli involving more intense heat (Leighton et al., 1987; Tyers, 1980; Hunter et al., 1990).

In addition, single fiber (electrophysiological) recordings of the saphaneous nerve in anesthetized rats (Kajander at al, 1994) and monkeys (Simone et al, 1994) have shown that A–δ and C "mechanoreceptors" are excited by noxious cold and that C nociceptors

are active at temperatures at or above 0° C, whereas, both A–δ and C nociceptors are active at temperatures below 0° C. It is interesting to take note of this action, especially since there also seems to be a discriminating difference at these temperatures for kappa opioids but not mu opioids.

The differences in efficacy observed in kappa opioids at least in the CWTF at 0° and -10° C may be partially explained by the difference in neuronal activation induced by the thermal stimulus. Thus, the idea that kappa agonists are not efficacious against intense thermal stimuli is argueable, since 1) -10° C seems to be a more intense stimulus than 0° C and kappa agonists seem to be more efficacious at the more intense thermal stimulus, and 2) data from electrophysiological recordings suggest that different temperatures activate different neuronal fibers. This evidence suggests that kappa receptors may be associated with more A–δ than C fibers, and that mu receptors are either associated with both or may have a closer association with C fibers. Although evidence for this hypothesis is not conclusive, it is an attractive hypothesis which needs to be investigated further.

CHAPTER 4

KAPPA ANTINOCICEPTIVE ACTIVITY OF SPIRADOLINE IN THE COLORECTAL DISTENSION ASSAY IN RATS.

Introduction

Spiradoline (U62066E) is a racemic mixture of the two enantiomers U63639 and U63640. The racemic mixture appears to have kappa opioid recepter activity in rodents (VonVoigtlander and Lewis, 1988; Meecham et. al., 1989) and primates (France et. al., 1994; Pitts and Dykstra, 1994). However, U63639, the levo-enantiomer has been shown to have slight affinity for mu receptors, although studies have not clearly shown whether this activity is agonistic or antagonistic (VonVoigtlander and Lewis, 1988). Previous results of spiradoline-induced antinociceptive activity in the cold-water tail-flick (CWTF) strongly indicated that only the U-63640 enantiomer (enantiomer selective for the kappa receptor) contributed to the analgesic activity of the racemic compound (Briggs et. al., submitted). Although antinociceptive effects of spiradoline seem to be mediated by kappa receptors in the CWTF, selectivity has not been established in other nociceptive assays, such as the colorectal distension (CRD) assay. Nociceptive assays have varying sensitivities to opioid agonists depending on the stimulus presented in the assay. Thus, to determine the selectivity of spiradoline in a visceral nociceptive assay, the racemic mixture and its enantiomers were tested in the colorectal distension (CRD) assay.

Methods

Subjects

Male Sprague Dawley rats weighing 300 to 500 grams were approved for use in the following experiments by the All-University Committee on Animal Use and Care. Rats were trained over a two-month period to accept a lubricated (K-Y® Jelly, Skillman, NJ) colonic balloon catheter (Pointe Medical, Crown Pointe, IN) inserted per rectum while

lying quietly in a towel snugly fitted around them. Training started for rats between the ages of 60 to 80 days. At approximately 6 weeks, rats accomodated to the catheter and towel restraint. Subjects were reinforced after training sessions by access to Cheerios® cereal "treats" and time to "play and socialize" on a large table-top among towels and plastic boxes and tunnels.

Drugs

The racemic mixture of spiradoline (U62066E), and U63639 (levo-enantiomer) and U63640 (dextro-enantiomer) isomers were generously provided by P.F. VonVoigtlander from The Upjohn Company, Kalamazoo, MI. Fentanyl citrate was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Methadone and Naloxone were purchased from Mallinckrodt, Mundelein, IL. All agonists and naloxone were dissolved or diluted in saline. The selective antagonists, beta-funaltrexamine (b-FNA) and nor-binaltorphimine (nor-BNI), which were generously provided by the National Institute on Drug Abuse, were dissolved in sterile water.

Procedure for Log Dose Response Patterns of Agonists in CRD

Trained rats were restrained in towels with colonic catheters in place as described in the methods section in earlier chapters. Nociceptive thresholds were determined by introducing a pressure stimulus into the colonic balloon catheter for not more than 1 second. Lower (non-threshold) pressures were randomly presented to a subject (extinction) as occasional increasing pressures were presented to determine the pressure to which a naive rat would consistently respond. A nociceptive threshold response to a "nociceptive" pressure included a moderate abdominal contraction resulting in a hunched

posture, which is termed a guarding response. Abdominal contractions were recorded by using a water-filled Neonatal #2 Disposa-cuff® (Critikon, Tampa, FL) which was fitted around the abdomen and connected with tubing to a pressure transducer coupled to a polygraph recorder (Grass Instruments, Inc., Quincy, MA). A maximum pressure stimulus was used as a cutoff level in situations of maximum levels of analgesia to prevent permanent tissue damage, (see the equation for maximum percent effect [MPE] under the Data Analysis sub-heading in this chapter).

After determining thresholds, catheters were removed and rats were released from towel restraint and given an injection SC of a coded drug (experimenter blinded). Five minutes later, rats were again prepared for nociceptive testing and responses to the stimulus were recorded at 15 minute intervals for one hour after injection.

Procedure for Naloxone Antagonism

Nociceptive thresholds (controls) were determined as previously described. Rats were then given a subcutaneous (SC) injection of saline or various doses of naloxone (0.01 to 0.8 mg/kg) followed by either fentanyl, 0.03 mg/kg, or spiradoline, 1.0 mg/kg. Nociceptive thresholds were again determined 15 and 30 minutes post-injection.

Procedure for Methadone Tolerance

Rats were treated every 12 hours with either methadone or saline. Over a two month period, doses of methadone were gradually increased to 9.2 mg/kg IP every 12 hours and maintained at that level during the time which experiments were conducted. Thus, rats became tolerant to 9.2 mg/kg intraperitoneal (IP) methadone during this study. Experiments were scheduled such that residual methadone contributed no antinociceptive

effect (control responses for methadone- or saline-treated subjects were not significantly different at the beginning of testing, $p < 0.05$). On test days, both methadone-tolerant rats and saline-treated rats were randomly put into two groups and nociceptive thresholds (controls) were determined as previously described. Doses of fentanyl or spiradoline were randomly administered to methadone-tolerant and saline-treated animals. Thereafter, nociceptive thresholds were determined at 15 minute intervals for 60 minutes. The methadone-tolerant group and saline group were randomly administered fentanyl or spiradoline. After 3 hours (time at which agonists at these doses were no longer active, unpublished observation), rats that had received fentanyl were given an injection of spiradoline and rats that had first received spiradoline were given an injection of fentanyl. Thereafter, rats were again tested at 15 minute intervals for 60 minutes. A blinded experimenter observed and recorded reponses.

Procedure for Enantiomers

Nociceptive thresholds were determined as described earlier, followed by SC injection of one of the following coded drugs: 1.0 mg/kg U62066E, 1.0 mg/kg U63639, 1.0 mg/kg U63640, or saline (vehicle/control). Nociceptive thresholds were again determined at 15 and 30 minutes post-injection.

Procedure for Selective Antagonism

Rats were pretreated 24 hours earlier with 8.0 mg/kg SC of b-FNA, a mu receptor antagonist shown at this dose and time of testing to produce selective mu antagonism (Ward et al., 1982; Dykstra et al., 1987b; Zimmerman et al., 1987). After the 24 hour pretreatment, rats randomly received either fentanyl (0.012 or 0.02 mg/kg) or spiradoline

(0.3 or 0.8 mg/kg). In addition, a second group of rats were pretreated 48 hours earlier with 10.0 mg/kg SC of nor-BNI, a kappa receptor antagonist shown at this dose and time of testing to be selective for and potent at the kappa receptor (Diop et al., 1994). After 48 hours, rats received either fentanyl (0.012 or 0.02 mg/kg) or spiradoline (0.3 or 0.8 mg/kg). A blinded experimenter observed and recorded responses 15 and 30 minutes post-injection.

Data Analysis

The ED_{50} doses of fentanyl and spiradoline were determined by using the linear regression function of Sigma Plot® (Jandel Corporation, San Rafael, CA). All statistical procedures were completed using Sigma Stat® (Jandel Corporation, San Rafael, CA). A one-way random ANOVA was used to determine significant differences in the naloxone antagonism study, in the enantiomer study, and in the selective antagonism study. Student-Neuman-Keuls method was used to determine significant differences between groups. Student's T-test was used to test for significant differences between responses of agonists in saline- and methadone-tolerant animals. Significance was set at $p < 0.05$.

For graphic representation, antinociceptive data were standardized as a maximum percent effect (MPE) (Harris and Pierson, 1964):

$$MPE = \frac{PDn - C}{Max - C} \times 100,$$

where PDn is the stimulus level to which a subject responds at n minutes post-injection. C is the stimulus level to which a naive subject normally responds. Max is the maximum allowable stimulus level presented to a subject.

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Results

Dose Response in CRD

Spiradoline was fully efficacious in CRD, as was fentanyl. The effective dose producing 50% antinociception (ED_{50}) for spiradoline was 0.56 mg/kg SC and the ED_{50} for fentanyl was 0.01 mg/kg SC (Figure 7). This result is in agreement with results of spiradoline tested in other nociceptive assays in the rat (VonVoigtlander and Lewis, 1988).

Naloxone Anatagonism

Results showed that naloxone (0.01 and 0.1 mg/kg) did not effect the fentanyl or spiradoline dose response pattern. Naloxone at 0.2 mg/kg fully antagonized fentanyl, but had no affect on spiradoline. Antagonism of spiradoline-induced antinociception was achieved with naloxone 0.8 mg/kg SC at 15 minutes (Figure 8), and full antagonism was observed 30 minutes post-injection (data not shown). Thus, antinociception by spiradoline was antagonized completely by naloxone at 0.8 mg/kg, a dose four times that required to antagonize antinociception by fentanyl in this same assay.

Methadone Tolerance

In a third procedure, rats were made tolerant to methadone (9.2 mg/kg IP) and tested for altered antinociceptive effects of fentanyl or spiradoline in CRD. Fentanyl-induced antinociception was significantly reduced, while spiradoline-induced antinociception was essentially unchanged for at least 60 minutes in the methadone-tolerant animals relative to non-tolerant control subjects (Figure 9).

Antinociceptive Activity of Spiradoline and its Enantiomers

After testing spiradoline and its enantiomers in CRD, results showed that U63640 (levo-enantiomer of spiradoline) produced antinociceptive levels similar to those of the racemic mixture 15 and 30 minutes post-injection, whereas U63639 (dextro-enantiomer) failed to affect the nociceptive response in the effective dose-range of the racemate (Figure 10).

Selective Antagonism

Antinociceptive effects of spiradoline and U-63640 were significantly reduced in animals pretreated with nor-BNI. However, fentanyl-induced antinociception (0.02 mg/kg) was also antagonized (Figure 11). In animals pretreated with b-FNA, the antinociceptive effect of spiradoline was significantly reduced, while the effect of fentanyl remained unchanged (Figure 11).

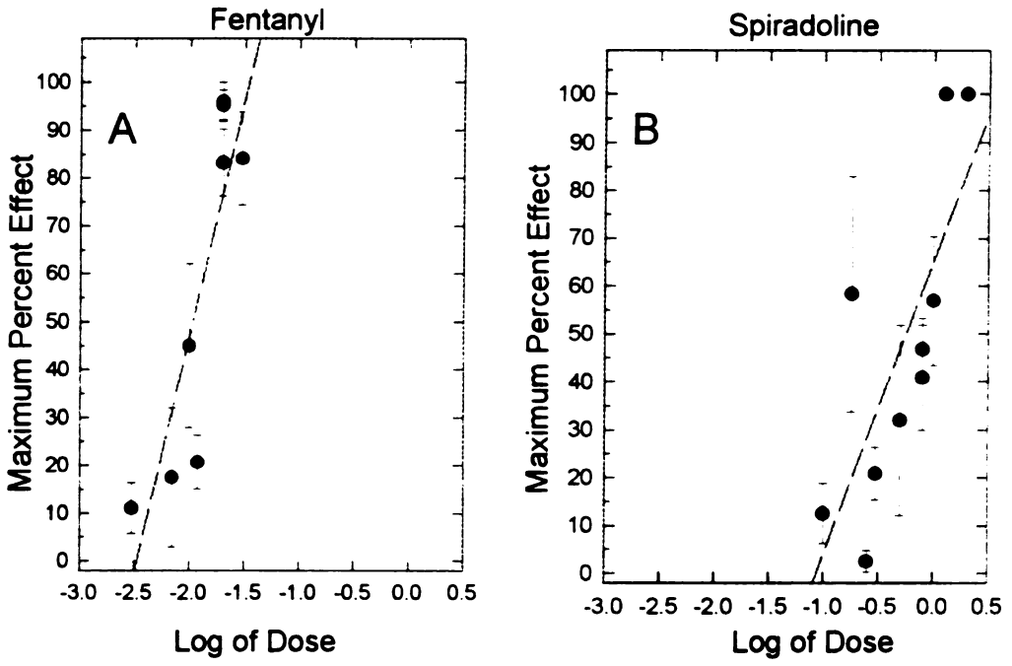

Figure 7. Antinociceptive dose-response for fentanyl and spiradoline in CRD.

Graph A: Mean (± SEM) for fentanyl dose response in the CRD assay at 15 minutes post-injection; ED_{50} = 0.01 mg/kg SC; n = 3 to16 subjects per dose. Graph B: Mean (± SEM) for spiradoline dose response in CRD at 15 minutes post-injection; ED_{50} = 0.56 mg/kg SC; n = 3 to 12 subjects per dose.

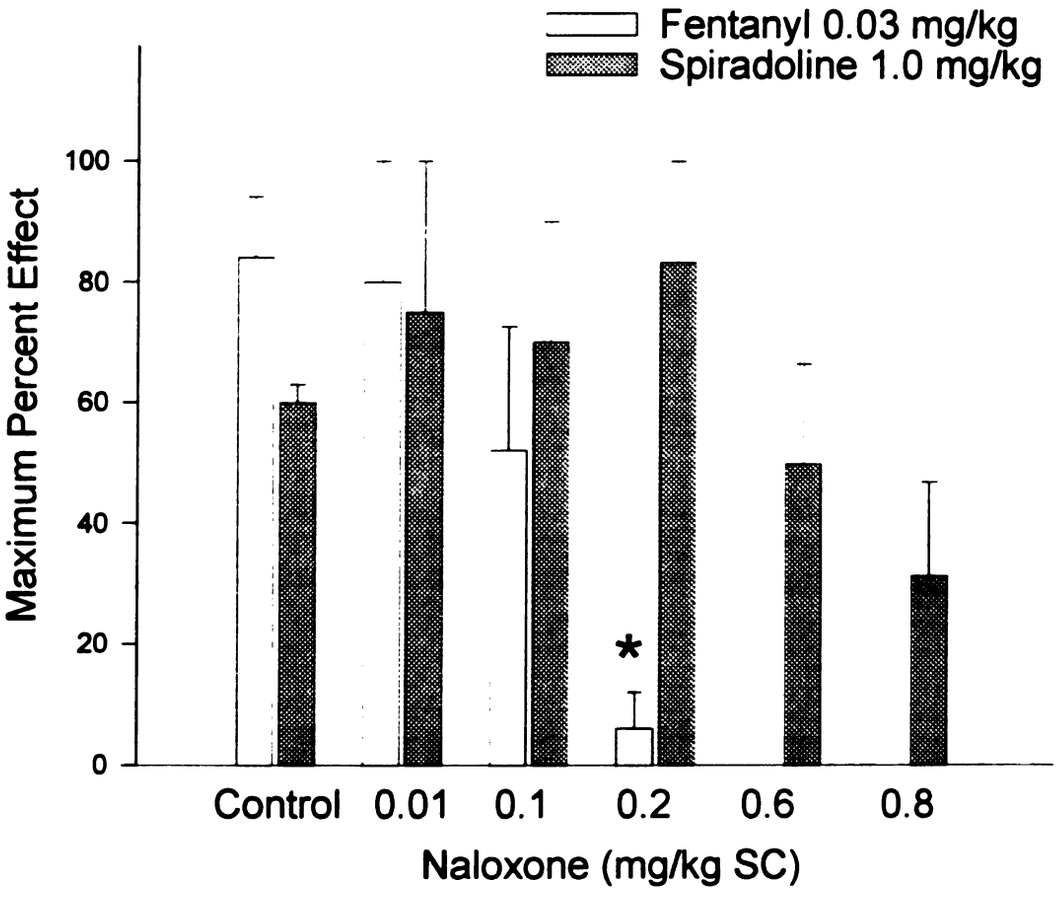

Figure 8. Naloxone antagonism of fentanyl- and spiradoline-induced antinociception. Mean (± SEM) of the level of MPE 15 minutes post-injection in subjects administered naloxone (N) in incremental doses (0.01, 0.1, 0.2, 0.6, 0.8 mg/kg) and either fentanyl 0.03 mg/kg SC or spiradoline 1.0 mg/kg SC. Asterisk indicates significant difference from control ($p < 0.05$).

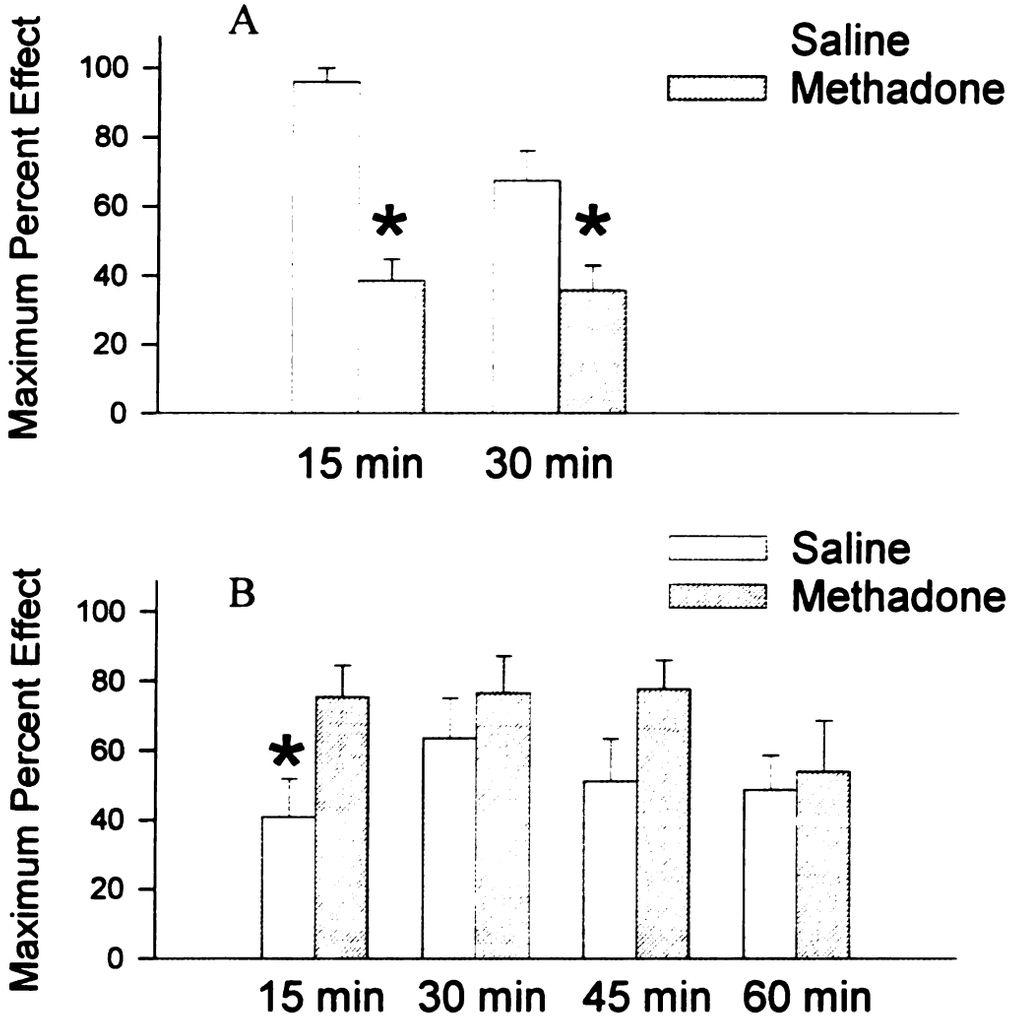

Figure 9. Antinociception of fentanyl and spiradoline in methadone-tolerant rats. Graph A: Mean (± SEM) of controls and of the level of antinociception (MPE) of fentanyl 0.02 mg/kg SC 15 and 30 minutes post-injection in non-tolerant (saline-treated) vs. methadone-tolerant subjects. Graph B: Mean (± SEM) of controls and of the level of antinociception (MPE) after spiradoline 0.8 mg/kg SC 15, 30, 45, and 60 minutes post-injection in non-tolerant (saline) vs. methadone-tolerant subjects. Asterisk indicates significant difference from non-tolerant (saline- treated) subjects ($p < 0.05$).

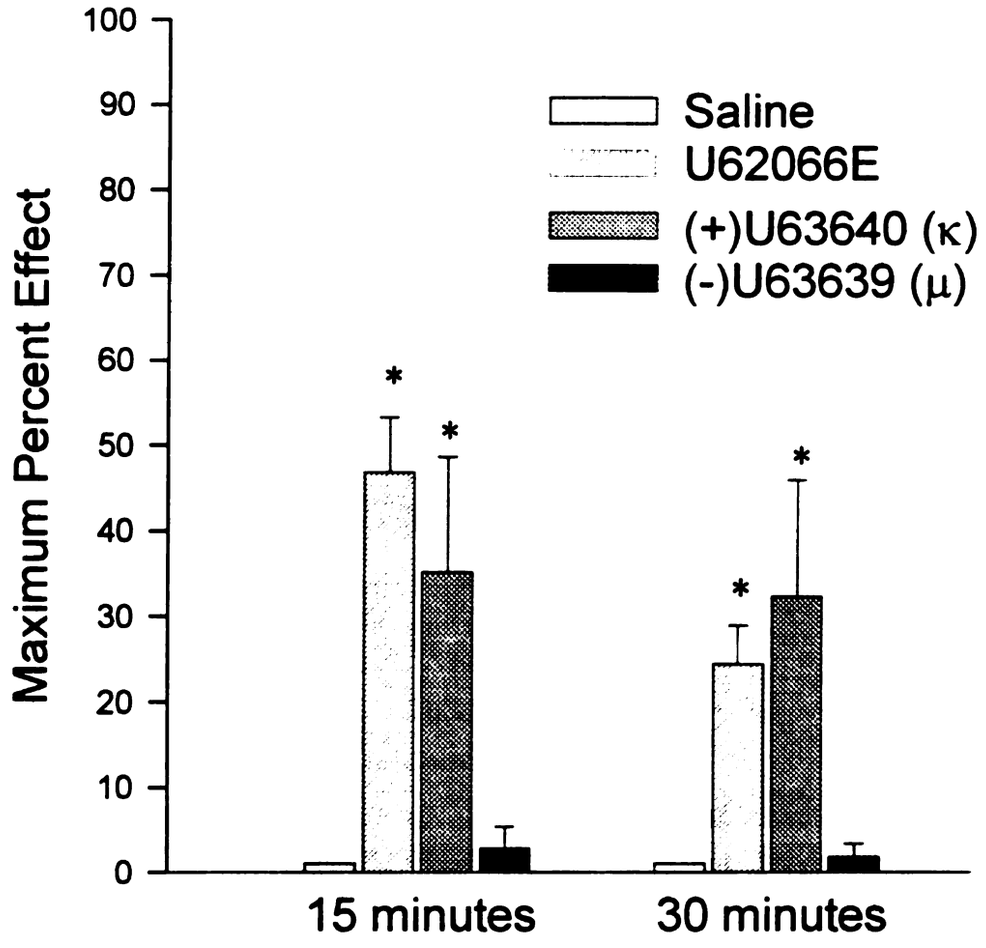

Figure 10. Antinociception of spiradoline and its enantiomers.

