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Endopeptidase-24.15 and opioid antinociception: Characterization and specificity of action

Kest, Benjamin, Ph.D.

City University of New York, 1991

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Endopeptidase-24.15 and Opioid Antinociception: Characterization and Specificity of Action

by

BENJAMIN KEST

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

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Abstract

Endopeptidase-24.15 and Opioid Antinociception:
Characterization and Specificity of Action

by

Benjamin Kest

Adviser: Dr. R. J. Bodnar

It is commonly accepted that extracellular proteolysis is the primary method of CNS opioid peptide inactivation. Much in vivo and in vitro evidence implicates aminopeptidase, ACE, and endopeptidase-24.11 as the primary participants. In vitro and in vivo studies with endopeptidase-24.15 demonstrate that it too possesses high affinity for several opioids, and that degradation could be halted by specific active-site directed inhibitors. Conclusive demonstration of the involvement of endopeptidase-24.15 in the metabolism of the opioids however requires the demonstration that its inhibition in vivo leads to changes in those functions which are dependent upon opioid peptide function. Thus, the aim of this dissertation was to assess the effects of endopeptidase-24.15 upon opioid-mediated pain inhibitory processess through the use of selective inhibitors. Three different but integrated lines of inquiry were pursued. First, the endopeptidase-24.15 inhibitors cFP-A(D)AF-pAB and cFP-AAF-pAB were compared to the endopeptidase-24.11 inhibitor cFP-F-pAB for alterations upon basal nociceptive thresholds. Second, inhibitor effects upon opioid peptide antinociception were assessed by comparing the ability of cFP-AAF-pAB and cFP-F-pAB to increase MERGL and met-enkephalin antinociception, and induce dynorphin A_{1-8} antinociception. Finally, the role of endopeptidase-24.15 in the phasically-activated, opioid-mediated pain inhibitory system was evaluated through inhibitor pretreatment prior to a cold-water swim stressor. Inhibitors of endopeptidase-24.15 produced time- and dose- dependent effects comparable to cFP-F-pAB upon basal nociceptive thresholds. While cFP-AAF-pAB co-administration was more effective in potentiating dynorphin A_{1-8}, MERGL, and opioid-mediated cold-water swim antinociception, cFP-F-pAB was more effective upon met-enkephalin antinociception. The general opioid receptor antagonists naloxone and naltrexone blocked basal threshold increases and dynorphin A_{1-8} and cold-water swim antinociception. Central pretreatment with the kappa antagonist nor-BNI, but not the mu antagonist ß-FNA, blocked cFP-AAF-

pAB-dynorphin A_{1-8} antinociception. Central cFP-AAF-pAB, alone or after co-administration with dynorphin A $_{1-8}$, did not produce motor impairment, suggesting changes in pain perception.The data suggest that endopeptidase-24.15 plays a modulatory role upon opioid-mediated antinociceptive processess, and that inhibitors of endopeptidase-24.15 may serve as useful scientific probes and clinical analgesics.

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This dissertation is dedicated to my wonderful, wonderful children,
Ushi, Sima, and Rachel,

...and Anna: "thy praises could fill 100 dissertations".

Sh'hecheyanu, v'kimanu, v'higiyanu l'zman hazeh

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List of Abbreviations

PAG ...
...periaqueductal grey.

cFP-AAF-pAB ...
...N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-Alanine-Phenylalanine-p-aminobenzoate.

cFP-A(D)AF-pAB ...
...N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-(D)Alanine-Phenylalanine-p-aminobenzoate.

cFP-F-pAB ...
...N-[1(RS)-Carboxy-3-phenylpropyl]-Phenylalanine-p-aminobenzoate.

β-FNA ...
...beta-Funaltrexamine.

nor-BNI ...
...nor-Binaltorphimine.

MERGL ...
...met-enkephalin-Arg^6-Gly^7-Leu^8

ED_{50} ...
...effective dose for a pharmacological effect in 50% of subjects.

MERGL ...
...met-enkephalin-Arg^6-Gly^7-Leu^8

I. STATEMENT OF AIMS

The interest in the biological functions of endogenous opioids had its origins in the age-old observation that morphine, extracted from the opium poppy, is without equal in relieving pain and anxiety. Based on the discovery (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973) that opiates fulfill these roles by interacting with multiple "opiate" receptors in the nervous system (Zukin and Zukin 1984; Pasternak and Wood 1986), there ensued a search for an endogenous ligand of the receptor. Those efforts resulted in the eventual discovery of a number of endogenous opioid peptides (Hughes et al. 1975; Guillemin 1976; Goldstein 1984) which are derived from three distinct gene families (see review: Akil et al. 1984), and which have been found to be widely distributed along the entire neuraxis. The production and regulation of the endogenous opioid peptides from their respective gene-related precursor prohormone are under the control of processing enzymes. This yields several opioid-active fragments from one precursor type, with a generally similar pharmacological profile. Further post-translational processing may also occur, affecting the differential distribution of opioid peptide subtypes. The selectivity of opioid peptides for receptor subtypes