Mean (± SEM) of the level of MPE 15 and 30 minutes post-injection in subjects administered either saline (control), U62066E, U63640(-), or U63639(+). Asterisk indicates significant difference from control ($p < 0.05$).

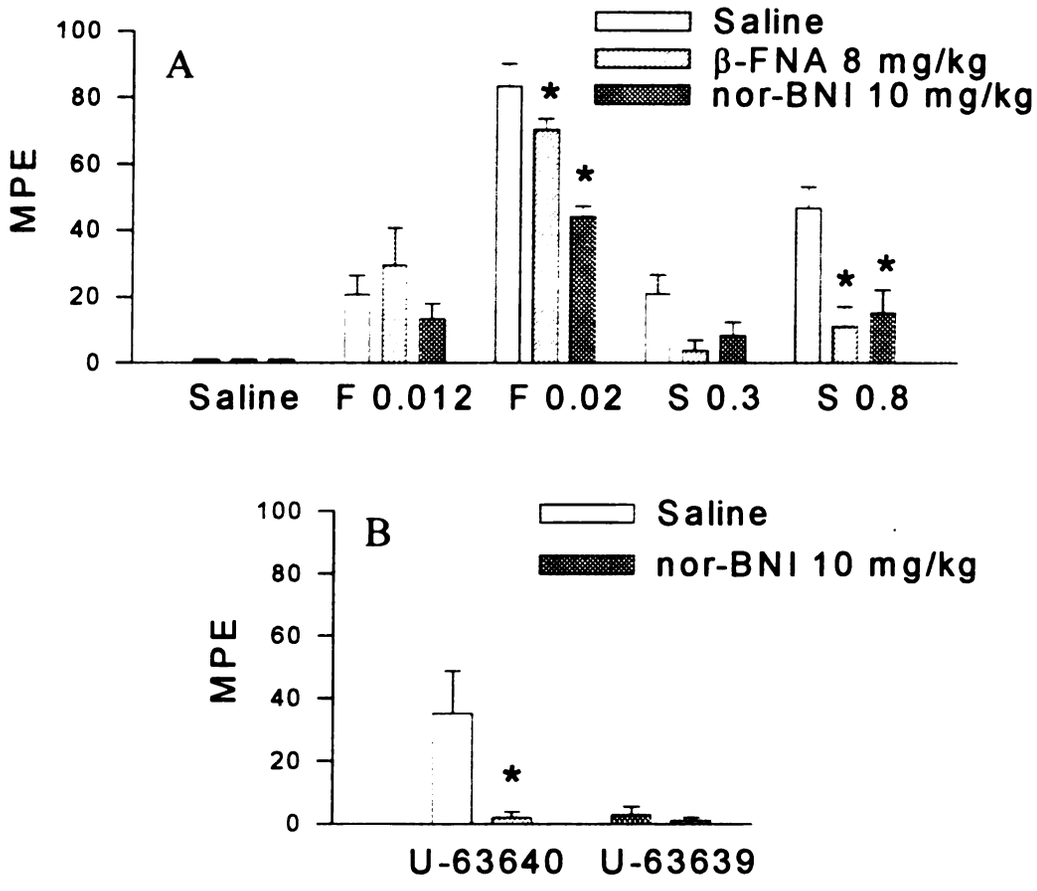

Figure 11. Antinociception of fentanyl, spiradoline, and enantiomers of spiradoline in saline, b-FNA, and nor-BNI pretreated rats.
Graph A: Mean (± SEM) of MPE in subjects pretreated with either saline, β-FNA (8.0 mg/kg), or nor-BNI (10.0 mg/kg) at 15 minutes post-injection of either saline, fentanyl (F) (0.012 or 0.02 mg/kg), or spiradoline (S) (0.03 or 0.8 mg/kg). Graph B: Mean (± SEM) of MPE in subjects pretreated with saline or nor-BNI at 15 and 30 minutes post-injection of either U63640 (0.8 mg/kg SC) or U63639 (0.8 mg/kg SC). Asterisk indicates significant difference from corresponding saline pretreatment ($p < 0.05$).

Discussion

Results indicate that fentanyl and spiradoline were fully efficacious in producing antinociception in the CRD assay. These results are in agreement with reports of mu and kappa agonists producing visceral antinociception (VonVoigtlander et. al., 1983; Ness and Gebhart, 1988; Sawyer et. al., 1991). Results from naloxone antagonism showed that fentanyl was antagonized with lesser amounts of naloxone (suggestive of mu receptor activity) and that antagonism of spiradoline required greater amounts of naloxone (indicative of kappa receptor activity). These results are in agreement with others who have shown that kappa receptor activity is antagonized by naloxone at doses which are 4 to 5 times that required to antagonize mu receptor activity (Ward and Takemori, 1983; Dykstra et al., 1987a; Dykstra and Massie, 1988). Results of testing spiradoline in methadone-tolerant rats also demonstrated kappa selectivity in that spiradoline-induced antinociception was equal to that observed in saline-treated rats for 60 minutes, whereas fentanyl-induced antinociception was significantly reduced in methadone-tolerant rats compared to saline-treated rats. In addition, results of testing the enantiomers and racemic mixture of spiradoline in the CRD revealed that the proposed mu enantiomer (U63639) produced no measureable antinociceptive effect, whereas the proposed kappa enantiomer (U63640) produced an antinociceptive effect very similar to the racemic mixture for a period of at least 30 minutes. Thus these results indicate that the antinociceptive effect of the racemic compound U62066E is predominantly from U63640 in the dose range tested.

In contrast to the afore-mentioned data, results of b-FNA and of nor-BNI antagonism were less clear. Since b-FNA demonstrated minimal antagonism of fentanyl and significant antagonism of spiradoline and nor-BNI demonstrated antagonism of spiradoline, U-63640 and fentanyl as well, it is possible that these antagonists perform differently in this assay in comparison to others. Reports in the literature and our work in previous experiments in the cold-water tail-flick (CWTF) nociceptive assay indicated that b-FNA is most potent and selective as an antagonist of mu receptor actvity 24 hours after administration and nor-BNI is most selective and potent as an antagonist at the kappa receptor 48 hours after administration (Ward et al., 1982; Dykstra et al., 1987; Diop et al., 1994; Briggs et al., submitted). After the 24 hour-pretreatment, b-FNA antagonized antinociceptive effects of fentanyl, 0.018 mg/kg SC, by 80% in CWTF, whereas the antinociceptive effect of spiradoline, 1.0 mg/kg SC, in b-FNA pre-treated rats was similar to that in untreated rats, ($p < 0.05$). After 48 hours pretreatment, nor-BNI did not affect fentanyl-induced antinociception but clearly antagonized spiradoline-induced antinociception. In the present study using the CRD assay, the same agonists were employed and the antagonists were administered in the same manner as in the CWTF. Thus, it is interesting that the same protocol for drug administration in the CWTF produced different results in the CRD assay.

In light of reports from others indicating that b-FNA and nor-BNI are selective antagonists, it is possible that in the CRD assay, b-FNA was selective for the mu receptor and nor-BNI was selective for the kappa receptor. This being the case, results may reflect dynamic receptor interactions of kappa receptors on the function of mu receptors as they

relate to nociceptive pathways. The notion of multiple opioid receptor interaction has been reported for mu-kappa and mu-delta interactions (Vaught and Takemori, 1979; Rothman et. al., 1988). Interactions related to antinociception have been shown for mu and kappa opioid agonists which produced synergistic levels of antinociception in the CRD assay (Sawyer et al., 1994). In contrast, those same agonists in the CWTF assay produced sub-additive levels of antinociception at least at higher doses (Briggs et. al., submitted). The mechanisms for these intriguing differences are unclear. Nevertheless, these differences suggest that an interesting interaction may exist between mu and kappa receptors in relation to the CWTF and CRD nociceptive systems.

In summary, although results from the selective antagonists in the CRD test may indicate non-selective activity of spiradoline, the antagonists themselves did not demonstrate total selectivity, and thus interpretation of these results was circumspect. In contrast, spiradoline clearly demonstrated selectivity for the kappa receptor in the other tests and the enantiomer U-63640 produced antinociceptive effects similar to spiradoline that were antagonized by nor-BNI. Thus, it is more likely that spiradoline is selective for the kappa receptor and that results from the specific antagonist tests represented effects of interactions between the two classes of opioid receptors.

CHAPTER 5

ANTINOCICEPTIVE INTERACTIONS OF MU AND KAPPA AGONISTS IN COMBINATION USING THE COLD-WATER TAIL FLICK PROCEDURE.

Summary

Attempts to find suitable pain relievers that produce less side effects have led to the study of kappa opioids individually or in combination with mu opioids. Kappa opioids may have an advantage over mu opioid agonists in that their side effects are less deleterious and actually to some degree reciprocal to those of mu opioids. Thus combinations of mu and kappa opioids could provide pain relief while reducing side effects, including dependency phenomena. Results of these studies showed that appropriate doses of mu and kappa agonists individually produced maximal levels of antinociception in the cold-water tail-flick. However, antinociceptive effects produced by mu and kappa agonists in combination were quite variable and reflected dose dependent interactions. At relatively lower doses, combinations produced additive antinociceptive effects, but higher doses of combinations produced antagonistic interactions. Mechanisms for these dose-dependent antinociceptive interactions remain to be elucidated. Several hypotheses of possible interactive mechanisms are discussed that need to be investigated further.

Introduction

Most of the analgesic therapies currently available to treat prominent pain syndromes consist of variations of mu opioid agonists. While mu agonists powerfully obtund nociception, side effects such as respiratory depression, nausea and vomiting, and addiction liability severely limit the usefulness of mu opioids. Opioids act dose-dependently, meaning that larger doses of opioids produce greater or more intensive effects. Thus, larger doses of mu opioids required to alleviate a strong nociceptive

stimulus are also likely to produce troublesome side effects. Attempts to find suitable pain relievers with fewer or less intense side effects have led to the potential use of kappa opioids. Some kappa opioids have sufficient pain-relieving activity, but this class also produces unwanted side effects: dysphoria, hallucinations, and diuresis. Recently, several laboratories have proposed the utility of combinations of mu and kappa opioids. The hypothesis that the combination of the two classes may exert greater analgesia with decreased side effects is appealing since mu and kappa opioids are similar in providing pain relief but they differ dramatically in types of side effects. Mu opioids induce euphoria (positive reinforcement), respiratory depression, constipation, urinary retention, and prominent tolerance and physical dependence to mu receptors. In contrast, side effects of kappa agonists include dysphoria, minimal changes in respiratory function, increased gut motility, diuresis, and a milder and qualitatively different form of physical tolerance and dependence than occurs with mu agonists. Thus, kappa opioid agents may counterbalance some mu related side effects in addition to providing additional pain relief.

Previous antinociceptive studies indicated possible synergistic interactions between mu (morphine, DAMGO, fentanyl, oxymorphone) and kappa agonists (spiradoline, enadoline, U-50,488) in the hot plate, tail flick, colorectal distension and paw withdrawal tests (Ren et al., 1985; Jhamandas et al., 1986; Kunihara, et al., 1989; Miaskowski et al., 1990; Sutters et al., 1990; Briggs et al., in preparation). Although these nociceptive assays demonstrated synergistic interactions, Schmauss et al. (1983) reported linear interactions of spinally injected mu and kappa agonists at low doses and antagonism at high doses. Schmauss and Herz (1987) and Song and Takemori (1991) also reported

antagonistic antinociceptive interactions between mu and kappa agonists. It appears that antinociceptive interactions of mu and kappa agonists may vary depending on the opioid dosage and nociceptive stimulus used. Hayes et al. (1987) showed that mu and kappa agonists had different pharmacological profiles depending on the nociceptive stimuli used in the test. Thus, the objective of this study was to test antinociceptive interactions of mu and kappa agonists given systemically in various dose combinations using the CWTF.

Cold stimuli have been used to study pain in human subjects (Kreh, et al., 1984) as well as animals (Pizziketti et al.1985; Tiseo et al., 1988). Furthermore, animal studies showed that the CWTF was specific for opioid agonists (ie., not affected by CNS depressants, aspirin, tylenol, and ethanol) and that it was sensitive to mu (morphine) and kappa agonists (dynorphin, U-50,488, and pentazocine) (Pizziketti et al., 1985). Furthermore, the CWTF is an interesting nociceptive model in that electrophysiological studies showed that at -10 ° C both A-delta and C fibers were excited, whereas at 0 ° C only C fibers discharged. Additional studies using the warm water tail flick showed that C fibers and possibly A-delta fibers fired but only C-fibers were active when tested with the hot water tail flick. Kappa opioids demonstrated antinociception only at -10 ° and in the warm water tail flick (when A-delta fibers are firing) whereas mu agonists produced antinociception at all temperatures (C fibers firing). Thus, the cold-water tail-flick nociceptive assay at -10 ° C was used to test the efficacy and potency of individual mu or kappa agonists and compare individual results to their combined antinociceptive effects.

Material and Methods

Subjects

Male Sprague Dawley rats weighing 300 to 500 grams were approved for use in the following experiments by the All-University Committee on Animal Use and Care of Michigan State University. All rats were trained over a two-month period to lie quietly in a towel which was snugly fitted around them. Training started for rats between the ages of 60 to 80 days. At approximately 6 weeks, rats accomodated to being restrained in the towels without struggling. The subjects were reinforced after training sessions by access to Cheerios® cereal "treats" and time to "play and socialize" on a large table-top among towels and plastic boxes and tunnels.

Drugs

Fentanyl citrate was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Oxymorphone was purchased from Mallinckrodt (Mundelein, IL). Spiradoline racemic (U62066E) was generously provided by P.F. VonVoigtlander from The Upjohn Company, Kalamazoo, MI. Enadoline (PD-129290 or CI-977) was generously supplied by David Downs , Parke-Davis Pharmaceutical Research, Ann Arbor, MI. All agonists were dissolved in saline. The selective antagonists, b-FNA and nor-BNI, which were generously provided by the National Institute on Drug Abuse, were dissolved in sterile water.

Procedure for Log Dose-Response Patterns of Agonists Individually or in Combination in CWTF

Trained rats were restrained in towels as described earlier while their tails were dipped into tap water at 27 to 30° Celsius (dummy stimulus) or a solution of ethylene glycol and water (1:1) maintained at -10° Celsius (nociceptive stimulus). Nociceptive thresholds were determined by dipping tails into the cold solution while a timer was used to measure the latency until the rat flicked its tail from the cold solution. Tail dips using tap water were also used to extinguish any conditioned pattern of tail flicking with the cold solution. After four tail dips at -10° C, latencies were averaged and the mean latency was used as a

baseline response. Most rats removed or "flicked" their tails in less than 3 seconds. After determining thresholds, rats were released from towel restraint and injected with a coded drug (experimenter blinded). Rats were again restrained and responses to the cold solution were recorded at 15, 30, 45, and 60 minutes after injection. The subjects' tails were never left in the cold solution for more than 60 seconds.

Procedure for Methadone Tolerance

Two groups of trained rats (n=15 each) were treated every 12 hours with either methadone or saline. Doses of methadone were gradually increased to 7.6 mg/kg, at which time experiments were conducted. Thus, rats became tolerant to 7.6 mg/kg i.p. methadone during this study. Experiments were scheduled such that residual methadone contributed no antinociceptive effect (Figure 14). On test days, both methadone tolerant rats and saline-treated rats were randomly put into two groups each. One methadone-tolerant group and one saline group were randomly administered fentanyl and spiradoline or fentanyl and enadoline and then tested. The protocol used to measure antinociceptive responses and results of resonses of individual agonists were reported in Briggs et al. (submitted). A blinded experimenter observed and recorded latency responses.

Procedure for Selective Antagonism

Trained rats were pretreated s.c. with 2.5 mg/kg b-FNA, a selective mu receptor antagonist. Twenty-four hours later, rats received either fentanyl (0.018 mg/kg), spiradoline (0.6 mg/kg), or a combination dose of each. Trained rats were also pretreated s.c. with 10.0 mg/kg nor-BNI, a selective kappa receptor antagonist. After 48 hours rats received fentanyl (0.018 mg/kg), spiradoline (0.6 mg/kg) or a combination dose of each. A blinded experimenter observed and recorded latency responses 15 and 30 minutes post-injection.

Data Analysis

The ED_{50} doses of fentanyl, oxymorphone, spiradoline and enadoline were determined by using the linear regression function of Sigma Plot® for Windows. All drug comparisons were tested using a random ANOVA. Student-Neuman-Keuls method was used to determine significant group differeneces. Significance was set at $p < 0.05$. For graphical representation, antinociceptive data were standardized as a maximum percent effect (MPE) (Harris and Pierson, 1964):

$$MPE = \frac{PDn - C}{Max - C} \times 100$$

where PDn is the stimulus level that a subject responds at n minutes post-injection. C is the stimulus level to which a naive subject normally responds. Max is the maximum stimulus level presented to a subject.

Analysis of antinociceptive responses of combination doses in comparison to theoretical additive sums of individual responses was accomplished by using the Z table (Steel and Torrie, 1984). First, to calculate theoretical additive sums of individual responses, the MPE of their effects were summed. The SEM of the theoretical sum was calculated by using the root mean square of the individual SEM's. Finally, the absolute difference between the theoretical and actual response was divided by the root mean square of the theoretical and actual SEM's. The calculated number was then compared to values on the Z table. Values corresponding to numbers in the table at $p < 0.05$ indicated significant deviation from additivity.

Results

Individual dose-response patterns in the CWTF for fentanyl, oxymorphone, spiradoline, and enadoline showed that each agonist produced maximal levels of antinociception (Figure 12). The following is a list of ED_{50}'s (mg/kg, SC) for the

agonists: fentanyl, 0.009 (0.003 - 0.02); oxymorphone, 0.044 (0.001 - 0.32); spiradoline, 0.56 (0.25 - 1.99); enadoline, 0.031 (0.01 - 0.1). Peak levels of antinociception occurred at 15 minutes post-injection for fentanyl and spiradoline and 30 minutes for oxymorphone and enadoline. Duration of antinociception was shortest for fentanyl (less than 60 minutes) while enadoline, spiradoline, and oxymorphone were approximately equal in duration (2-3 hours).

Antinociceptive effects of combination doses of mu and kappa agonists peaked at 15 minutes and returned to control levels as early as 30 minutes post-injection (Figure 13). By comparing actual antinociceptive responses of the combinations at 15 minutes to their theoretical additive (linear) sum, results showed a trend towards antagonistic interactions (sub-additive) (Figure 14). Actual antinociceptive responses of the combinations at 30, 45, or 60 minutes produced flat dose-response patterns in comparison to their additive sum (data not shown), clearly indicating antagonistic interactions.

Results of direct dose comparisons between individual and combined doses of fentanyl and spiradoline showed additive (linear) and antagonistic (sub-additive) interactions (Figure 15). Lower doses (fentanyl 0.004 and 0.009 mg/kg; spiradoline 0.16 and 0.3 mg/kg) in combination produced additive interactions at 15 minutes and sub-additive interactions at 30 minutes when compared to individual levels of antinociception. In contrast, higher doses (fentanyl 0.018; spiradoline 0.6) produced sub-additive interactions at 15 and 30 minutes when compared to individual antinociceptive effects. Results of direct dose comparisons between individual and combined doses of fentanyl and enadoline showed additive (linear), antagonistic (sub-additive), and synergistic (supra-

additive) interactions (Figure 16). Fentanyl and enadoline at lower doses (fentanyl 0.004; enadoline 0.008) produced additive interactions at 15 minutes and synergistic interactions at 30 minutes when compared to individual effects. In contrast, fentanyl and enadoline at higher doses (fentanyl 0.018; enadoline 0.04) produced antagonistic interactions at 15 and 30 minutes.

Combinations of fentanyl and spiradoline or enadoline that produced sub-additive interactions were tested in methadone-tolerant animals. Fentanyl-induced antinociception was significantly reduced whereas spiradoline and enadoline produced similar antinociceptive levels in methadone-tolerant rats in comparison to saline-treated rats (Figure 17). Results of combination doses showed that antinociceptive responses in methadone-tolerant subjects reflected additive interactions at 15 and 30 minutes post-injection (Figure 17). Unfortunately, antinociceptive effects of fentanyl returned to baseline at 30 minutes, thus further analysis at 45 and 60 minutes was not possible. However, if the baseline effect of fentanyl at 45 and 60 minutes were used with the individual effects of spiradoline or enadoline and compared to the combination effect, then the interactions for fentanyl and spiradoline or enadoline at 60 minutes would be synergistic (fentanyl-spiradoline, $p = 0.0174$; fentanyl-enadoline, $p = 0.0446$). In another comparison, the combination of fentanyl and spiradoline in saline-treated animals was significantly greater than in methadone-tolerant subjects at 45 minutes. The combination of fentanyl and enadoline was significantly greater in saline-treated animals than in methadone-tolerant subjects at 15 minutes.

Using selective antagonists b-FNA and nor-BNI, the combination dose of fentanyl and spiradoline was tested to determine mu and/or kappa contributions to the antinociceptive effect of the combination (Figure 18). In subjects pretreated with b-FNA, antinociceptive effects of fentanyl were unchanged and the combination dose was minimally but not significantly affected (antagonism) at 15 minutes but not at 30 minutes, $p < 0.05$. In subjects pretreated with nor-BNI, antinociceptive effects of spiradoline were antagonized at 15 and 30 minutes but the combination dose was unaffected, $p < 0.05$.

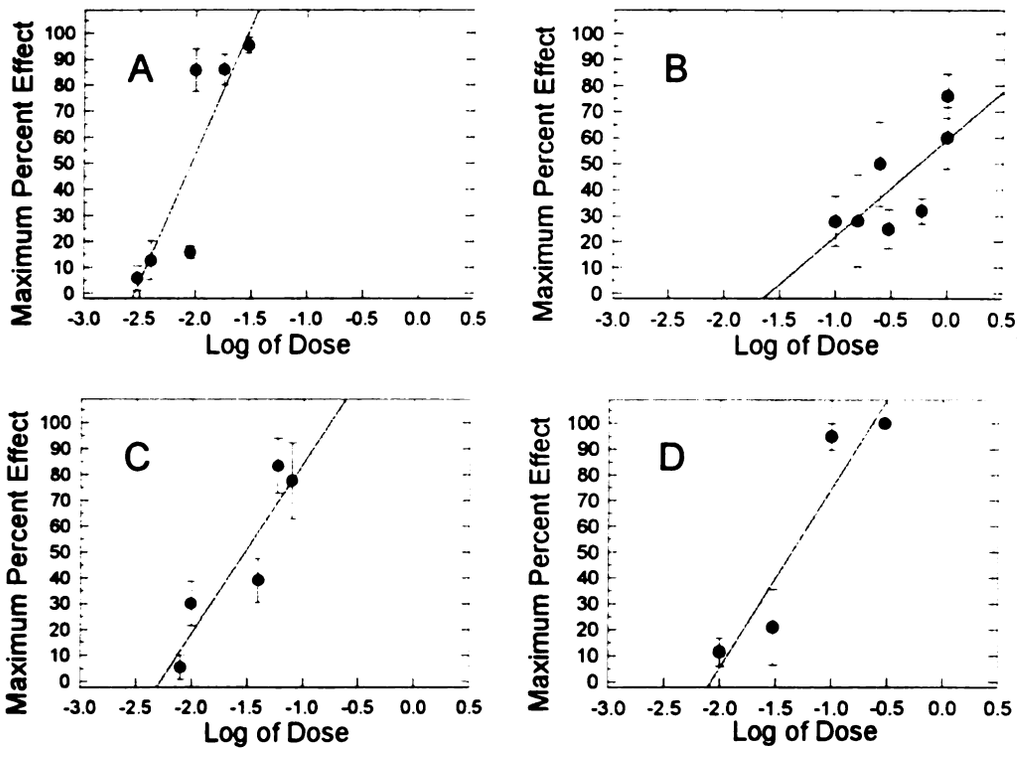

Figure 12. Antinociception of fentanyl, spiradoline, enadoline, and oxymorphone. Graph A: Mean (± SEM) for fentanyl dose responses in the CWTF assay at 15 minutes post-injection; ED50 = 0.009 (0.003-0.02) mg/kg SC, n = 3 to 11 subjects per dose. Graph B: Mean (± SEM) for spiradoline dose responses in the CWTF assay at 15 minutes post-injection; ED50 = 0.56 (0.25-1.99) mg/kg SC, n = 3 to 20 subjects per dose. Graph C: Mean (± SEM) for enadoline dose responses in the CWTF assay at 45 minutes post-injection; ED50 = 0.031 (0.01-0.1) mg/kg SC, n = 5 to 13 subjects per dose. Graph D: Mean (± SEM) for oxymorphone dose responses in the CWTF assay at 30 minutes post-injection; ED50 = 0.044 (0.001-0.32) mg/kg SC, n = 3 to 5 subjects per dose.

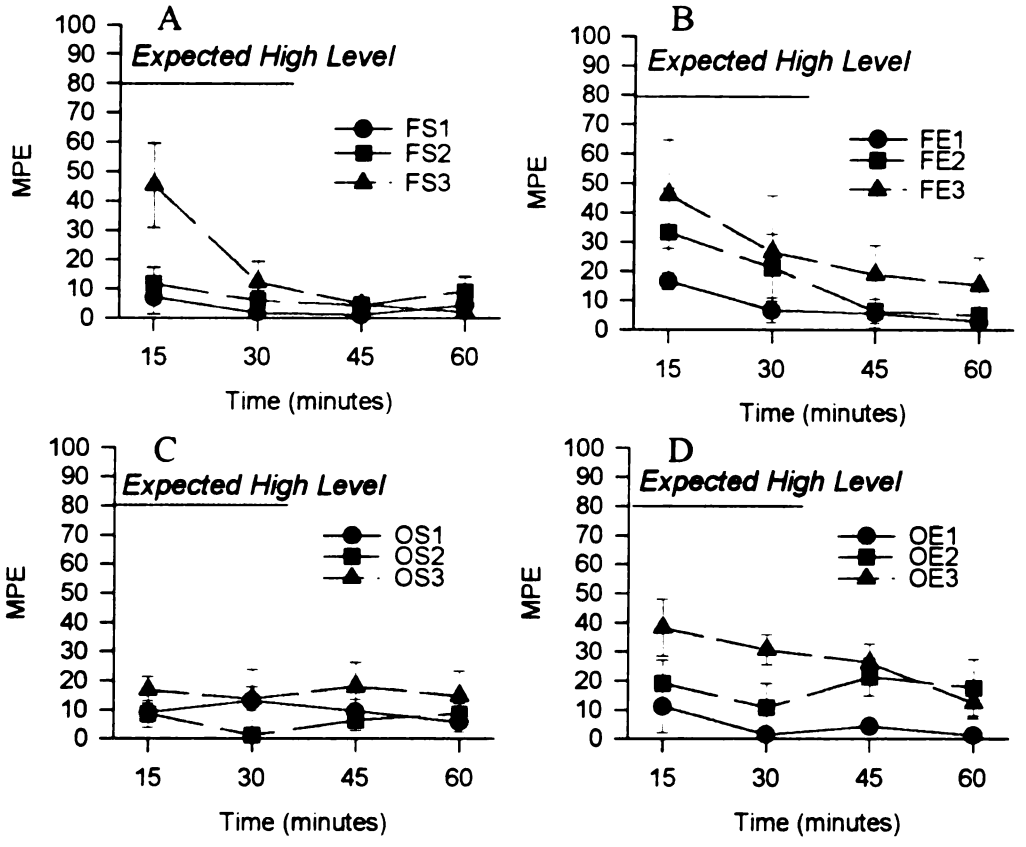

Figure 13. Antinociception of combination doses of fentanyl-spiradoline, fentanyl-enadoline, oxymorphone-spiradoline, and oxymorphone-enadoline.
Graph A: Mean (± SEM) of MPE for fentanyl-spiradoline combination (FS) in the CWTF assay. FS1 dose 0.002 + 0.075 mg/kg SC; FS2 dose 0.0045 + 0.15 mg/kg SC; FS3 dose 0.009 + 0.3 mg/kg SC. Graph B: Mean (± SEM) of MPE for fentanyl-enadoline combination (FE) in the CWTF assay. FE1 dose 0.002 + 0.005 mg/kg SC; FE2 dose 0.0045 + 0.01 mg/kg SC; FE3 dose 0.009 + 0.02 mg/kg SC. Graph C: Mean (± SEM) of MPE for oxymorphone-spiradoline combination (OS) in the CWTF assay. OS1 dose 0.01 + 0.075 mg/kg SC; OS2 dose 0.02 + 0.15 mg/kg SC; OS3 dose 0.04 + 0.3 mg/kg SC. Graph D: Mean (± SEM) of MPE for oxymorphone-enadoline combination (OE) in the CWTF assay. OE1 dose 0.01 + 0.005 mg/kg SC; OE2 dose 0.02 + 0.01 mg/kg SC; OE3 dose 0.04 + 0.02 mg/kg SC.

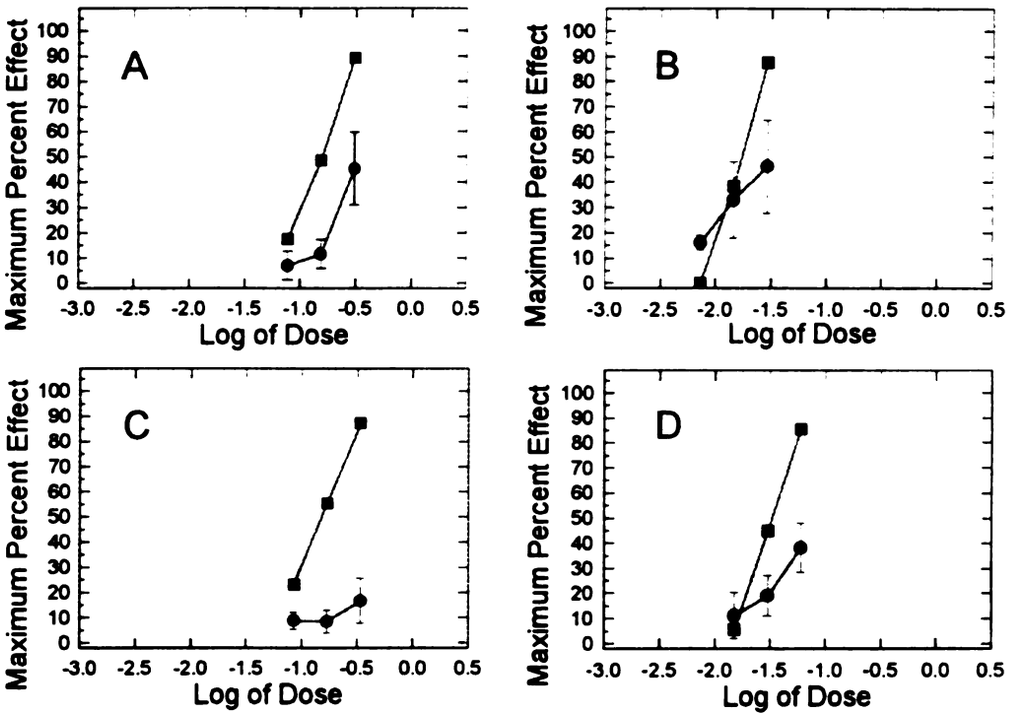

Figure 14. Log dose-response patterns of antinociceptive effects of combinations vs. theoretical additive sum of individual doses.

Mean (± SEM) of MPE in the CWTF assay for the combinations fentanyl-spiradoline (Graph A), fentanyl-enadoline combination (Graph B), oxymorphone-spiradoline (Graph C), oxymorphone-enadoline (Graph D). Actual response (filled circles) vs. expected response of the additive sum of doses (filled squares).

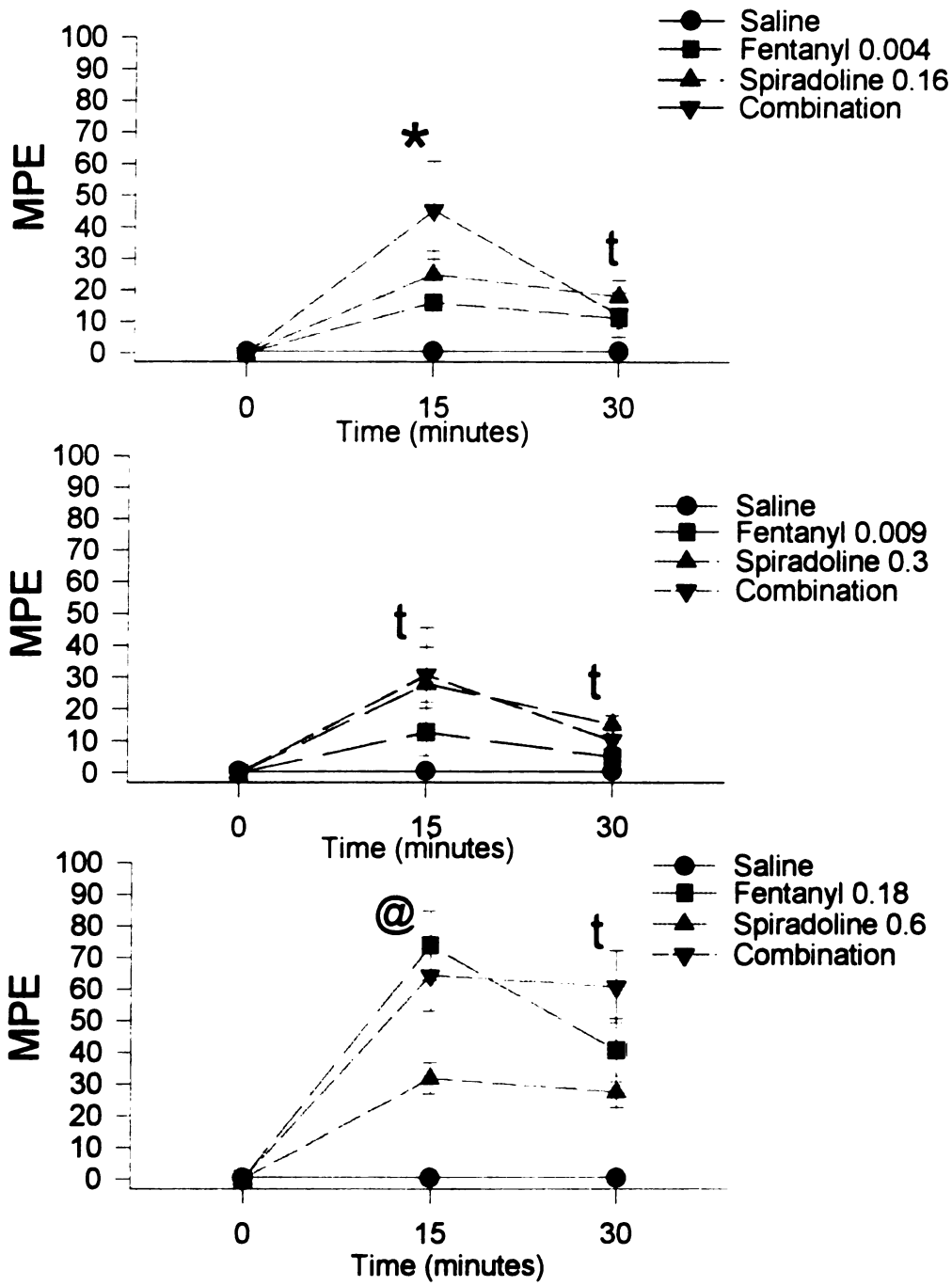

Figure 15. Direct comparisons of the mean (± SEM) of antinociception (MPE) of fentanyl, spiradoline, and their combination vs. their individual effects.
Asterisk indicates additive effect, t indicates subadditive effect, @ indicates antagonistic effect, $p < 0.05$.

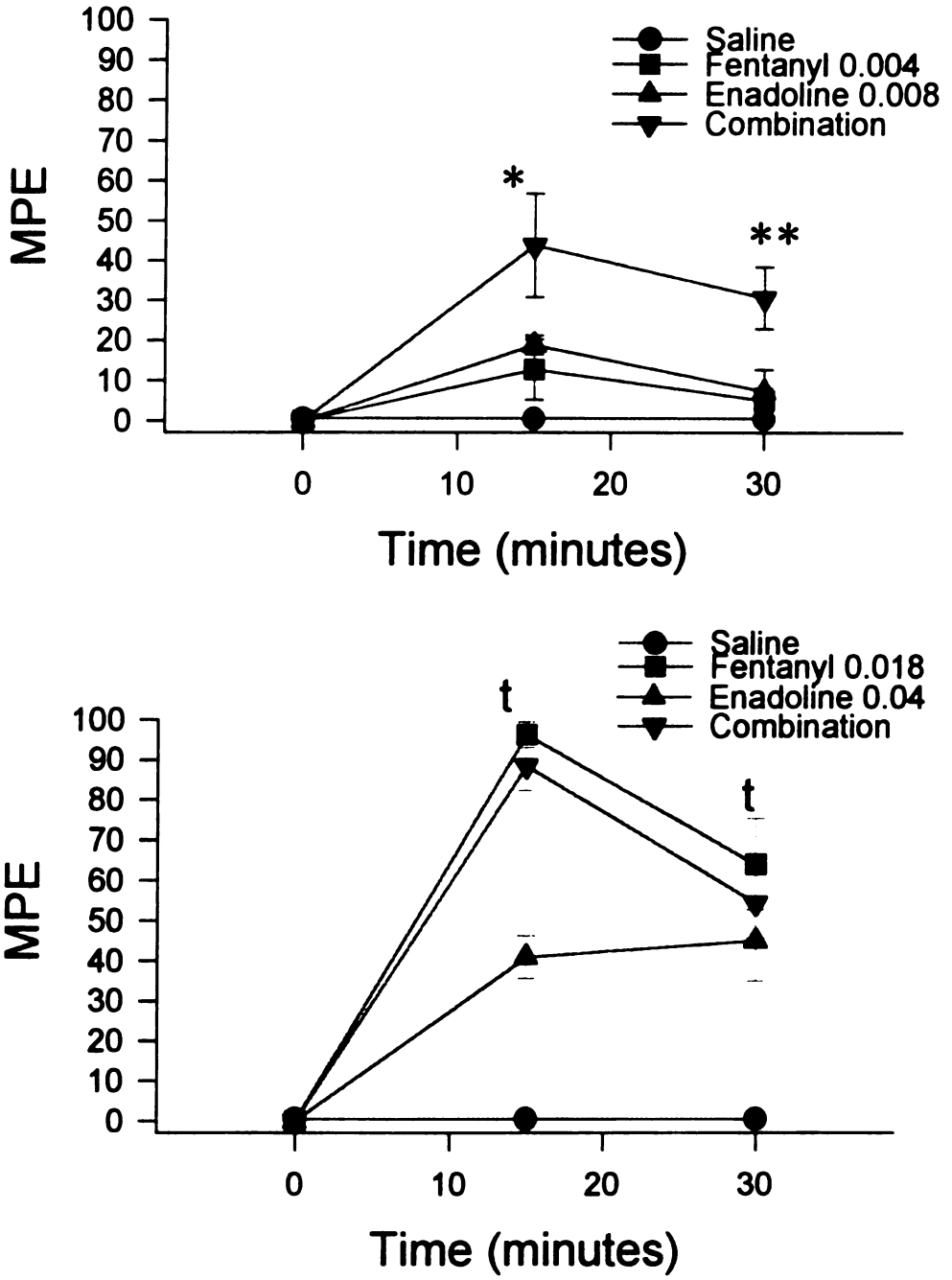

Figure 16. Direct comparison of mean (± SEM) antinociceptive effects (MPE) of fentanyl and enadoline in combination vs. their individual effect.

Asterisk indicates additive effect, double asterisk indicates synergistic effect, t indicates subadditive effect, $p < 0.05$.

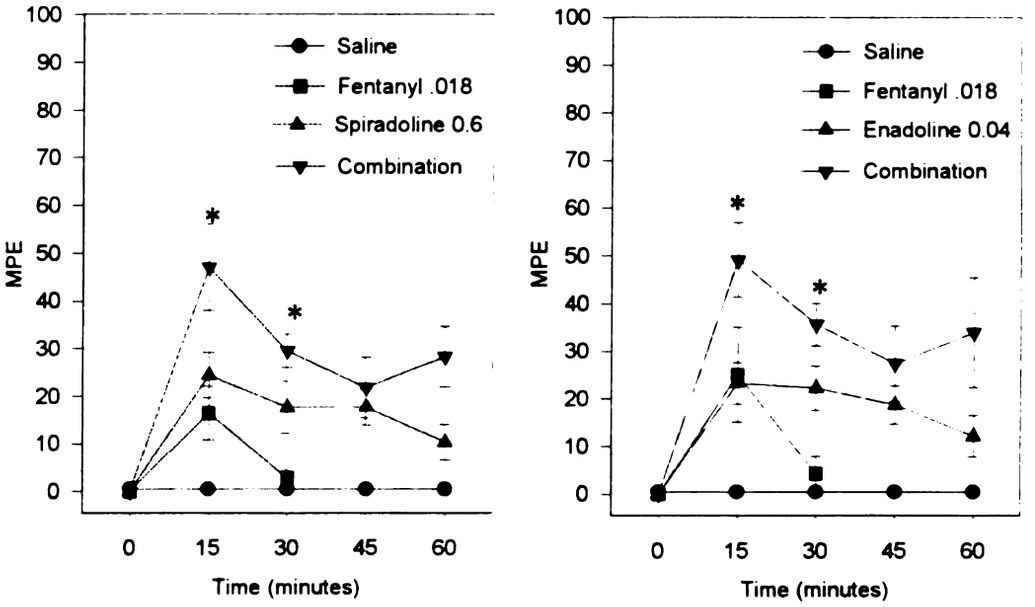

Figure 17. Antinociceptive responses, mean (± SEM) of MPE, of fentanyl, spiradoline, enadoline and combinations of fentanyl-spiradoline and fentanyl-enadoline in methadone-tolerant rats.
Asterisk indicates additive effect, $p < 0.05$.

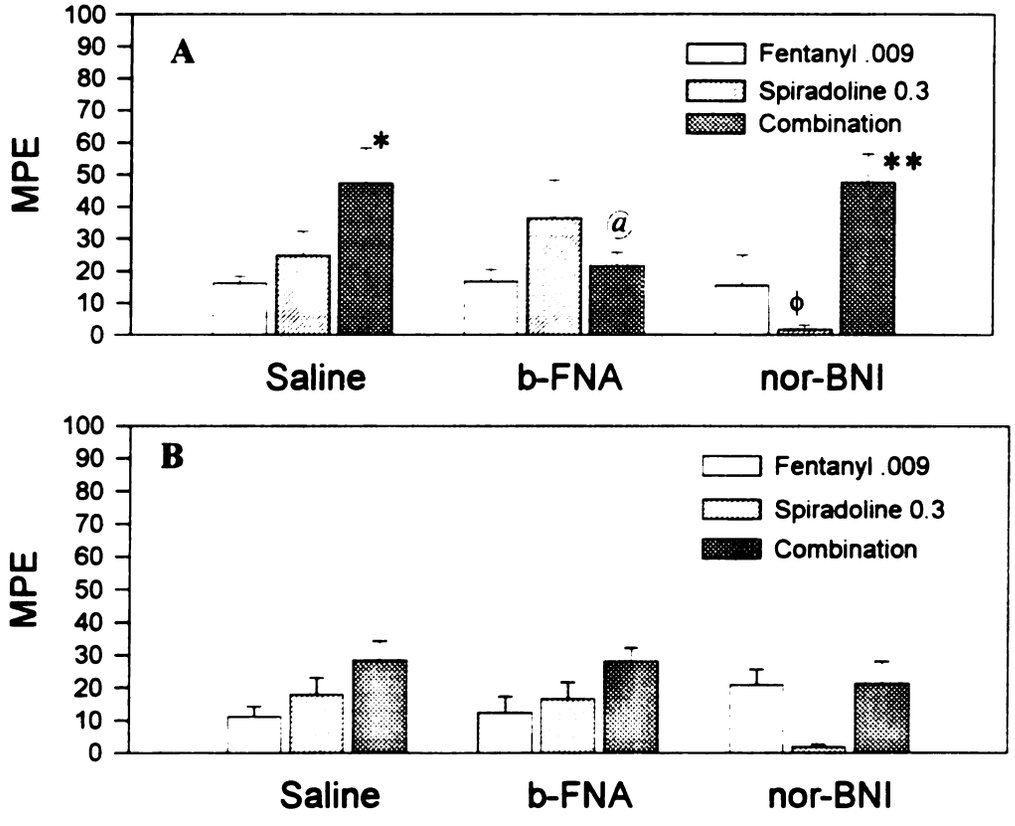

Figure 18. Antinociceptive responses, mean (± SEM) of MPE, of fentanyl, spiradoline, and their combination in saline, b-FNA or nor-BNI pretreated subjects. Graph A shows effects at 15 minutespost injection, Graph B shows effects at 30 minutes post injection. Asterisk indicates additive effect, double asterisk indicates synergistic effect, @ indicates antagonistic effect, and ϕ indicates selective antagonism, $p < 0.05$.

Discussion

Results of individually testing agonists in CWTF showed that mu and kappa agonists produced antinociception with similar potency and efficacy. These similarities have been reported for intrathecally administered dynorphin and morphine in rats (Han and Xie, 1982; Herman and Goldstein, 1985). Thus, it seems that both mu and kappa agonists have a dose-dependent interaction in the CWTF. Results also showed that lower doses of each agonist in combination demonstrated additive and even synergistic interactions, whereas higher doses produced sub-additive interactions. This biphasic interaction was also shown by Schmauss et al. (1983) and antagonistic interactions were reported by Schmauss and Herz (1987) and Song and Takemori (1991). It is evident that mu and kappa opioids in combination do not act as full agonists, as they otherwise do individually.

There are at least three possible hypotheses that may explain mechanisms involved in antinociceptive interactions of mu and kappa agonists. One could be related to distinct receptor actions in the periphery, spinal cord, or in supraspinal areas. Each agonist demonstrated supraspinal and spinal antinociception in other studies (Sasson and Kornetsky, 1986; Dykstra et al., 1987; Leighton et al., 1987; Unterwald et al., 1987; Millan et al., 1989; Piercey and Einspahr, 1989; Horan et al., 1991), and since agonists were administered SC peripheral receptor binding would be possible. Of the three areas, data seem to support the notion that interactions are more likely to occur in the spinal cord. Antinociceptive interactions could be related to the neuroanatomical association of kappa receptors with A-delta fibers and mu receptors with C fibers in the spinal cord. Previous experiments showed that dynorphin produced no antinociception in response to

"hot" thermal stimuli and thus antagonistic interactions between morphine and dynorphin were concluded to be mediated by kappa receptors interfering with mu receptors (Schmauss and Herz, 1987; Song and Takemori, 1991). In contrast, experiments of this paper showed antagonistic antinociceptive interactions of mu and kappa agonists that had previously produced maximal levels of antinociception when tested individually. These results suggest that antinociceptive actions of mu and kappa receptors interacted with each other in some occlusive manner. This hypothesis may be more reasonable especially in light of results from methadone-tolerant rats that showed additive interactions of combinations that were previously sub-additive (antagonistic). In addition, nor-BNI antagonized spiradoline antinociception, but did not affect the combination dose.

Regarding a hypothesis related to interactions at supra-spinal areas, not much is known about mu and kappa receptor interactions in these sites. However, one study showed that microinjection of ethylketocyclazocine in the periaqueductal gray and locus coeruleus produced synergistic interactions. In contrast, microinjection of EKC into either the PAG or the LC failed to elicit antinociception and antagonized analgesic actions of co-administered morphine or DSLET (Bodnar et al., 1991).

Another potential relates to the notion that only certain opioid receptor subtypes may be involved in antinociceptive interactions. The kappa agonists in these studies have been proposed as kappa1 agonists (Nock et al., 1988a; 1989; Zukin et al., 1988; de Costa et al., 2989; Paul et al., 1990; Horan et al., 1991; Unterwald et al., 1991; Horan et al., 1993). Fentanyl and oxymorphone have been shown to act on both mu subtypes, mu1 and mu2 (Jang and Yoburn, 1991; Ferrante, 1993). Possibly mu1 receptors interfere with

kappa1 receptors. The rationale for this idea comes from results in this study that showed combination doses producing sub-additive interactions in saline-treated rats, but later additive interactions in methadone-tolerant rats. Other studies have shown that mu2 receptors are more resistant to tolerance development than mu1 receptors (Ling et al., 1989). These data support the idea that mu1 receptors in methadone-tolerant rats were not able to produce antinociception, but mu2 receptors were still active , since mu2 receptors in the spinal cord could mediate antinociceptive effects (Ling and Pasternak, 1983; Ling et al., 1985). This interpretation suggests that mu1 receptors interfere with kappa1 receptor-mediated antinociception.

Although the mechanisms for these antinociceptive interactions have not yet been clarified, they represent interesting puzzles that may have clinical as well as theoretical significance. In any case, the CWTF proved to be a useful nociceptive model that can be utilized in studying antinociceptive effects and interactions of mu and kappa agonists. Furthermore, these studies demonstrated that great differences exist between various nociceptive stimuli as evidenced by differing pharmacological profiles of opioid agonists and antagonists in various nociceptive tests.

CHAPTER 6

ANTINOCICEPTIVE INTERACTIONS OF MU AND KAPPA AGONISTS IN COMBINATION USING COLORECTAL DISTENSION.

Summary

Morphine and other mu agonists have disadvantages as analgesics in severity of side effects (e.g., repiratory depression) and liability of dependence. Attempts to find alternate treatments that produce less side effects and less dependence have led to the study of kappa opioids individually or in combination with mu opioids. Kappa opioids do not induce severe respiratory impairments or addicting influences, and in fact produce dysphoria and other actions that are "opposite" to those of mu opioids. In addition, kappa opioids have been shown to be efficacious in allaying visceral nociception, often comparable to that of mu agonists. Thus, it is possible that combinations of mu and kappa opioids could relieve visceral pain while reducing side effects of the individual drugs. Results of these studies showed that mu and kappa agonists individually produced maximal levels of antinociception in the colorectal distension assay. In combination, mu and kappa agonists produced either additive or synergistic antinociceptive interactions. These interactions were significantly antagonized by beta-funaltrexamine and nor-binaltorphimine, indicating that both mu and kappa receptor activity was involved. The discrete mechanisms of these antinociceptive interactions remain to be elucidated. Results thus far demonstrated that combinations of mu and kappa agonists produced additive as well as synergistic antinociception depending upon the dose and temporal factors. Colorectal distension is considered to be a useful visceral nociceptive model in studying antinociceptive drug interactions. These results suggest that there is great potential for the application of combined use of mu and kappa opioids for the control of visceral nociception.

Introduction

Most analgesic therapies currently available for control of severe pain involve the use of mu opioid agonists. Although agonists powerfully attenuate nociception, side effects such as respiratory depression, nausea and vomiting, and dependency liability severely limit their usefulness (Erian and Shih, 1987). Opioid effects are generally dose-dependent. Thus, larger doses of mu opioids required to alleviate prominent nociception are also likely to produce troublesome side effects. Attempts to find suitable substitutes that produce less side effects have led to the potential use of kappa opioids. Kappa opioids may have useful pain-relieving contributions in their own right, but also have distressing side effects (e.g., dysphoria). The proposal to use mu and kappa opioids in combination is appealing since mu and kappa opioids both provide pain relief by different mechanisms but they exhibit different and to some extent opposing side effects. Side effects of mu opioids include euphoria, prominent respiratory depression, constipation, urinary retention, and tolerance and physical dependence to mu receptors (Pasternak and Wood, 1986). In contrast, side effects of kappa receptor activity include dysphoria, moderate changes in respiratory function, increased gastrointestinal motility, diuresis, and a weaker tolerance and physical dependence (Martin, 1984; Woods and Winger, 1987). Thus, kappa opioids may reciprocally oppose mu-related side effects while augmenting the pain relief. Furthermore, mu and kappa agonists have been shown to be efficacious in attenuating visceral nociception (VonVoigtlander, et al., 1983; Quirion, 1984; Schmauss and Yaksh, 1984; Ness and Gebhart, 1988; Sawyer et al., 1991). Kappa agonists in one study were shown to be more efficacious than mu agonists (Upton et al., 1982). This premise that mu and kappa opioids may be combined to augment pain relief is of course dependent upon the contingency that selective kappa agonists, without significant mu antagonist activity, be chosen.

Previous antinociceptive studies indicated possible synergistic opioid interactions between morphine and spiradoline in the hot plate and tail flick assays (Kunihara et al., 1989). Isobolographic analyses showed "powerful synergy between otherwise inactive doses" of mu (morphine, DAMGO) and kappa (U-50,488) in a paw withdrawal test (Miaskowski et al., 1990; Sutters et al., 1990). In addition, Ren et al. (1985) and Jhamandas et al. (1986) showed that kappa agonists potentiated antinociceptive effects of mu. It is likely that antinociceptive interactions of mu and kappa agonists will vary depending on the opioid dosage and nociceptive stimulus used. Hayes et al. (1987) showed that mu and kappa agonists had different pharmacological profiles depending on the nociceptive stimuli used in the test. Thus, the objective of this study was to test antinociceptive interactions of mu and kappa agonists in various dose combinations using the colorectal distension (CRD) procedure.

Methods

Subjects

Male Sprague Dawley rats weighing 300 to 500 grams were approved for use in the following experiments by the All-University Committee and Care of Michigan State University. All rats were trained over a two-month period to accept a lubricated (K-Y® Jelly, Skillman, NJ) colonic balloon catheter (Pointe Medical, Crown Point, IN) inserted per rectum while lying quietly in a towel which was snugly fitted around them. Training started for rats between the ages of 60 to 80 days. At approximately 6 weeks, rats accomodated to the catheter and being restrained in the towels without struggling. Subjects were reinforced after training sessions by access to Cheerios cereal "treats" and time to "play and socialize" on a large table-top among towels and plastic boxes and tunnels.

Drugs

Fentanyl citrate was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Oxymorphone was purchased from Mallinckrodt (Mundelein, IL). Spiradoline racemic (U62066E) was generously provided by P.F. VonVoigtlander from The Upjohn Company, Kalamazoo, MI. Enadoline (PD-129290 or CI-977) was generously supplied by David Downs , Parke-Davis Pharmaceutical Research, Ann Arbor, MI. All agonists were dissolved in saline. The selective antagonists, b-FNA and nor-BNI, were generously provided by the National Institute on Drug Abuse. The antagonists were dissolved in sterile water.

Procedure for Log Dose-Response Patterns of Agonists Individually and in Combination in CRD

Rats were restrained in towels with catheters in place as described earlier. Nociceptive thresholds were determined by introducing a pressure stimulus into the colonic balloon catheter for not more than 1 second. Lower (non-threshold) pressures were randomly and frequently presented to a subject (extinction) as increasing pressures were occasionally presented to determine the pressure to which a naive rat would consistently respond. A nociceptive threshold response to a "nociceptive" pressure included a moderate abdominal contraction resulting in a hunched posture, which is termed a guarding response. Abdominal contractions were recorded by using a water-filled Disposa-cuff (Critikon, Tampa, FL) which was fitted around a subject's abdomen and connected with tubing to a pressure transducer coupled to a polygraph recorder (Grass Instruments, Inc., Quincy, MA). A maximum pressure stimulus was used as a cutoff level in situations of maximum levels of analgesia to prevent permanent tissue damage (see the equation for maximum percent effect [MPE] under the Data Analysis sub-heading).

After determining thresholds, catheters were removed and rats were released from towel restraint and given a subcutaneous injection of a coded drug (experimenter blinded).

Rats were again prepared for nociceptive testing and responses to the stimulus were recorded at 15 minute intervals for one hour after injection.

Procedure for Selective Antagonism

Trained rats were pretreated 24 hours earlier with 8.0 mg/kg SC of b-FNA, a mu receptor antagonist shown at this dose and time of testing to produce selective mu antagonism (Ward et al., 1982; Dykstra et al., 1987). After the 24 hour pretreatment, rats randomly received either fentanyl (0.012 mg/kg), spiradoline (0.3 mg/kg), or their combination. A second group of trained rats were pretreated 48 hours earlier with 10.0 mg/kg SC of nor-BNI, a kappa receptor antagonist shown at this dose and time of testing to be selective for and potent at the kappa receptor (Diop et al., 1994). After 48 hours, rats received either fentanyl (0.012 mg/kg), spiradoline (0.3 mg/kg), or their combination. A third group of trained rats were randomly pretreated 24 or 48 hours earlier with saline and later received either fentanyl (0.012 mg/kg), spiradoline (0.3 mg/kg), or their combination. A blinded experimenter observed and recorded responses 15 and 30 minutes post-injection.

Data Analysis

The ED_{50} doses of fentanyl, oxymorphone, spiradoline and enadoline were determined by using the linear regression function of Sigma Plot (Jandel Corporation, San Rafael, CA). Using Sigma Stat (Jandel Corporation, San Rafael, CA), drug comparisons were tested with a random ANOVA. Student-Neuman-Keuls method was used to determine significant group differeneces. Significance was set at $p < 0.05$. For graphical representation, antinociceptive data were standardized as a maximum percent effect (MPE) (Harris and Pierson, 1964):

$$MPE = \frac{PDn - C}{Max - C} \times 100$$

where PDn is the stimulus level that a subject responds at n minutes post-injection. C is the stimulus level to which a naive subject normally responds. Max is the maximum stimulus level presented to a subject.

Analysis of antinociceptive responses of combination doses in comparison to theoretical additive sums of individual responses was accomplished by using the Z table (Steel and Torrie, 1984). First, to calculate theoretical additive sums of individual responses, the MPE of their effects were summed. The SEM of the theoretical sum was calculated by using the root mean square of the individual SEM's. Finally, the absolute difference between the theoretical and actual response was divided by the root mean square of the theoretical and actual SEM's. The calculated number was then compared to values on the Z table. Values corresponding to numbers in the table at $p < 0.05$ indicated significant deviation from additivity.

Results

Individual dose-response patterns in the CRD for fentanyl, oxymorphone, spiradoline, and enadoline showed that each agonist produced maximal levels of antinociception (Figure 19). The following is a list of ED_{50}'s (mg/kg, SC) of the agonists: fentanyl, 0.01 (0.006-0.016); oxymorphone, 0.078 (0.02-0.126); spiradoline, 0.56 (0.25-1.26); enadoline, 0.077 (0.04-0.2). Peak levels of antinociception occurred at 15 minutes post-injection for fentanyl and spiradoline and 30 minutes for oxymorphone and enadoline. Duration of antinociception was shortest for fentanyl (less than 60 minutes) while enadoline, spiradoline, and oxymorphone were relatively equal in duration (2-3 hours).

Antinociceptive effects of combination doses of mu and kappa agonists peaked at 15 minutes and maintained antinociceptive levels for at least 60 minutes. By comparing actual antinociceptive responses of the combinations at 15 minutes to their theoretical additive (linear) sum, results showed a trend towards additive interactions (Figure 20). Results of direct dose comparisons between individual and combined doses of fentanyl and spiradoline showed additive (linear) and synergistic interactions (Figure 21). Results of direct dose comparisons between individual and combined doses of fentanyl and enadoline also showed additive interactions (Figure 21).

Using selective antagonists b-FNA and nor-BNI, a combination dose of fentanyl and spiradoline that produced synergistic antinociception was tested to determine mu and/or kappa contributions to the antinociceptive effect (Figure 22). In subjects pretreated with b-FNA, antinociceptive effects of fentanyl were unchanged but the combination dose was significantly affected (antagonism) at 15 minutes in comparison to their respective saline-controls, $p < 0.05$. In subjects pretreated with nor-BNI, antinociceptive effects of spiradoline were unaffected at 15 and 30 minutes but the combination dose was significantly reduced in comparison to corresponding saline controls, $p < 0.05$.

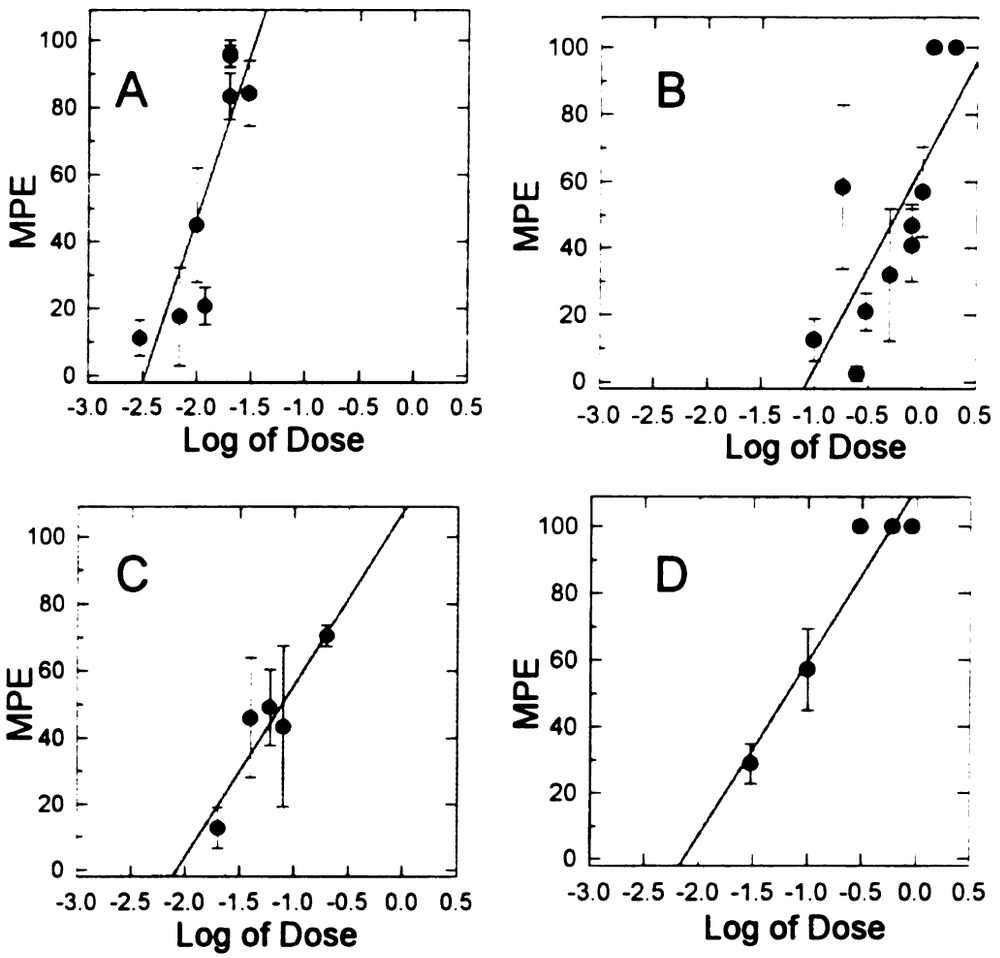

Figure 19. Antinociception of fentanyl, spiradoline, enadoline, and oxymorphone. Graph A: Mean (± SEM) for fentanyl dose response in the CRD assay 15 minutes post-injection; ED_{50} = 0.01 (0.006-0.016), n = 3 to 9 subjects per dose. Graph B: Mean (± SEM) for spiradoline dose response in the CRD assay 15 minutes post-injection; ED_{50} = 0.56 (0.25-1.26), n = 5 to 0 subjects per dose. Graph C: Mean (± SEM) for enadoline dose response in the CRD assay 30 minutes post-injection; ED_{50} = 0.077 (0.04-0.2), n = 2 to 2 subjects per dose. Graph D: Mean (± SEM) for oxymorphone dose response in the CRD assay 30 minutes post-injection; ED_{50} = 0.078 (0.02-0.126), n = 5 to 8 subjects per dose.

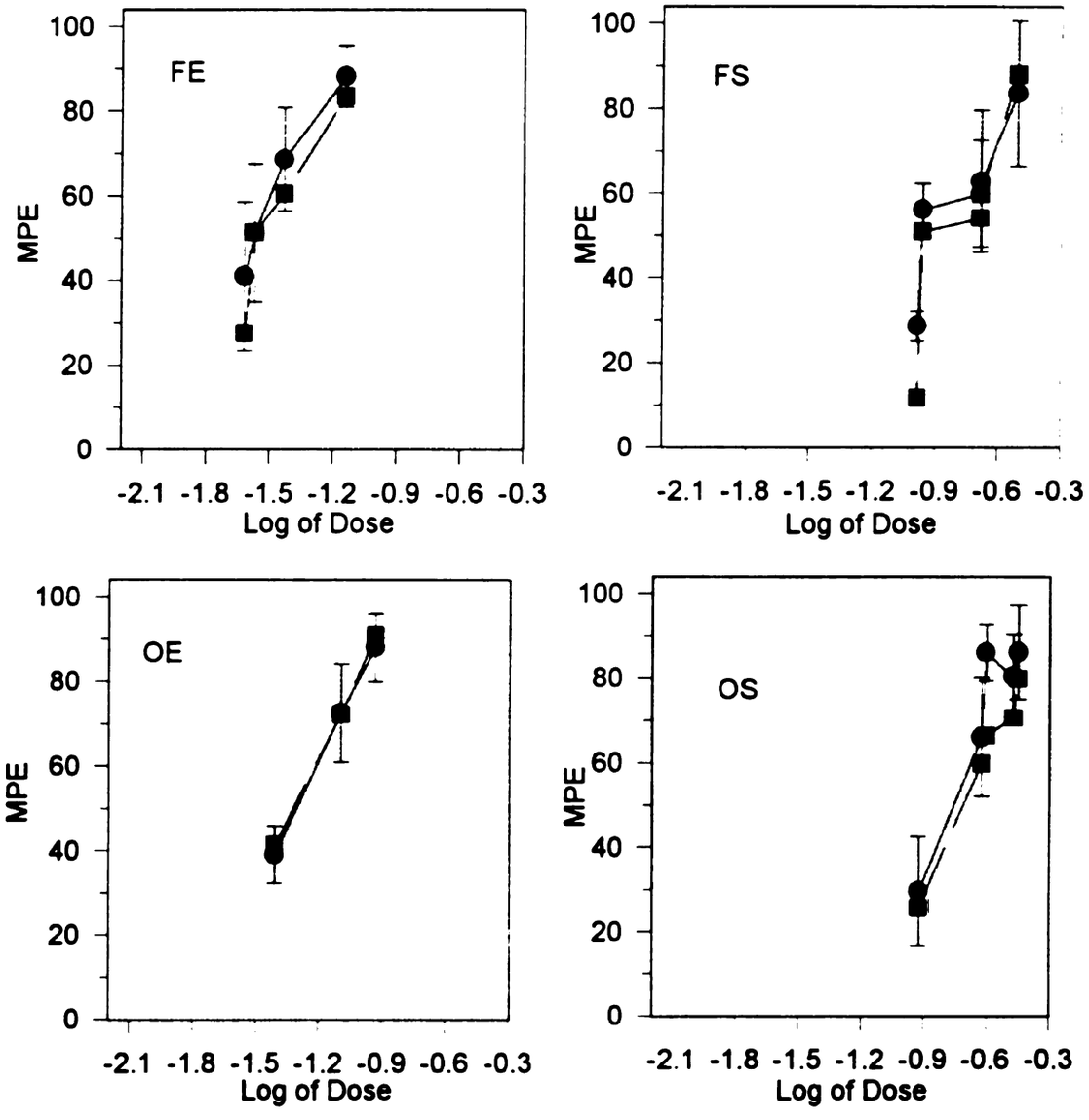

Figure 20. Mean (± SEM) of additive theoretical responses (MPE) vs. combination responses (MPE) of fentanyl-enadoline, fentanyl-spiradoline, oxymorphone-enadoline, oxymorpone-spiradoline.

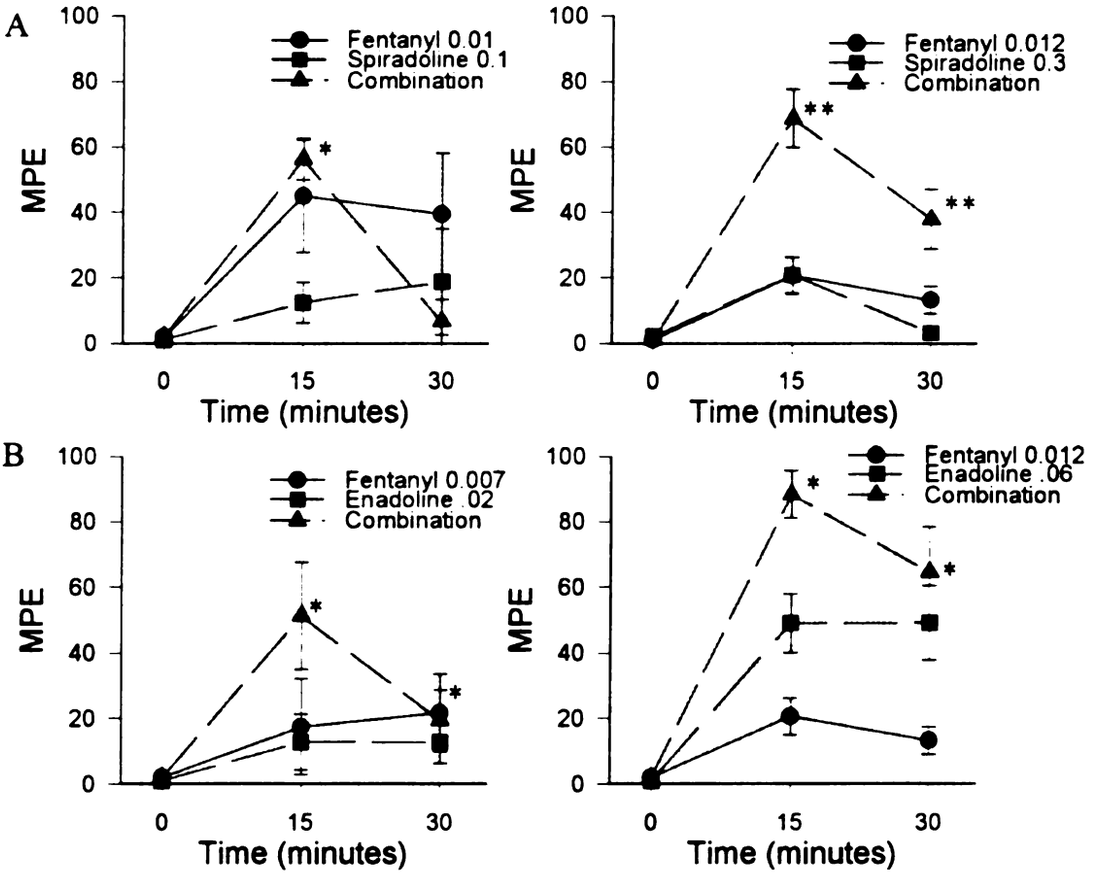

Figure 21. Direct comparisons of antinociceptive effects of fentanyl-spiradoline and fentanyl-enadoline vs. their individual effects.
Graphs A: Mean (± SEM) of responses (MPE) of single and combination doses for fentanyl and spiradoline. Graphs B: Mean (± SEM) of responses (MPE) of single and combination doses for fentanyl and enadoline. * indicates additive interaction, $p < 0.05$. ** indicates supra-additive (synergistic) interaction, $p < 0.05$.

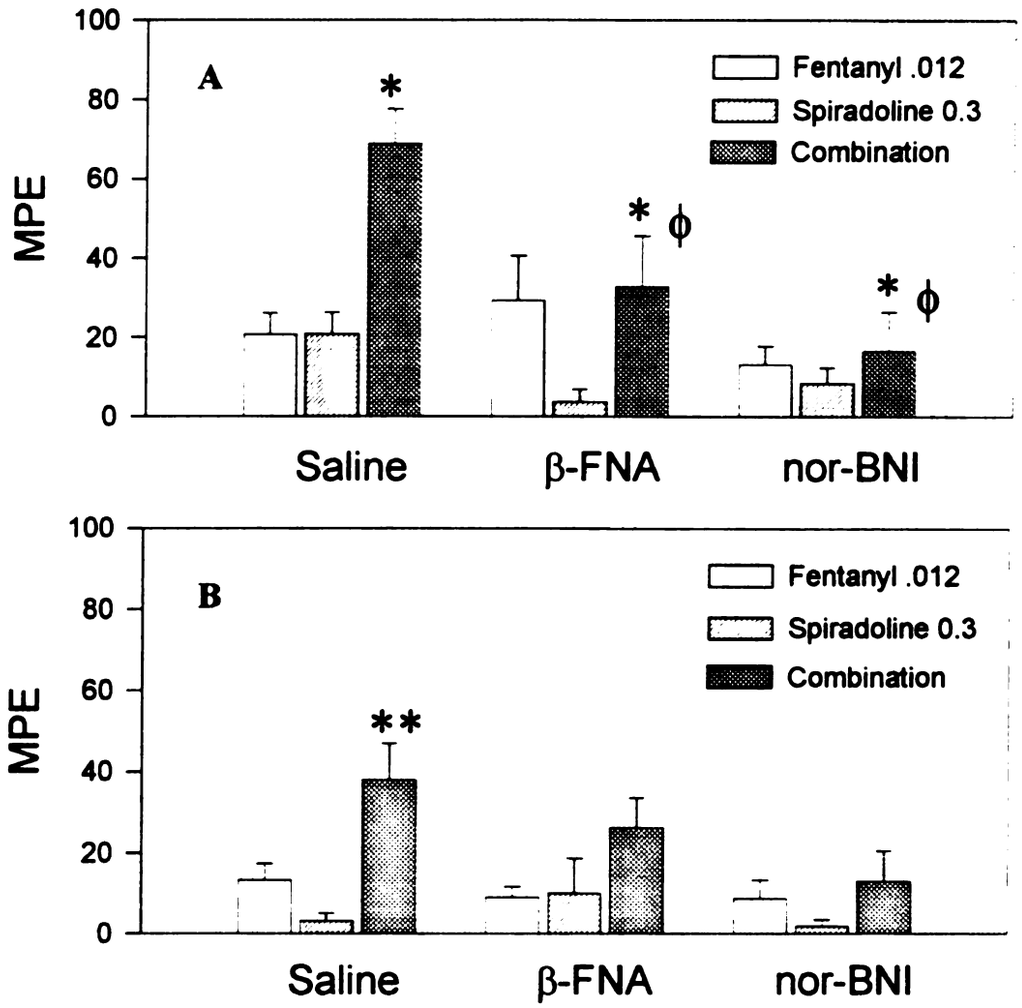

Figure 22. Antinociceptive response at 15 and 30 minutes of fentanyl, spiradoline, and their combination in saline-, b-FNA-, and nor-BNI-pretreated rats.
Mean (± SEM) of responses (MPE) of fentanyl 0.012, spiradoline 0.3, or their combination 15 (Graph A) and 30 minutes (Graph B) post-injection in either saline-, β-FNA-, or nor-BNI-pretreated subjects. Asterisk indicates supra-additive interaction (synergism) and significant difference from single-drug treatments; double asterisk indicates supra-additive effect compared to single-drug treatment and a significant difference from all other treatments; ϕ indicates selective antagonism of combination dose compared to combination dose in saline-pretreated subjects, $p < 0.05$.

Discussion

Results of individually tested agonists in CRD showed that mu and kappa agonists produced antinociception with similar potency and efficacy. When mu and kappa agonists were tested in combination, antinociceptive results reflected additive and synergistic interactions. These interactions were antagonized by β-FNA and nor-BNI, antagonism indicative of mu and kappa receptor activity. Interestingly, results of the antagonists on fentanyl- and spiradoline-induced antinociception did not show significant antagonism. The low level of antinociception produced by the agonists individually complicated statistical analysis; spiradoline antinociception was visibly reduced in nor-BNI-pretreated animals. (Relatively lower doses of fentanyl and spiradoline were required in order to give allowance for the possibility of synergistic antinociceptive effects of their combination. Analysis of antinociceptive effects over 100% could not be measured nor analyzed.) In contrast, relatively higher doses of fentanyl and spiradoline were tested in β-FNA- and nor-BNI-pretreated rats. Interestingly, doses of fentanyl (0.02 mg/kg, SC) and spiradoline (0.8 mg/kg, SC) producing 50% to 80% antinociception (MPE) in CRD were both antagonized by the same doses of b-FNA and nor-BNI used in the present experiment. Thus, antinociceptive levels of individual agonists in the present experiment were likely too low for statistical comparison. In regard to the non-selective antagonism of fentanyl and spiradoline, results were suggested to be related to mu and kappa receptor interactions rather than non-selectivity of the antagonists (Briggs et al., submitted). Rationale for this conclusion was based on numerous reports on the selectivity of fentanyl and b-FNA for mu receptors and spiradoline and nor-BNI for kappa receptors (Jang and

Yoburn, 1991; Dykstra et al., 1988; Diop et al., 1994; Briggs et al., in preparation; Briggs et al., in preparation).

If the antagonists were selective for their respective receptors, then antagonism of antinociceptive effects of individual and combined doses of fentanyl and spiradoline would indicate that mu and kappa receptors are closely integrated in nociceptive pathways that are related to the nociceptive stimulus of CRD. Antinociceptive interactions between mu and kappa receptors could occur in the periphery, spinal cord, or in supraspinal areas. Each agonist demonstrated supraspinal and spinal antinociception in other studies (Ling and Pasternak, 1983; Sasson and Kornetsky, 1986; Dykstra et al., 1987; Leighton et al., 1987; Unterwald et al., 1987; Millan et al., 1989; Piercey and Einspahr, 1989; Horan et al., 1991) and since agonists were administered SC, peripheral receptor binding would be possible. Unfortunately, the association between neural pathways for visceral nociception and specific opioid receptor modulation have not been clearly identified. However, studies have characterized at least three different types of neurons responsive to visceral nociception that are distributed throughout the superficial and deeper laminae of the dorsal spinal cord. Most of these neurons were shown to have long ascending projections that were localized to ventrolateral quadrants in the brain. In addition, most of these neurons were subject to descending inhibitory influences while others were apparently subject to tonic descending facilitory influences (Ness and Gebhart, 1988b; Gebhart and Ness, 1990; Ness and Gebhart, 1990). Coincidentally, mu and kappa receptors were shown to exist in the periphery, spinal cord, and supraspinal sites (Mansour et al., 1988; Stein, 1993). Thus,

visceral antinociception could possibly be modulated by mu and kappa receptors at numerous sites.

Although the mechanisms for these antinocieptive interactions have not yet been deciphered, the CRD proved to be a useful nociceptive model that can be utilized in studying antinociceptive interactions of mu and kappa agonists. Furthermore, these studies demonstrated that great differences exist between various nociceptive stimuli (thermal vs. visceral) as evidenced by differing pharmacological profiles of opioid agonists and antagonists.

CHAPTER 7

INTERACTION BETWEEN MU AND KAPPA OPIOID AGONISTS ON PATTERNS OF PLACE CONDITIONING.

Summary

Mu and kappa opioid agonists in combination have produced synergistic antinociception in both the rat and cat (Kunihara et a., 1989; Briggs, et al., 1992; Sawyer et al., 1994). While achieving analgesia with lesser doses, a concomitant reduction in side effects (ie. mu euphoria, kappa aversion) of the agents in combination would be clinically beneficial. To determine interactions of mu and kappa opioid agonists on motivational processes, rats were trained in a place-conditioning X-maze. Fentanyl-trained rats demonstrated a place preference of 75% which decreased to 64% ($p < 0.05$) after subsequent training with a combination of fentanyl and endadoline. Enadoline-trained rats showed a slight place preference (55%) which remained unaltered after subsequent training with the combination of enadoline and fentanyl (54%, $p > 0.05$). Alley entrances (per 10 minute session) decreased significantly from 89.9 ± 3.46 in fentanyl-trained subjects to 68 ± 4.42 after training with the combination of fentanyl and enadoline. Activity of enadoline-trained animals (67.4 ± 3.91) was unaltered after combination with enadoline and fentanyl (67.1 ± 3.99). Thus, a combined dosing schedule of fentanyl and enadoline was capable of attenuating a fentanyl induced place preference and also produced no increase in place preference in comparison to enadoline alone.

Introduction

The positively reinforcing effects of mu opioid agonists are well-known, as are the aversive influences of many kappa opioid agonists (Woods et al., 1982; Mucha and Herz, 1985; Bals-Kubik et al., 1989). The opposing motivational effects, at least for conditioned place responses of mu (preference) and kappa (aversion) agonists, appear to be exerted at different sites in the brain (Shippenberg et al., 1988). The rewarding effects have been associated with mu receptor activation in the ventral tegmental area (VTA) of the brain, which is the origin of the mesolimbic-mesocortical dopamine systems (Bozarth, 1986; Wise, 1989). The aversive influences have been related to kappa receptors in VTA and limbic-cortical terminals of cell bodies having their origin in VTA (Pfeiffer et al., 1986; Bals-Kubik et al., 1993; Narita et al., 1993).

Other studies have demonstrated neurochemical interactions of mu and kappa opioid agonists on the activity of brain dopaminergic systems (DiChiara and Imperato, 1988; Devine et al., 1993). The interactions with mesolimbic-mesocortical dopamine systems have been proposed to involve presynaptic mu receptor activity to curtail gamma-aminobutyric acid (GABA) interneuronal inhibition of VTA dopaminergic cell bodies and presynaptic kappa receptors on dopaminergic nerve terminals in nucleus accumbens to decrease dopamine release (Spanagel et al., 1992). These neurochemical effects of mu and kappa opioids are consistent with effect of these drugs on motivational behaviors (Pfeiffer et al., 1986; Wise, 1989; Bals-Kubic et al., 1993).

Both mu and kappa agonists are antinociceptive or analgesic but with different spectra of efficacy as relating to the type of nociceptive insult (Yaksh, 1986; Zvartau and Kovalenko,

1986; VonVoigtlander and Lewis, 1988; Briggs et al., 1994). For visceral types of nociception, both mu and kappa agonists can be very effective as analgesics (VonVoigtlander et al., 1983; Ness and Gebhart, 1988; Sawyer et al., 1991). Since mu and kappa agonists have different spectra of undesirable side effects (euphoria vs. dysphoria, prominent respiratory depression vs. weak depression or increased respiratory activity, antidiuresis vs. diuresis; see Negus et al., 1990; Spanagel et al., 1992; France et al., 1994), their combination may promote additive or supra-additive analgesia while reducing the side effects of each class. In fact, combinations of these agents have induced supra-additive antinociception experimentally (Kunihara et al., 1989; Sawyer et al., 1994; and unpublished observations). Thus, combinations of more selective mu and kappa agonists may have utility in the clinical management of certain types of pain, particularly if superior pain control is accompanied by a reduced risk of physical and psychological dependencies and respiratory embarrassment.

In this study we have addressed the hypothesis that motivational influences of mu and kappa agonists, when the two classes are combined in doses that are analgesic separately as well as in combination, may interact to mutually attenuate euphorigenic effects of the mu opioid and dysphoric qualities of the kappa opioid. This hypothesis was tested using conditioned place preference or aversion in an X-maze (Rech et al., 1984) utilizing food-reinforced alley changes to enhance activity levels and choice opportunities.

Method

Subjects

Thirty-five male Sprague-Dawley rats (230-400 gm) were approved for these studies by the All-University Committee on Animal Use and Care (MSU). The animals were housed

two per container in plastic animal boxes in temperature, humidity, and light-controlled quarters (illumination from 7:00 a.m. to 7:00 p.m.). Food and water were provided al lib, except when animals were fasted for 12 hours prior to training and testing in the X-Maze.

Drugs

Fentanyl citrate (purchased from Elkins-Sinn, Inc., Cherry Hill, NJ) was utilized as a mu-selective agonist (Negus et al., 1990). The dose of fentanyl studied was 0.01 mg/kg, found to be a dose producing 30% of the maximal possible analgesia (ED_{30}) in previous studies of a visceral nociceptive response (colorectal distension, Briggs et al., 1994). Enadoline (PD-129290 or CI-977, generously supplied by Dr. David Downs, Parke-Davis Pharmaceutical Research, Ann Arbor, MI) has been characterized as a specific kappa opioid agonist (Hunter et al., 1990). The dose of enadoline [salt] was 0.02 mg/kg, which represented an ED_{20} in our previous analgesic testing (Briggs et al., 1994). We have also tested the combination of fentanyl and enadoline in these doses in several nociceptive procedures (see previous chapters), and found that the combination produced supra-additive analgesia based upon the calculated additive effects of the drugs tested singly.

Place Conditioning Apparatus and Procedure

The subjects were conditioned and tested in an X-maze (Rech et al., 1984) which was adapted to develop conditioned preference to alleys previously associated with drugs having positively-reinforcing effects and to develop conditioned aversion to alleys associated with agents inducing aversive influences. Place conditioning studies of rewarding and aversive drug effects have been commonplace in recent years, including the determination of motivational influences of various types of opioids (Shippenberg et al., 1988; Bals-Kubik et al., 1989, 1993;

Carr et al., 1989; Pchelintsev et al., 1991). The X-maze consisted of a square-shaped central arena 29 cm on each side and 19 cm high with 4 alleys radiating therefrom at 90° angles (Figure 23). Alleys were 35 cm long and 13 cm in width and height with openings from the central arena into each alley of 9 cm wide and 10 cm high. Top panels over the alleys were hinged to facilitate removal of a subject. The central arena was covered by a loose panel of clouded plexiglass allowing introduction and observation of responses of a subject. The central arena was painted neutral gray and the alleys were painted alternately black and white. Black alleys (2 and 4) had a screen mesh (textured) floor; white alleys (1 and 3) had a smooth floor. A 3 cm hole in the distal end of each alley allowed access to a cup into which was introduced a 45 mg food pellet (Noyes Precision Food Pellets, Lancaster, NH) as a subject traversed the alley. The maze was cleaned with dilute detergent solution between sessions. In order to restrain rats from entering certain alleys during training sessions, baffles painted neutral gray to match walls in the centeral arena were used to block off alley entrances.

To determine that rats did not have an initial preference for black or white alley colors, ten naive rats were prepared for testing. All subjects were deprived of food for 12 hours before testing and injected with saline s.c. 15 min. before entering the maze. For 10 minutes rats were allowed to traverse alleys in a random pattern to receive food pellets as reinforcers. These rats made approximately 30-40 alley entries per 10-min session and showed no preference for white vs. black alleys (48.5% vs. 51.5%, $p>0.05$). Therefore, our previous experience with this paradigm (unpublished results), that drug-naive rats showed no preference as to alley color, was confirmed.

Twenty-five naive rats in three groups (9 for fentanyl, 8 for enadoline, and 8 for saline) were then trained in the X-maze for place conditioning patterns. The three groups were conditioned as follows. After 12-hours food deprivation, each subject was injected with a coded dose and then retained in a neutral cage for 15 minutes prior to being placed into the maze for 10 minutes; thus, the observer was blinded to treatments. Saline-trained rats (controls) were injected with saline on days 1, 4, 5, 8 and 9 and were allowed to traverse white alleys. On days 2, 3, 6, 7, 10, and 11 these rats were injected with saline and allowed to traverse black alleys. Fentanyl-trained rats were injected with saline on days 1, 4, 5, 8 and 9 and were allowed to traverse black alleys while on days 2, 3, 6, 7, 10, and 11 these rats were injected with saline and allowed to traverse white alleys. Enadoline-trained rats were injected with saline on days 1, 4, 5, 8 and 9 and were allowed to traverse white alleys. On days 2, 3, 6, 7, 10, and 11 rats in this last group were injected with saline and allowed to traverse black alleys. After this conditioning period, the first test for any place conditioning was accomplished on day 12 by injecting the subject with saline and 15 minutes thereafter placing it in the maze for 10 minutes with all alley entrances open. The following day animals were conditioned in the same manner for 7 additional days, drug administered on days 13, 14, 17 and 18 and saline injected on days 15, 16 and 19. On day 20 a second test for place conditioning was performed as described above.

In testing for drug interactions, the same fentanyl- and enadoline-trained subjects were conditioned as in the above paradigm for an additional 10 days, except that rats received a dose of both fentanyl and enadoline. Saline-trained rats (controls) were conditioned as originally described for the additional 10 days. Thus, drug was administered on days 21, 22, 25, 26, 29,

and 30 while saline was administered on days 23, 24, 27, and 28. On day 31 subjects were again tested as previously described. By comparing results from day 31 with those from day 20, any effect of the drug combination that would modify a conditioned place preference or aversion induced during single-drug conditioning can be determined.

Another criterion of drug motivational effects is locomotor activity patterns of treated subjects. Tilson and Rech (1973) and Wise (1988, 1989) have indicated that drugs having positively reinforcing effects tend to increase locomotor response levels, whereas drugs exerting aversive effects tend to reduce activity levels (Rech, 1968; Heath and Rech, 1985). Therefore, we compared the number of total alley entrances from each test day to determine if the various treatments altered this index of locomotor responsivity. An alley entrance almost invariably related to the subject traversing the whole alley. Pauses in activity by an animal seldom occurred in the central arena.

To determine each subject's alley preferences during test days, alley entrances associated with either drug or saline experiences were individually summed and compared using the Binomial test (Zar, 1984). To compare groups of animals for significant place conditioning, differences in the sums of drug and saline alley entrances were determined using a Chi-Square test. Differences in activity levels (total number of entrances into each alley) for the various test days were determined by a one-way analysis of variance followed by Student Newman-Keuls procedure to isolate groups with significant differences. In all cases, $p < 0.05$ was taken as the index of significance.

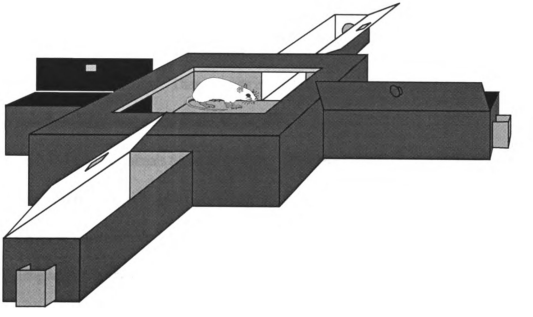

Figure 23. The X-maze is shown with opened hinged doors over aisles.
Note that two aisles opposite each other are painted white and two aisles are black. The center square area is painted "neutral" gray. After the rat is placed in the square "neutral" area, a plexiglass cover is placed over the top. Rats remain in the maze for 10 minute periods.

Results

Place Conditioning

Saline-trained rats (controls) did not demonstrate place conditioning on any of the three test days for either white (51%, 49%, 52%) or black alleys (49%, 51%, 48%) (Table 1). In contrast, fentanyl-trained rats demonstrated a significant preference for drug-associated alleys on the first test day with a score of 66% (Table 1). Additional training with fentanyl dosing increased this preference to a significantly greater level on the second test day with a score of 75%. Subsequent training with fentanyl combined with enadoline resulted in a reduced preference in this group on the third test day, compared to the preference level expressed on the second test day. These results indicate that effects of enadoline appear to interfere with the conditioned place preference activity of fentanyl.

Enadoline-trained animals failed to manifest either place preference or place aversion to drug-associated alleys on the first test day (Table 1). However, after additional training with enadoline, a slight preference for drug-associated alleys (55%) was realized on the second test day. Training involving the combination of enadoline and fentanyl administered over the next 10 days did not significantly alter the place preference of these subjects on the third test day (54% for drug-associated alleys, still a significant preference, but not different from the second test day score).

Table 2. Place conditioning trends as percent of alley entrances into drug-associated alleys vs. saline-associated alleys for saline (controls), fentanyl, enadoline, and the combination treatments.

Test Day	First			Second			Third		
Treatment	Sal	F	E	Sal	F	E	Sal	F&E	E&F
% drug association	49	66*	47	51	75*†	55*	48	65*‡	54*
% saline association	51	34	53	49	25	45	52	35	46

* Subjects showed significant conditioned place preference for drug-associated alleys, $p < 0.05$.

†Demonstrated higher level of conditioned place preference than for those of all other test days, $p = 0.00002$.

‡Demonstrated higher level of conditioned place preference than that of E&F on the third test day, $p = 0.0002$.

Alley Entries

The number of alley entrances recorded on test days support indications of conditioned place preference effects of these two opioids and their combination as described above. Total alley entrances in saline-trained rats gradually increased with training from a mean of 60 to 73 to 82 alley entrances (Table 2). Total alley entrances in fentanyl-trained rats increased from a mean of 80.3 on the first test day to 89.9 on the second test day as did the place preference influence of fentanyl (Table 2). After fentanyl-trained rats were conditioned with the combination of fentanyl and enadoline, a significant reduction in alley entrances was noted on the third test day (89.9 vs. 68.6). The number of alley entrances generated by the enadoline-trained subjects on the first test day (mean of 59.0) was at the level expected of animals that had received only saline over the training period. On the second test day, the number of alley entrances increased to a level that was significantly greater than that of the first test day, in agreement with the slight place preference shown by these animals at that time. After subsequent training with the combination of enadoline and fentanyl in rats initially trained on enadoline, the number of alley entrances in this group did not change on the third test day as compared to the score on the second test day. This last result again correlates with the place-preference pattern exhibited by these rats on the second and third test days.

Table 3. Mean (SEM) of alley entrances into drug-associated alleys vs. saline-associated alleys for saline (control), fentanyl, enadoline, and their combination.

Test Day	First			Second			Third		
Treatment	Sal	F	E	Sal	F	E	Sal	F&E	E&F
Drug association	30	53.3*	28	37	67.7†	37.1	39.75	44.4	35.9
Saline association	30.9	27	31	36	22.2	30.3	42.4	24.1	31.3
Mean Total	60.8	80.3	59	73.6	89	67.4	82.1	68.6	67.1
(± SEM)	2.1	4.2	4.9	5.7	3.5	3.9	5.3	4.4	4.0

*Entered significantly more alleys than enadoline on the first test day, $p < 0.05$.

†Entered significantly more alleys than all other treatments on all test days, except fentanyl on the first test day, $p = 0.00002$.

Discussion

The objective of this study was to explore the interactions of the mu-selective opioid agonist fentanyl and the kappa-specific opioid agonist enadoline as to their presumable opposing effects on the motivational brain mechanisms in rats. Fentanyl was shown to promote place-preference responding in the X-maze after only 12 days of training (while receiving fentanyl only 6 of those days). Additional training for 7 days strengthened the place preference of fentanyl. These results are in good agreement with other authors that fentanyl has potent positively reinforcing influences similar to that of other mu agonists and that preference for environments previously associated with these mu agonists is readily conditioned (Bozarth, 1987; Bals-Kubik et al., 1988, 1993; Shippenberg et al., 1988; Neisewander et al., 1990; Pchelintsev et al., 1991; Kuzmin et al., 1992).

Place conditioning studies of effects of kappa agonists have demonstrated aversive effects of this class (Shippenberg and Herz, 1987; Bals-Kubik et al., 1989; Funada et al., 1993). The results we obtained with enadoline, a kappa-specific agonist (Hunter et al., 1990), during the first phase of place conditioning indicated neither preference nor aversion. The second phase of training with enadoline produced a slight preference. The difference between our results with enadoline and reports of conditioned aversive responding to other kappa-selective agonists such as U-50,488H and U-69503 (Shippenberg et al., 1988; Bals-Kubik et al., 1989) may relate to peculiar properties of the less well-studied enadoline. The dose of enadoline (0.02 mg/kg) may have been less than that producing a prominent aversion. In studies of enadoline antinociceptive properties (Briggs et al., 1994), we noted that irritability and vocalization on handling occurred with enadoline-dosed rats at doses of 0.04 mg/kg and above.

Additionally, the difference in our observation of enadoline-trained rats in the second phase of training may be related to the place-conditioning procedures. We used food pellets in hungry subjects to increase rate of alley entrances and facilitate choice behavior, whereas other authors relied on exploratory activity. The use of food-reinforced responding may have modified motivational influences of the kappa agonist, since kappa-opioid agonists appear to facilitate food intake (Jackson and Cooper, 1986; Levine et al., 1990). Furthermore, nor-binaltorphimine (nor-BNI) (a selective kappa antagonist) blocked the facilitation by food restriction of hypothalamic self-stimulation (Carr and Papadouka, 1994). Thus, it seems that further study of enadoline and place-preference procedures is required to resolve the issue of its potential for aversive effects.

When fentanyl and enadoline were co-administratered in fentanyl-trained rats during the third training session (days 21-30) and then tested for place preference or aversion on the third test day, the previous level of preference induced by fentanyl alone (75%, second test day) was significantly reduced (65%). If enadoline had no capacity to interact with fentanyl, the third phase of training with the combined drugs should have induced an even greater level of place preference for fentanyl-associated alleys on the third test day due to the additional training with fentanyl. In contrast to results of fentanyl-trained rats after the last 10 days of conditioning with the drug combination, place condtioning results of enadoline-trained rats on the third test day (54%) were unchanged from those of the second test day (55%). Again, if enadoline had no influence on place conditioning effects of fentanyl, the score for drug-associated alleys on the third test day in enadoline-trained rats should have been similar to the score of fentanyl-trained rats on the first test day (66%, after 12 days training with fentanyl

alone). Since the third test day score in enadoline-trained rats was less than the first day score in fentanyl-trained rats, the results suggest an interference of conditioned place-preference influences of fentanyl by combined treatment with enadoline.

The molecular basis for the place-conditioning interactions of these drugs described here probably involves opposite effects of mu and kappa opioid agonists on mesolimbic-mesocortical dopaminergic systems (see Bals-Kubic et al., 1993; Devine et al., 1993), which would be consistent with other evidence relating to rewarding and aversive effects of drugs (Spyraki et al., 1982; Stolerman, et al., 1985; Wise, 1988, 1989). Since levels of locomotor responses are often correlated with rewarding/aversive activity of drugs (Rech, 1968; Tilson and Rech, 1973; Heath and Rech, 1985;Wise, 1988, 1989), the number of alley entrances per 10 minute session for the various treatments was measured as a reflection of response level. In fentanyl-trained rats, a higher level of responding was noted in fentanyl-associated alleys than in saline associated alleys and in comparison to saline-trained rats. Furthermore, the higher level of responding decreased significantly after the third phase of training in these subjects with the drug combination. Enadoline-trained rats showed no significant difference between the number of saline- and endaoline-associated alleys in comparison to each other and those of saline-trained rats. After training with the drug combination in enadoline-trained rats, the level of responding for drug-associated alleys on the third test day (Table 2) did not differ from that of enadoline alone (second test day). It is clear that these results on response levels are very well correlated with those of place conditioning patterns and yield another index of the rewarding influence of fentanyl as well as the attenuating effects of combining enadoline with fentanyl.

CHAPTER 8

ANTINOCICEPTIVE POTENTIAL OF AN OXYMORPHONE-BUTORPHANOL COMBINATION IN CATS IN THE COLORECTAL DISTENSION ASSAY.

Summary

Administration of a mu opioid agonist (oxymorphone) and a mixed kappa opioid agonist (butorphanol) may produce additive, synergistic, or antagonistic antinociception. To determine antinociceptive effects, using the colorectal distension assay as a model of visceral nociception, 7 cats were blindly administered individual and combined intravenous (IV) doses of each agent. Results showed that combined doses of 0.05 and 0.10 mg/kg of each agent demonstrated synergistic antinociceptive interactions. Further study of the 0.05 mg/kg dose (0.10 mg/kg total drug) included testing various ratios of oxymorphone and butorphanol (1:1, 1:2, 1:3, 2:1, 3:1). Statistical tests showed that all of the various ratio combinations produced levels of antinociception that were not significantly different. These results indicate that oxymorpohone and butorphanol given together produce additive and synergistic antinociception. In addition, acepromazine (ACE), a phenothiazine-tranquilizer, was tested to determine if it could increase the level of antinociception when administered in combination with oxymorphone and butorphanol. Results showed that ACE was without effect when tested alone. However, ACE significantly increased the magnitude and duration of antinociception when administered with oxymorphone and butorphanol as compared to the combination of oxymorphone and butorphanol. Furthermore, physiological parameters including respiratory rate, mean arterial pressure, and pulse rate were unaffected by these drug combinations. We conclude that oxymorphone and butorphanol in combination produced additive and synergistic levels of antinociception, and that ACE in conjunction with oxymorphone and

butorphanol produced even greater and longer levels of antinociception than the two-drug combination.

Introduction

Oxymorphone is a commonly used analgesic, but it has limited use in cats at doses exceeding 0.1 mg/kg due to their hypersensitivity to mu opioids (Short, 1987). Butorphanol is also a commonly used analgesic, but its effectiveness is dose-limited. Butorphanol is efficacious as an analgesic, but its peak antinociception plateaued at approximately 50% to 80% of the level of a mu agonist (unpublished observation). Thus, increasing doses of butorphanol do not necessarily provide increased levels of antinociception.

In comparison to morphine antinociception (standard analgesic effect to which most agonists are compared), oxymorphone, a mu agonist, has been shown to be ten times more potent than morphine (Jaffe and Martin, 1990). Butorphanol, an agonist-antagonist, was shown to be 17 times more potent than morphine (Pircio et al., 1976; Martin, 1984). Characterization of oxymorphone as a mu agonist has been well accepted, but attempts to identify butorphanol-mediated effects have been difficult. More recent reports characterized butorphanol as a mu agonist and a kappa agonist with intermediate efficacies at both receptors (mu: Shannon and Holtzman, 1977; Zimmerman et al., 1987; Schaefer and Holtzman, 1981; White and Holtzman, 1983; Picker et al., 1989; kappa: Pircio et al., 1976; Leander, 1982; 1983; Woods and Gmerek, 1985; Leander et al., 1987; Picker et al., 1990). However, pharmacological profiles of butorphanol seem to depend on the species and on the assays used in testing since butorphanol also produced antagonistic actions at

mu and kappa receptors (Martin, 1979; Young and Woods, 1982; Leander, 1983; France and Woods, 1985; Negus et al., 1989; Picker et al., 1990). Although butorphanol has shown itself to be a difficult opioid to clearly characterize, its ability to relieve moderate to severe pain has been well documented (Horan and Ho, 1989; Short, 1987).

As was mentioned earlier, species differences play a significant role in pharmacological profiles of opioids. The cat is notorious for displaying hyperexcitability in response to mu agonists. Thus, an analgesic remedy for moderate to severe pain in the cat is a confounding problem: increased pain and suffering require either more potent drugs or increased doses; both scenarios increase behavioral hyperexcitabiliy in cats. Usually oxymorphone or butorphanol are used as analgesics for moderate to severe pain in cats (Short, 1987). In Europe buprenorphine is also used as an analgesic for cats, drud that is an agonist-antagonist similar to butorphanol in that receptor-mediated effects have not been clearly identified (Cowan et al., 1977; Dum and Herz, 1981; Sadée et al., 1982). Inasmuch as oxymorphone and butorphanol may provide adequate pain relief, we believe there may be a more effective application of these opioids in cats. For instance, preliminary results in our laboratory showed that buprenorphine or oxymorphone in combination with butorphanol produced synergistic levels of antinociception as measured by the colorecetal distension assay in rats. If oxymorphone and butorphanol produce at least additive levels of antinociception, then side effects of each drug may be reduced since lower doses of each agonist would be used. Furthermore, mu and kappa opioid agonists have "opposite" side effects (ie., respiratory depression vs. minimal changes; urinary retention vs. diuresis; constipation vs. increased motility) (Short, 1987). There is a

possibility that opposing side effects of each drug may create a reciprocal reduction in total side effects. Thus, to characterize antinociceptive interactions of butorphanol and oxymorphone, the colorectal distension assay was used as a model of moderate visceral nociceptive stimulus in cats. Certain physiological parameters were also recorded.

Materials and Methods

Subjects

Four male and four female neutered adult cats of mixed breed weighing a mean of 4.36 kg were used in these studies, which were approved by the All-University Committee on Animal Use and Care. Subjects were housed in a temperature and humidity controlled room with food and water provided ad libitum, except for a 12-hour fast before a study. Each animal was socialized and trained to become accustomed to the noninvasive monitoring devices.

Instrumentaion

Before studies, cats were instrumented with a specially designed silastic balloon catheter (Cook Veterinary Products, Spencer, IN) lubricated with (K-Y® Jelly, Skillman, NJ) and inserted per rectum. The open end of the balloon catheter was connected with rubber tubing to a one gallon plastic jug pressurized to selected pressures (mmHg). Nociceptive thresholds were determined by inflating the colonic balloon catheter for 30 second periods. Deflation of the balloon catheter was accomplished by releasing pressurized air out through a three-way stopcock. When inflated at threshold volumes, the balloon catheter exerted pressure (measured in mmHg) on the visceral mucosa, which induced a minimum level of discomfort due to distention of the gut lumen. Behavioral

changes indicative of discomfort included stretching of hind limbs, contracting abdominal muscles, arching of back, or breathing pattern changes.

Respiratory rates were recorded by a pneumotachygraph using a water filled Disposa-cuff (Neonatal #2 or #3, Critikon, Tampa, FL) sewn to velcro strips making a "belt" which was fitted around the cats rib cage. The Disposa-cuff was connected to a plethysmograph (Grass Instruments, Inc., Qunicy, MA) which recorded changes in volume (gross movements). The belt did not restrict normal respiratory movements and facilitated recordings of both frequency and amplitude of breathing patterns.

A Dynamap Veterinary Blood Pressure monitor (Critikon 8300) was connected to the forelimb using a Disposa-cuff (Neonatal #2 or #3). This device recorded systolic, diastolic, and mean arterial blood pressure and pulse rate. Other behavioral observations were recorded by a blinded observer.

Protocol

Studies were conducted five days per week with resting periods of 6 to 7 days between studies for individual cats. In preparation for a study, cats were instrumented with the Disposa-cuff belt, blood pressure cuff and colonic balloon. A command was given to lie on the table in lateral/sternal recumbancy. Control measures were taken for blood pressure, pulse rate, respiratory rate, and control (threshold) responses to balloon inflation were also determined. Threshold responses were verified at least two times to establish predrug control levels.

Data Analysis

For graphic representation and data analysis, antinociceptive data were expressed as the pressure difference (mmHg) between pre- and post-drug threshold responses. Analysis of antinociceptive responses of combination doses in comparison to theoretical additive sums of individual responses was accomplished by using the Z table (Steel and Torrie, 1984). First, to calculate theoretical additive sums of individual responses, the mean pressure differences were summed. The SEM of the theoretical sum was calculated by using the root mean square of the individual SEM's. Finally, the absolute difference between the theoretical and mean of the actual response was divided by the root mean square of the theoretical and actual SEM's. The calculated number was then compared to values on the Z table. Values corresponding in the table at $p < 0.05$ indicated significant deviation from additivity.

Results

Individual Antinociceptive Effects

Individual doses of oxymorphone (0.025, 0.05, 0.10 mg/kg IV) produced dose-dependent increases in threshold responses (Figure 24). Higher doses of oxymorphone produced behavioral excitability which confounded nociceptive threshold measurements. Individual doses of butorphanol (0.025, 0.05, 0.10, 0.20 mg/kg IV) also produced dose-dependent increases in threshold responses (Figure 24).

Antinociceptive Effects of Combinations

The combination doses of oxymorphone and butorphanol (0.05 and 0.1 mg/kg each IV) produced synergistic antinociception compared to the additive sum of their individual doses. The combination dose of oxymorphone and butorphanol (0.2 mg/kg

each, IV) produced additive antinociception compared to the additive sum of their individual doses (Figure 25).

Further testing of various ratios (1:1, 1:2, 1:3, 2:1, 3:1) of the combination of oxymorphone and butorphanol (total drug 0.1 mg/kg IV) showed that all ratio combinations produced levels of antinociception that were not significantly different, $p < 0.05$.

Acepromazine (0.05 mg/kg IV) was also tested in the CRD and demonstrated no antinociception (Figure 26). However, when the same dose of ACE was administered with the combination of oxymorphone and butorphanol (0.05 mg/kg each IV), antinociceptive levels were significantly greater (Figure 26) and lasted longer (data not shown) than those of oxymorphone and butorphanol (0.05 mg/kg each IV), $p < 0.05$.

Physiological Parameters

Respiratory rates, pulse rates, and MAP as measured for 60 minutes were not significantly affected by individual doses or combination doses of oxymorphone or butorphanol as compared to saline controls, $p < 0.05$. Furthermore, ACE and the combination of ACE plus oxymorphone and butorphanol as measured for 60 minutes did not affect respiratory rates, pulse rates or MAP, $p < 0.05$.

Based on subjectively graded observations, cat behaviors did not seem to be affected by any drug or dose as compared to saline controls. During studies, cats were described as calm, playful, and affectionate (with the exception of oxymorphone 0.2 mg/kg IV, which produced such excitability in two cats that other parameters could not be measured [data not shown]). In addition, subjective sedation scores (0 = no sedation, 1 =

slight, 2 = moderate, 3 = marked) were used to describe the level of observed sedation. Throughout all studies, sedation was given a score of less than 2, regardless of drug or dose.

Other behaviors noted included salivation and pupil dilation. At no time in any study were cats observed salivating. However, pupillary dilation was observed with all drugs and doses, but not in saline controls.

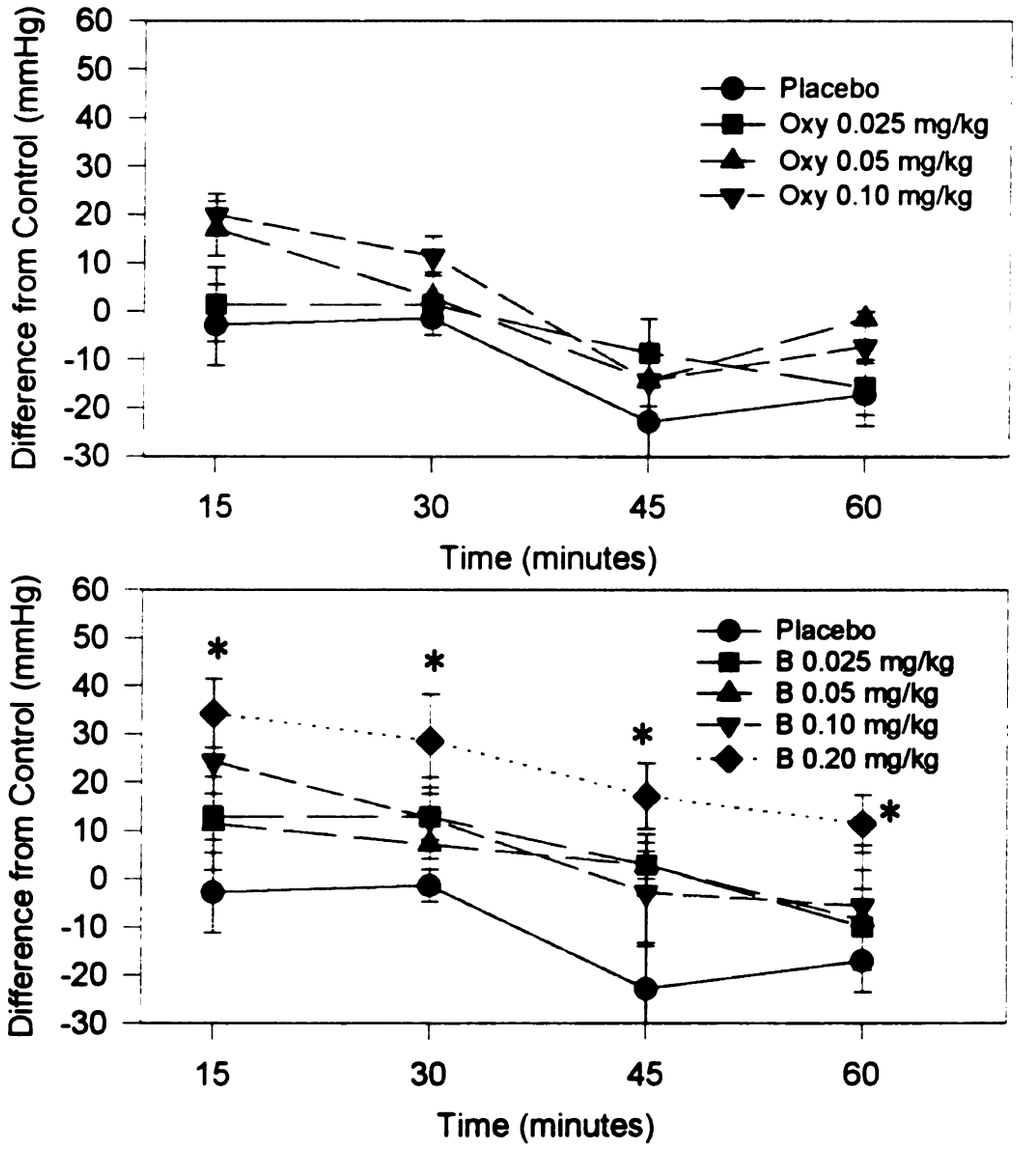

Figure 24. Mean (± SEM) of the pressure difference between pre- and post-drug nociceptive thresholds for oxymorphone and butorphanol.
Asterisk indicates significant level of antinociception, $p < 0.05$.

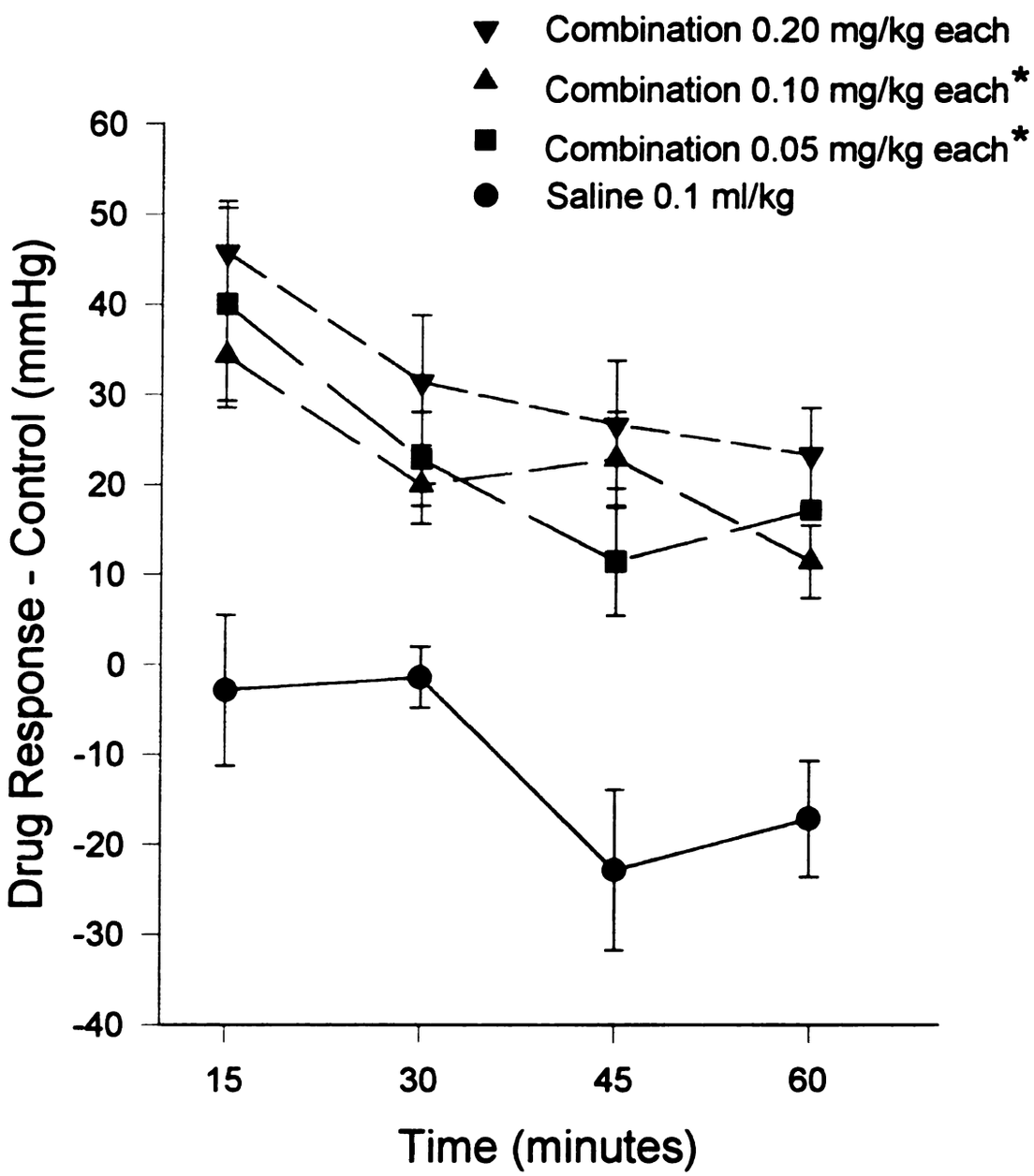

Figure 25. Mean (± SEM) of the pressure difference between pre- and post-drug nociceptive thresholds for oxymorphone-butorphanol combinations.
All drug combinations produced significant antinociception, $p < 0.05$. An asterisk beside a combination dose indicates synergistic antinociception as compared to the sum of individual dose effects, $p < 0.05$.

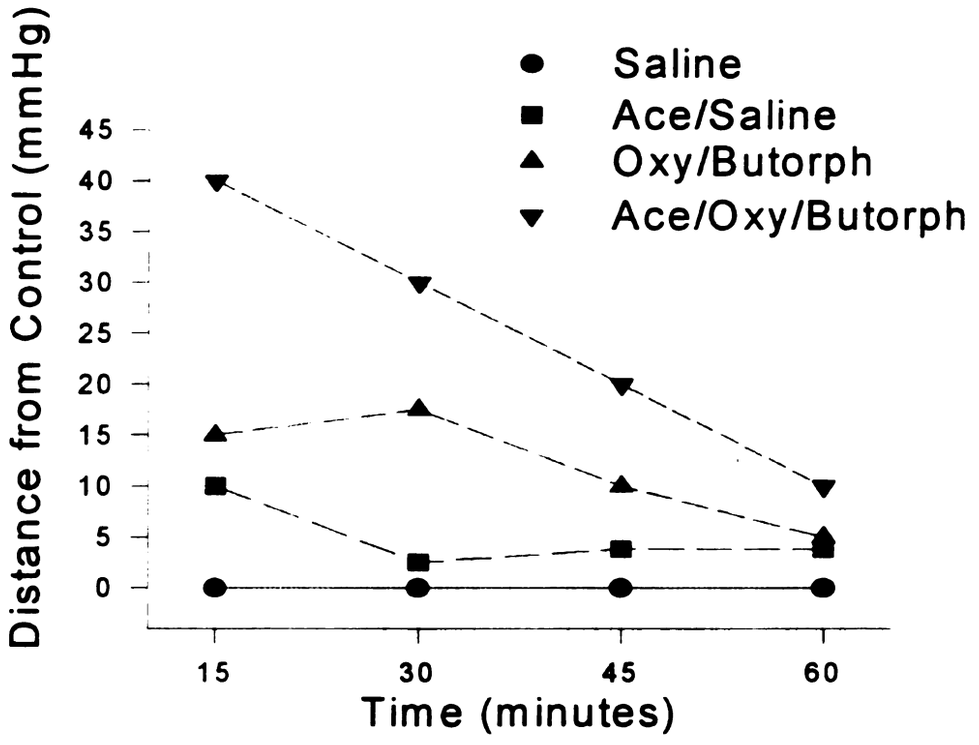

Figure 26. Pressure difference between pre- and post-drug nociceptive thresholds for saline, ACE-saline, Oxymorphone-Butorphanol, and ACE-Oxymorphone-Butorphanol. Mean (± SEM) of the pressure difference between pre- and post-drug nociceptive thresholds for IV doses of saline, ACE (0.05 mg/kg)-saline, oxymorphone-butorphanol (0.05 mg/kg each), and ACE (0.05 mg/kg)-oxymorphone-butorphanol combination (0.05 mg/kg each). Asterisk indicates significant antinociception, $p < 0.05$.

Discussion

Oxymorphone and butorphanol in combination produced superior levels of antinociception without causing significant changes in physiological parameters. Furthermore, the use of a neuroleptic, such as acepromazine, significantly enhanced the magnitude and duration of antinociception of the oxymorphone-butorphanol combination. Also, physiological parameters were not significantly affected by the addition of ACE to the oxymorphone-butorphanol combination.

Regarding drug ratios in combination, results indicated that the dose ratios tested were similar to the 1:1 ratio combination. Although these data did not show any differences, other ratio combinations not yet tested may produce significant changes in level of antinociception.

In summary, it was found that oxymorphone and butorphanol are especially effective as an analgesic combination against visceral pain and that ACE significantly enhances their analgesic effects. Furthermore, we conclude that oxymorphone, butorphanol, and ACE as a combined pain remedy are relatively safe in 1) that their combination produced minimal to moderate sedation (no hyperexcitability), and 2) they did not significantly affect respiratory rates, pulse rates, or MAP.

CHAPTER 9

FINAL SUMMARY AND DISCUSSION

Colorectal Distension as a Model of Visceral Nociception

Before antinociceptive studies of opioid combinations could begin, there were two assumptions that required testing. The first assumption was that the CRD assay was an appropriate visceral nociceptive assay useful for testing opioids. Subjects treated with a mu opioid, oxymorphone, demonstrated maximal levels of analgesia while motility and pressure recordings (tone of the colon) in these subjects were similar to saline- or atropine-treated rats (Figure 1 and Table 1). Thus, the CRD assay proved to be an effective means of testing opioid-induced visceral antinociception.

Selectivity of Spiradoline as a Kappa Opioid Agonist

The second assumption requiring testing was that spiradoline was a selective kappa opioid agonist. Results from studies using antagonists naloxone, β–FNA, and nor-BNI (Figures 3 and 6), methadone-tolerant rats (Figure 4), and spiradoline enantiomers (Figure 5) indicated that spiradoline-induced antinociception in the CWTF was mediated by kappa receptors but not mu receptors. In contrast, antinociceptive effects of spiradoline and its enantiomers (Figures 10) in CRD demonstrated selective kappa receptor activity when tested in naloxone-treated (Figure 8) or methadone-tolerant rats (Figure 9), but studies using β–FNA or nor-BNI pretreated rats were less clear (Figure 11). Antagonism of antinociceptive effects in the CRD indicated that the β–FNA and nor-BNI did not demonstrate selectivity for their respective receptors. The antagonist pretreatment for CRD was completed in the same manner as in the CWTF and as reported in the literature. In addition, the same agonists tested in the CRD were selectively antagonized in the CWTF. Thus, the non-selective antagonism of the antagonists was suggested to be due to

mu and kappa receptor interactions in nociceptive pathways of the CRD. Although the antagonists did not demonstrate selectivity in the CRD, spiradoline-induced antinociception was concluded to be mediated via kappa receptor activation in the CRD based on data from naloxone antagonism, methadone-tolerant rats, and the enantiomers. Therefore, spiradoline-induced antinociception in the CRD and CWTF was concluded to be mediated via kappa receptor activation.

Mechanisms of Opioid Combinations

After the two assumptions were tested, antinociceptive testing of opioid combinations began. In the rat, all agonists individually produced maximal levels of antinociception when tested in the CRD (visceral nociceptive model) and CWTF (cutaneous nociceptive model). However, opioid combinations in the CRD and CWTF differed dramatically. Combinations in the CRD produced additive and synergistic antinociceptive interactions. In contrast, combinations in the CWTF produced dose-dependent antinociceptive interactions. Stated more specifically, relatively lower doses of opioid combinations produced additive (and at one time point synergistic) antinociceptive interactions, whereas, relatively higher doses of combinations produced sub-additive and antagonistic interactions.

In the case of the observed synergy in the CRD, the most likely mechanisms involved are interactions between the individual mu and kappa receptors. The receptors have been found on most if not all nociceptive neurons (Atweh and Kuhar, 1977; Fields et al., 1980; Slater and Patel 1983; Allerton et al., 1989). Thus, opioid receptors can have effects on A-δ and C fibers in the periphery, on the dorsal root ganglia and synapses, on

interneurons in the dorsal horn, on projection neurons, in supraspinal centers including the PAG, PVG, and raphe nuclei, and on the descending serotonergic pathways terminating in the dorsal horn. Synergistic interactions of mu and kappa opioids could be due to the fact that mu and kappa receptors utilize different ionic channels and both types of channels function to reduce total neurotransmitter release. As an example, recent evidence demonstrated a reduction in substance P release which required simultaneous co-activation of mu and kappa receptors (Collin et al., 1992). Although mechanisms of the combined effects of mu and kappa opioids have not been defined, individual actions may give clues as to possible interactions. Mu receptors increase potassium ion conductance which hyperpolarizes the nociceptive membrane potential. A steady hyperpolarization of the membrane reduces cellular receptivity to excitatory input by removing the inactivation of fast transient voltage-gated potassium channels (Jahnsen and Llinás, 1982). Consequently depolarization is more difficult resulting in a decreased propagation and duration of action potentials (Werz and Macdonald, 1982; Frank, 1985; Russell et al., 1987). Thus, neurotransmitter release is less feasible. Kappa receptors also function to reduce nociceptive activity by preventing neuronal release of excitatory neurotransmitters (algesic agents) from central and/or peripheral primary afferent endings (Lembeck and Donnerer, 1985; Yaksh, 1988). Mechanisms related to this effect are most likely due to presynaptic depression of voltage-sensitive Ca-conductance of the N-type (Gross and Macdonald, 1987). This action decreases calcium ion flow into the cell. Additionally, kappa receptors have been proposed to decrease postsynaptic actions of excitatory neurotransmitters (glutamate), but these mechanisms are not fully understood (Kolaj et al.,

1995). That is, kappa receptor activity may bias glutamate receptor conformation to reduce activation, may interfere in some manner with glutamate second-messenger systems, or may act in some independent way to dampen excitability of glutamate-receptor neurons. Since mu and kappa receptor activity decrease neurotransmitter release via separate mechanisms, it is possible that the combined ionic changes intracellularly and those affecting the membrane potential and would render the neuron virtually unresponsive to nociceptive impulses.

It is not yet possible to thoroughly define opioid receptor interactions, but data from these studies give evidence that mu and kappa receptors are mutually sensitive to each other's activity. For instance, when a selective mu antagonist, β-FNA, was present the analgesic effects of both fentanyl and spiradoline and their combination in CRD were reduced. In addition, when a selective kappa antagonist, nor-NBI, was present the analgesic effects of fentanyl and spiradoline and their combination were again decreased. Assuming that the antagonists were selective, these data indicate that analgesic effects mediated by mu receptors are affected by antagonism of kappa receptors. Additionally, analgesic effects mediated by kappa receptors were also affected by antagonism of mu receptors. Furthermore, the opioid combination was partially antagonized by β-FNA and nor-NBI. This taken together with the observation of synergy in the CRD suggests that mu and kappa receptors may be coupled or intimately linked in some manner in which they are capable of enhancing each other's analgesic effect. Although there are examples of receptor coupling between mu and delta opioid receptors (Werz and Macdonald, 1983a,b; Holaday et al, 1991) and there are some studies suggesting mu and kappa receptor

coupling, that latter studies do not clearly demonstrate this action (Holaday et al, 1991). In addition to the possibility of receptor cooperativity, downstream actions of each receptor may enhance each other's antinociceptive effects. Both mu and kappa receptor activity involve G_i or G_o G-proteins, adenylate cyclase, phosphorylation, and many changes in ionic conductance. Recent evidence suggests that changes in downstream actions involving Ca^{++} concentrations, second messenger systems and protein kinases can produce prolonged changes in membrane excitability (Kolaj et al., 1995).

In the case with data from the CWTF, individual mu and kappa opioid agonists produced maximal levels of antinociception (Figure 2). These results indicate that either mu or kappa receptor activity attenuates nociceptive activity induced by the CWTF. Individual mechanisms of these antinociceptive effects may be similar to those previously mentioned for CRD antinociception. In addition to results of individual agonists, pretreatment with either mu or kappa antagonists selectively reduced antinociceptive effects of mu and kappa opioids respectively (Figure 6). These results also demonstrate that either mu or kappa receptor activity reduces nociceptive input from the CWTF stimulus. In contrast, combined mu and kappa receptor activity did not reduce nociceptive input from the CWTF. Although combination doses in relatively lower quantities produced at least additive interactions (Figures 15 and 16), in higher quantities, combination doses produced sub-additive and antagonistic interactions (Figures 15 and 16). These observed interfering interactions of mu and kappa receptors in CWTF antinociception could be related to characteristics of nociceptive neurons.

As explained earlier, opioid combinations produced additive and synergistic interactions in the CRD, whereas in the CWTF, opioid combinations in higher doses produced less than additive interactions. This observation is interesting in that the same agonists individually produced similar results but agonists in combination produced seemingly opposite effects. Although the CRD and CWTF are similar in that they produce nociception, they produce different types of nociception: CRD is visceral and CWTF is somatic. This important difference has many ramifications. First, nociceptors responsive to distension or stretch may produce profoundly different spike train potentials than a nociceptor responsive to thermal changes at -10 C. These spike patterns may have varying frequencies and amplitudes, thus receptor actions may have varying effects on the propagation of the signal and the specific neuronal mechanisms of excitability.

Second, nociceptive neurons (A-δ and C fibers) of the two nociceptors (distension vs. thermal) may be qualitatively different. For instance, there are variations in the type and density of ion channels in cells throughout the nervous system. Also, there are differences in the distribution of channel types within individual cells. For example, in some neurons, continuous hyperpolarization of the membrane makes the cell more excitable. Hyperpolarization removes the inactivation of some voltage-gated calcium channels (Koester, 1991). Coincidentally, mu receptor activation produces hyperpolarization and kappa receptor activation inhibits inward calcium ion flow through channels. This coincidence may partially explain the dose-dependent antinociceptive interactions. Antinociception may be accomplished through mu receptors by hyperpolarizing membranes even though some voltage-gated calcium channels are

inactivated because the cell membrane is hyperpolarized and less likely to depolarize and propagate action potentials. So, even though calcium ions are permitted into the cell (with the chance of facilitating neurotransmitter release), the membrane is already hyperpolarized. Thus, there may be no action potentials to excite the neuron to release neurotransmitters. Antinociception produced by kappa opioids is achieved by decreasing Ca^{++} flow or inward current into the cell. This action decreases the probability of action potential propagation and prevents release of excitatory neurotransmitters. Even if an action potential were generated in a nociceptive neuron, kappa receptor activity would prevent further propagation of the stimulus by preventing presynaptic release of neurotransmitters. Although as mu and kappa receptor activity individually attenuate nociception, their combined effect in certain types of nociceptive neurons may cause interference. For example, it may be possible for kappa receptor activity at calcium channels to in some way disrupt the hyperpolarized "inhibitory balance" induced by mu receptors. Studies have shown that changes in calcium concentrations have profound effects on morphine (mu) induced antinociception; increased calcium concentrations reduce antinociceptive effects (Harris et al., 1976; Sanghvi and Gershon, 1977; Illés et al., 1980). In this case, kappa receptor activity would seem to "interfere" with the antinociceptive effects of mu opioids. It is possible that nociceptors and nociceptive neurons excited by the CWTF respond to hyperpolarization as described. In the case of lower doses of mu opioids, hyperpolarization may be lessened, thus causing less inactivation of voltage-gated calcium channels. In these circumstances, kappa receptor activity may be able to elicit its response without interfering with mu related activity. In

contrast, higher doses of mu opioids may produce greater levels of hyperpolarization and in turn produce more inactivation of voltage-gated calcium channels. In this situation, kappa receptor activity could be either significantly compromised or altered in a way that would interfere with the mu receptor induced ionic changes.

At this point, data from these studies support the hypothesis that simultaneous kappa receptor activity affects mu receptor induced antinociception. Data from methadone-tolerant rats indicated that by minimizing available mu receptors (by producing tolerance to mu receptors) the combination effect produced an additive interaction (Figure 17). However, the combination effect in methadone-tolerant rats was less than that in non-tolerant rats, even though the combination in non-tolerant rats was sub-additive (Figure 15). Thus, without available mu receptors, the combination effect was additive but also was reduced in comparison to the effect in control animals. It could be that in methadone-tolerant animals, mu receptor induced hyperpolarization was reduced (due to development of tolerance relating to mu receptors). In this case, there would be less kappa and mu receptor interactions since mu receptors had been down regulated. In conclusion, from this rationale, it seems that incremental, equipotent doses of mu and kappa opioids are antagonistic or sub-additive at least for certain antinociceptive mechanisms. In contrast, lower doses or doses in unequal ratios of mu and kappa opioids produce additive interactions since there is less concomitant activity of mu and kappa receptors; an effect similar to that observed with the lower doses of mu and kappa opioids in the CWTF.

In addition to the CWTF data from the methadone study, results from the selective antagonists indicated mu receptors contribute more to the combination effect than kappa receptors (Figure 18). Similar to the methadone study, when the number of available mu receptors were decreased, the combination effect was decreased in comparison to its effect in non-tolerant animals. In contrast, when kappa receptors were antagonized, the combination effect remained unchanged. These data support the hypothesis that although kappa agonists are efficacious alone, they disrupt the hyperpolarized "inhibitory balance" mediated by mu receptor activity.

In addition to neuronal differences between nociceptive models (CRD vs. CWTF), there are also differences in responses to a stimulus depending on the intensity or duration of the stimulus. The CWTF stimulus of -10° C produced nociceptive information that was distinct from other temperatures. As mentioned earlier, more C fibers are activated than A-δ fibers by thermal stimuli above approximately 30° C and below 0° C, whereas C fibers and A-δ fibers are activated by temperatures between 30° and 45° C and below 0° C. Coincidentally, mu opioid agonists are effective in producing antinociception at temperatures associated with C fiber activity and kappa opioid agonists are effective at temperatures associated with A-δ fiber activity. Thus it seems that although mu and kappa receptors have both been found presynaptically on primary afferents (C and A-δ fibers), there could be higher proportions of mu receptors on C fibers and kappa receptors on A-δ fibers. This hypothesis is supported by *in vitro* studies of Werz et al. (1987) which showed that the distribution of receptor types among neurons was variable. The data indicate that mu or kappa receptors individually attenuate nociceptive transmission, but in

combination, especially at higher doses, their interactions interfere with each other. The interference observed with higher doses could be related to differences in C and A-δ fiber activity and the relative mu and kappa receptor populations of each fiber.

The hypothesis of kappa receptor activity interfering with mu receptor mediated antinociception may be more applicable to C fibers than A-δ fibers for the CWTF model. Support for this proposed hypothesis comes from studies that demonstrated kappa receptor interference in situations where kappa opioids produced no antinociception. Previous experiments showed that dynorphin (endogenous kappa ligand) produced no antinociception in response to "hot" thermal stimuli; this effect was likely due to the lack of A-δ fiber activity, thus kappa opioids would have no effect. However, morphine produced significant analgesia that was reduced by the addition of dynorphin. This antagonistic interaction was concluded to be mediated by kappa receptors interfering with mu receptors (Schmauss and Herz, 1987; Song and Takemori, 1991). The situation described above is similar to results from the CWTF in that kappa opioid activity seemed to interfere with mu activity, but with the CWTF (-10 C), kappa agonists produced analgesia; kappa mediated antinociception is most likely mediated through A-δ fibers. Based on the previous discussion of hyperpolarization and the CWTF, kappa mediated interference most likely affected activity in C fibers which seem to have a closer association with mu opioids than kappa opioids. During a state of hyperpolarization in a C fiber, functions of kappa receptors could be signficantly changed since the voltage gated calcium channels are inactivated. Interference of mu opioid antinociception could be due to kappa receptor mediated ionic changes that are incompatible with mu receptor

activity. From these observations, kappa receptors are capable of interfering with mu receptor activity regardless of their own antinociceptive effect.

Besides A-δ and C fibers, individual and combined antinociceptive effects of mu and kappa receptors may produce their effects at many other sites. Receptors have been shown to exist throughout the nervous system. These sites include the periphery (C and A-δ fibers--as previously discussed in the case of the CWTF) or they may occur in the spinal cord or in supra-spinal centers. In the spinal cord interactions could occur at the first synapse of the A-δ and C fiber in lamina I or throughout synaptic junctions located in the lamina. At synaptic junctions, the release of neurotransmitters (e.g., an endogenous peptide, substance P) may be presynaptically reduced by opioid activity. Interference in the dorsal horn between mu and kappa opioid receptors may decrease their individual ability to minimize the amount and frequency of neurotransmitters released at synapses in the dorsal horn.

From the dorsal horn, projection neurons carry nociceptive impulses in spinothalamic pathways toward either the thalamus or reticular formation of the brainstem which activate neurons in the periventricular grey (PVG) and periaqueductal grey (PAG). From the PVG and PAG, information continues toward the raphe nuclei which sends serotonergic fibers back down through the spinal cord to the dorsal horn where they synapse with projection neurons and incoming nociceptive primary afferents. This serotonergic descending pathway acts to reduce nociceptive impulses from further propagation. Throughout these neuronal pathways, opioid induced antinociceptive modulation may occur in spinothalamic pathways as they ascend, but opioids produce

more profound effects at supra-spinal centers such as the PVG, PAG, raphe nuclei and the descending bulbospinal inhibitory pathway. Thus at sites where mu and kappa receptors are prevelant, their combined activity may produce more interference in CWTF antinociception.

Motivational Effects of Fentanyl and Enadoline

Further studies of mu and kappa opioids included testing their individual and combined motivational effects on behavior in the X-maze. Fentanyl (mu opioid-euphoric) produced significant place conditioning, whereas enadoline (kappa opioid-dysphoric) produced minimal or no place conditioning. Interestingly, the combination of fentanyl-enadoline significantly decreased the level of place conditioning in rats previously dosed with fentanyl (which had demonstrated significant place conditioning). The combination of fentanyl-enadoline did not alter previous place conditioning effects of rats dosed with only enadoline. A second indicator of motivational effects on behavior is the activity level of a subject. Rats dosed with fentanyl entered significantly more alleys than those dosed with enadoline or the combination of fentanyl-enadoline. This result indicated that the combination dose reduced the activity level associated with fentanyl alone. Thus, based on these results from the X-maze, the combination of a mu-kappa opioid (fentanyl-enadoline) has less motivational effects than a mu opioid alone. These results support the hypothesis of neurochemical interactions of mu and kappa opioids on motivation. Rewarding effects have been associated with mu receptor activation in the ventral tegmental area of the brain, which is the origin of the mesolimbic-mesocortical dopamine systems (Bozarth, 1986; Wise, 1989). Aversive influences have been related to kappa

receptors in VTA and limbic-cortical terminals of cell bodies having their origin in VTA (Pfeiffer et al., 1986; Bals-Kubik et al., 1993; Narita et al., 1993) and on dopaminergic nerve terminals in the nucleus accumbens which decrease dopamine release (Spanagel et al., 1992). Results from these studies coupled with the present results strongly indicate that motivational interactions between mu and kappa opioids may attenuate the positively reinforcing effects of mu opioids, thus reducing the abuse potential associated with narcotics (which are usually mu opioids).

Application of Opioid Combinations in Cats

Additional studies of opioid combinations in cats in CRD revealed similar findings to those of rats in CRD. Oxymorphone and butorphanol produced additive and synergistic antinociceptive interactions (Figure 25). Also, this opioid combination produced minimal changes in physiological parameters such as respiratory rate, pulse rate, and mean arterial pressure. Had similar levels of analgesia been attempted using individual doses of either oxymorphone or butorphanol, side effects associated with each drug would have precipitated unacceptable changes in physiological and behavioral variables. The onset of these untoward side effects would require a reduction in dose of drug, thus analgesia would be comprimised. Results from these studies indicated that opioid combinations produce superior levels of antinociception without troublesome side effects. Since side effects of the combination are minimized, greater levels of analgesia may be achieved.

In addition to the use of opioids for pain relief, neuroleptics are often used in conjunction with opioids to enhance analgesia. Neuroleptics decrease anxiety and have

been shown to be beneficial in pain therapy (Woolf, 1983; Pascoe, 1992). In the current study, acepromazine tested alone produced no analgesic effect, whereas when added to the opioid combination, acepromazine significantly enhanced peak analgesic effect and duration of effect (Figure 26). These results are in agreement with those previously mentioned. The mechanisms involved with these effects remain unclear, but neuroleptics have been proposed to enhance opioid analgesia in two ways. Neuroleptics have been shown to block dopamine auto-receptors (D2 receptors) and thus decrease negative feedback onto the neuron, thus increasing dopamine release (Burt et al., 1976). Also, chronic dosing with neuroleptics markedly increased methionine-enkephalin within the brain (Hong et al., 1978). Although this study in the cat did not elucidate mechanisms related to neuroleptic-enhanced opioid analgesia, results indicate that the CRD is an appropriate model to further study effects of neuroleptics on opioid induced analgesia.

Future Directions

Results of this thesis showed that mu and kappa opioids can be used in a manner that decreases addiction liability, produces minimal changes in physiological variables and most importantly, provides superior relief from visceral pain relative to either single agent alone at similar doses. These new advancements of opioid use in pain management have immediate application in some clinical situations. However, to fully utilize potential benefits of this opioid combination, receptor interactions and sites of their interactions should be investigated further. Experimental designs should include *in vitro* and *in vivo* preparations.

In-vitro studies should include measurement of ionic changes and membrane potentials of peripheral nociceptive neurons during individual and combined activation of mu and kappa receptors. Additionally, this experiment could be conducted with *in vitro* preparations pretreated with opioid antagonists, channel blockers, or inhibitors of second messenger systems. Assuming results of the first experiment demonstrate mu and kappa opioid interactions, the second approach could help identify whether interactions occur at the receptor, ionic channel, or with second messenger systems. A similar approach with these experiments could be conducted on neurons from the dorsal horn, spinal cord, supra-spinal connections, and descending serotonergic neurons. Experiments using *in-vitro* preparations from various sites could help elucidate areas where mu and kappa receptor interactions occur. Information of this type would identify key sites where opioid treatments would be most efficacious.

In vivo preparations should include electrophysiological measurements of visceral nociceptive neurons in the periphery, dorsal horn and spinal cord. These types of neurons have already been identified and characterized, but individual and combined effects of opioids have not been studied. Again, results from these types of experiments could elucidate specific sites where mu and kappa opioid interactions are most prominent. A second type of *in vivo* studies should include peripherally acting agents. Similar to the experiments in this thesis with naloxone, subjects could be pretreated with a peripherally acting antagonist, naloxone-hydrobromide. This approach would inhibit peripheral activity of mu and kappa opioid receptors. Thus, activity and interactions of mu and kappa receptors would be limited to areas in the spinal cord and in supra-spinal centers.

Additional approaches similar to this scheme could include intrathecal and/or intracerebroventricular injections. However, these invasive techniques, especially intrathecal injections of kappa opioids, produce inconsistent results. Thus, invasive techniques may not be as useful as other pharmacological approaches.

In addition to investigations of opioid combinations, experiments should also include protocols using neuroleptics. Results of a neuroleptic, acepromazine, in combination with opioids in the cat demonstrated significant increases in antinociceptive effects. As explained earlier, proposed mechanisms for this effect involve dopamine receptors (D2) and increased methioninie-enkephalin levels in the brain. *In vitro* or *in vivo* preparations pretreated with a D2 antagonist may help determine the role of dopamine with mu and or kappa receptor activity. It is possible that neuroloptics could affect mu and or kappa receptors individually or interactions of mu and kappa receptors. Although the proposed hypothesis of increased methionine-enkephalin levels is interesting, it is a difficult theory to continue studying since this phenomenon requires chronic manipulation. Thus, using D2 antagonists to investigate interaction of neuroleptics and opioids seems to be a more efficient strategy to determine mechanisms related to this combination effect.

In contrast to potential antinociceptive use of opioid combinations for visceral pain, results from the CWTF strongly indicate that opioid combinations are not efficacious for this type of nociception. This result provides more evidence confirming the notion that different types of pain have distinct mechanisms requiring specialized intervention. An important observation from results of opioid combinations in CRD and CWTF is that one pain therapy should not be used as a general remedy for all pains; the CWTF being a most

striking example. To further identify nociceptive mechanisms of the CWTF and opioid effects on these mechanisms would also require *in vivo* and *in vitro* experiments. *In vivo* electrophysiological recordings have been accomplished on nociceptive neurons excited by thermal stimuli. In addition to further characterization of these neurons, pharmacological protocols including mu and kappa opioids should be tested. Another *in vivo* preparation to employ is to pretreat subjects with naloxone-hyrdrobromide. This peripherally acting antagonist could help determine if mu and kappa opioid interactions occur in the periphery vs. the spinal cord or supraspinal centers. If mu and kappa opioid interactions were inhibited by naloxone hydrobromide, indicative of peripheral action, then the previously proposed hypothesis involving hyperpolarization of C fibers would be supported.

Further experiments involving *in vitro* preparations similar to those discussed for the CRD could help identify sites of interaction for the CWTF. However, temperature changes (stimulus intensity) must be carefully monitored during measurements of second messenger activity, ionic channel currents, and membrane potentials of thermal nociceptive fibers excited by the CWTF since different temperatures produce significantly different results. The use of opioid antagonists, channel blockers, and inhibitors of second messenger systems would serve as tools in determining if opioid combination interactions occur at the receptor, ion channel, or in second messenger cascade. Again, nociceptive neurons from the periphery, dorsal horn, spinal cord, supraspinal areas, and descending serotonergic neurons would be appropriate *in vitro* preparations.

In summary, experimental emphasis for the CRD and CWTF should be on the mechanisms involved and the location of opioid interactions. With answers to these

questions, pain management will be much more effective in that therapies could potentially be developed specifically for individual types of pain. And in cases of multiple types of pain, therapies can be developed which would provide pain relief for the various pains without producing antagonistic or "interfering" antinociceptive effects.

To gain an understanding of how these opioid combinations could have "opposite" analgesic effects in the CRD and CWTF, a review of mechanisms will be helpful. Mu and kappa opioid receptors mediate their effects by acting through voltage and or Ca^{++} dependent potassium channels and voltage-dependent Ca^{++} channels, respectively. Opioid induced ionic changes (potassium and calcium) decrease the excitability of nociceptive neurons and decrease action potential duration thus propagation of action potentials may be inhibited (Werz and Macdonald, 1982; Frank, 1985; Russell et al., 1987). In addition, ionic changes (calcium) can prevent neuronal release of excitatory neurotransmitters (algesic agents) from central and/or peripheral primary afferent endings (Lembeck and Donnerer, 1985; Yaksh, 1988). Mu and kappa opioid receptors have been found on peripheral, spinal, and supra-spinal neurons. Thus the previously described mechanisms occur throughout the neuronal networks of the body.

In addition to opioid receptor mechanisms, it is important to know that CRD and CWTF employ different types of nociceptive stimuli. For instance in the CWTF, the stimulus is a somatic type of pain, whereas the CRD is a visceral type. Furthermore, the CWTF stimulus of -10° C produced distinct nociceptive information from that of other temperatures. As mentioned earlier, more C fibers are activated than A-δ fibers by thermal stimuli above approximately 30° C and below 0° C, whereas C fibers and A-δ

fibers are activated by temperatures between 30° and 45° C and below 0° C. Coincidentally, mu opioid agonists are effective in producing antinociception at temperatures associated with C fiber activity and kappa opioid agonists are effective at temperatures associated with A-δ fiber activity. Thus it seems that although mu and kappa receptors have both been found presynaptically on primary afferents (C and A-δ fibers), there could be higher proportions of mu receptors on C fibers and kappa receptors on A-δ fibers. This hypothesis is supported by *in vitro* studies of Werz et al. (1987) which showed that the distribution of receptor types among neurons was variable. In addition to the differences associated with C and A-δ fibers, specific thermal coding patterns related to the frequency and modality of spike potentials may exist for various temperatures (Emmers, 1981). The data indicate that mu or kappa receptors individually attenuate nociceptive transmission, but in combination, especially at higher doses, their interactions interfere with each other. The interference observed with higher doses could be related to differences in C and A-δ fiber activity and the relative mu and kappa receptor populations of each fiber. Data from these studies on methadone-tolerant rats indicated that by minimizing available mu receptors, the combination effect produced an additive interaction. However, the combination effect in methadone-tolerant rats was less than that in control rats, even though the combination in control rats was sub-additive. Thus, without available mu receptors, the combination effect was additive but also was reduced in comparison to the effect in control animals. It could be that in methadone-tolerant animals, the combination dose produced an effect that was similar to a dose with a higher kappa:mu ratio. In this case, there would be less kappa and mu receptor interactions since

mu receptors were down regulated. In conclusion from this rational, it seems that incremental, equipotent doses of mu and kappa opioids are antagonistic or sub-additive. In contrast, lower doses or doses in unequal ratios of mu and kappa opioids produce additive interactions since there is less concomitant activity of mu and kappa receptors; an effect similar to that observed with the lower doses of mu and kappa opioids in the CWTF.

In addition to the data from the methadone study, results from the selective antagonists indicated mu receptors contribute more to the combination effect than kappa receptors. Similar to the methadone study, when the number of available mu receptors were decreased, the combination effect was decreased in comparison to its effect in control animals. In contrast, when kappa receptors were antagonized, the combination effect remained unchanged. These data indicate that although kappa agonists are efficacious alone, their contribution to the combination effect may be minimal. This observation in light of the fact that C and A-δ fiber activity is stimulus dependent and that mu and kappa mediated antinociception is also stimulus dependent indicates that nociceptive pathways associated with the CWTF may be specifically integrated in some fashion. Although mu or kappa opioids alone or low doses of mu and kappa opioids in combination may produce additive interactions, it seems that increased activity of kappa receptors or increased activity of both receptors interfere with antinociceptive interactions of the other.

Interference between mu and kappa receptors may be associated with receptors, second messenger systems, and/or ionic changes in nociceptive membrane potentials.

Receptor interactions could include conformational changes which may affect affinity or efficacy. (This effect may be possible if it is limited to nociceptive pathways associated with CWTF since the opioid combination in CRD was additive and synergistic.) Interactions may also occur with second messenger systems in that although the receptors are expressed independently it is possible for two different receptors to use a common channel, G-protein, or second messenger. Werz and Macdonald (1983a,b) have reported that delta receptors are coupled to mu receptors, but at this time, others et al have studied kappa and mu receptor coupling and have not shown evidence for this theory. Lastly, kappa receptor interference may be due to kappa mediated ionic changes that are incompatible for mu receptor activity. Although mu and kappa receptors have separate mechanisms, the effects of one receptor may interfere with the mechanism of the other receptor. Again, it is important to remember that all of these activities may be specifically related to those spike potential patterns in neurons excited by the CWTF stimulus. This is important since the opioid combination produced much different results in nociceptive pathways excited by the CRD stimulus.

These types of actions just described (receptor, second messenger, membrane potentials) can occur in the periphery (C and A-δ fibers) or they may occur in the spinal cord or in supra-spinal centers. In the spinal cord interactions could occur at the first synapse of the A-δ and C fiber in lamina I or throughout synaptic junctions located in the lamina. At synaptic junctions, the release of neurotransmitters (e.g., substance P, an endogenous peptide) may be presynaptically reduced by opioid activity. Interference in the dorsal horn between mu and kappa opioid receptors may decrease their individual

ability to minimize the amount and frequency of neurotransmitters released at synapses in the dorsal horn.

From the dorsal horn, projection neurons carry nociceptive impulses in spinothalamic pathways toward either the thalamus or reticular formation of the brainstem which activate neurons in the periventricular grey (PVG) and periaqueductal grey (PAG). From the PVG and PAG, information continues toward the raphe nuclei which sends serotonergic fibers back down through the spinal cord to the dorsal horn where they synapse with projection neurons and incoming nociceptive primary afferents. This serotonergic descending pathway acts to reduce nociceptive impulses from further propagation. Throughout these neuronal pathways, opioid induced nociceptive modulation may occur in spinothalamic pathways as they ascend, but opioids produce more profound effects at supra-spinal centers such as the PVG, PAG, raphe nuclei and the descending bulbospinal inhibitory pathway.

It is obvious that many places exist throughout the CNS where opioid mediated effects can occur. Although the observed sub-additive analgesic interactions of mu and kappa opioids in the CWTF were not expected, it is not the first case of a mu-kappa sub-additive interaction. Previous experiments showed that dynorphin (endogenous kappa ligand) produced no antinociception in response to "hot" thermal stimuli; this effect was likely due to the lack of A-δ fiber activity, thus kappa opioids would have no affect. However, morphine produced significant analgesia which was reduced by the addition of dynorphin. This antagonistic interaction was concluded to be mediated by kappa receptors interfering with mu receptors (Schmauss and Herz, 1987; Song and Takemori,

1991). The situation described above is similar to results from the CWTF in this study in that kappa opioid activity seemed to interfere with mu activity, but with the CWTF, kappa agonists produced analgesia. Thus, it seems that kappa receptors may interfere with mu receptor activity regardless of their own antinociceptive effect.

In addition to analgesia, mu and kappa opioids produce various other effects on different systems. For example, mu and kappa opioids produce opposing effects in regard to urinary control, drug discrimination procedures, behavioral motivation, and withdrawal symptoms. Thus mu and kappa opioid intereference observed in the CWTF may be a unique example of how mu and kappa receptors may oppose each others effects in producing analgesia. It is again important to remember that these interactions may be specifically limited to thermal nociception.

Although the opioid combination produced drastically different effects in the CRD and CWTF, the mechanisms involved with the additivity and synergy observed in the CRD may be similar to and as numerous as those mentioned for CWTF. The data from the selective antagonists in the CRD seem to indicate that mu and kappa opioid receptors are sensitive to each others activity. When a selective mu antagonist, b-FNA, was present, the analgesic effects of both fentanyl and spiradoline and their combination were antagonized. In addition, when a selective kappa antagonist, nor-NBI, was present the analgesic effects of fentanyl and spiradoline and their combination were again antagonized. Assuming that the antagonists were selective, these data indicate that analgesic effects mediated by mu receptors are affected by antagonism of kappa receptors. Additionally, analgesic effects mediated by kappa receptors are also affected by antagonism of mu receptors.

Furthermore, the opioid combination was partially antagonized by b-FNA and nor-NBI. This taken together with the observation of synergy in the CRD suggests that mu and kappa receptors may be coupled or intimately linked in some manner in which they are capable of enhancing each others analgesic effect.

The possible mechanisms responsible for these interactions are numerous. Interactions could occur at the receptor level, whereby in visceral nociceptor specific primary afferents mu and kappa receptors cooperate by either enhancing receptor binding or efficacy, by utilizing second messenger systems in a most optimum manner, or by enhancing each others effects as related to ionic changes of the nociceptive membrane potential. In regard to the last option, it seems reasonable that mu and kappa receptor ionic changes would produce synergistic effects based on the fact that each receptor utilizes a different ionic channel and that both types of channels work together to reduce total neurotransmitter release in at least three ways. First, mu receptors increase potassium ion conductance which hyperpolarizes the nociceptive membrane potential which makes depolarization more difficult (decrease action potential propagation) and decreases the action potential duration. Thus the neuron is less likely to release neurotransmitters. Kappa receptors also work to reduce neurotransmitter release by decreasing calcium ion flow into the cell, thus decreasing action potentials. Since mu and kappa receptors utilize separate mechanisms that both decrease neurotransmitter release, it is possible that the combined ionic changes affecting the membrane potential would render the neuron virtually unresponsive to nociceptive impulses.

Another hypothesis for mu-kappa interactions is that nociceptors responsive to stretch or distension are considerably different from other types of nociceptors. As an example, data from the CWTF indicated that nociceptive afferents were temperature- and opioid receptor-dependent. It seemed that higher mu and kappa receptor populations were associated with C and A-δ fibers, respectively and kappa receptor activity was proposed to interfere with mu activity. In contrast, the CRD model excites both types of nociceptive afferents and mu and kappa opioids are equally effective in producing analgesia against a wide range of pressures (range of stimulus). Furthermore, the ratio of visceral primary afferents (A-δ: C fibers) is 1:8 or 1:10 but at the dorsal root the ratio is 2:1 (Janig and Morrison, 1986). Also, opioid combinations in CRD produced additive and synergistic interactions that were equally antagonized by a mu or kappa antagonist. These results indicated that mu and kappa receptors mutually affected each others activity. Thus it seems that nociceptive afferents from the viscera (specifically the colon) may respond to opioids differently than nociceptive afferents responsive of other types of stimuli because receptor populations may be different and receptor interactions may be different.

In conclusion, the differing results of opioid combinations between the CRD and CWTF support the hypothesis mentioned previously that visceral and somatic pain are distinctly different from each other. Also, these results indicate that nocieptive pathways of the CRD and CWTF are modulated differently by mu and kappa opioid combinations.

Further studies of mu and kappa opioids included testing their individual and combined motivational effects on behavior in the X-maze. Fentanyl (mu opioid-euphoric) produced significant place conditioning, whereas enadoline (kappa opioid-dysphoric)

produced minimal or no place conditioning. Interestingly, the combination of fentanyl-enadoline significantly decreased the level of place conditioning in rats previously dosed with fentanyl (which had demonstrated significant place conditioning). The combination of fentanyl-enadoline did not alter previous place conditioning effects of rats dosed with only enadoline. A second indicator of motivational effects on behavior is the activity level of a subject. Rats dosed with fentanyl entered significantly more alleys than enadoline or the combination of fentanyl-enadoline. This result indicated that the combination dose reduced the activity level associated with fentanyl alone. Thus, based on these results from the X-maze, the combination of a mu-kappa opioid (fentanyl-enadoline) has less motivational effects than a mu opioid alone. These results support the hypothesis of neurochemical interactions of mu and kappa opioids on motivation. Rewarding effects have been associated with mu receptor activation in the ventral tegmental area of the brain, which is the origin of the mesolimbic-mesocortical dopamine systems (Bozarth, 1986; Wise, 1989). Aversive influences have been related to kappa receptors in VTA and limbic-cortical terminals of cell bodies having their origin in VTA (Pfeiffer et al., 1986; Bals-Kubik et al., 1993; Narita et al., 1993) and on dopaminergic nerve terminals in the nucleus accumbens which decrease dopamine release (Spanagel et al., 1992). Results from these studies coupled with the present results strongly indicate that motivational interactions between mu and kappa opioids may attenuate the positively reinforcing effects of mu opioids, thus reducing the abuse potential associated with narcotics (which are usually mu opioids).

Additional studies of opioid combinations in cats in CRD revealed similar findings to those of rats in CRD. Oxymorphone and butorphanol produced additive and synergistic antinociceptive interactions. Also, this opioid combination produced minimal changes in physiological parameters such as respiratory rate, pulse rate, and mean arterial pressure. Had similar levels of analgesia been attempted using individual doses of either oxymorphone or butorphanol, the side effects associated with each drug would have precipitated unacceptable changes in physiological parameters. The onset of these untoward side effects would require a reduction in dose of drug, thus analgesia would be comprimised. Results from these studies indicated that opioid combinations produce superior levels of antinociception without troublesome side effects. Since side effects of the combination are minimized, greater levels of analgesia may be achieved.

In addition to the use of opioids for pain relief, neuroleptics are often used in conjunction with opioids to enhance analgesia. Neuroleptics decrease anxiety and have been shown to be benenficial in pain therapy (Woolf, 1983; Pascoe, 1992). In the current study, acepromazine tested alone produced no analgesic effect, whereas when added to the opioid combination, acepromazine significantly enhanced peak analgesic effect and duration of effect. These results are in agreement with those previously mentioned. The mechanisms involved with these effects remain unclear, but neuroleptics have been proposed to enhance opioid analgesia in two ways. Neuroleptics have been shown to block dopamine receptors (D2 receptors) and thus decrease negative feedback onto the neuron, thus increasing dopamine release (Burt et al., 1976). Also chronic dosing with neuroleptics markedly increased methionine-enkephalin within the brain (Hong et al.,

1978). Although this study in the cat did not elucidate mechanisms related to neuroleptic-enhanced opioid analgesia, results indicate that the CRD in the cat is an appropriate model to further study effects of neuroleptics on opioid induced analgesia.

In summary, combinations of mu and kappa opioids more effectively produce visceral antinociception than when administered individually. Furthermore, mu and kappa opioid combinations reduce or minimize mu and kappa related side effects including physiological changes and motivational changes in behavior.

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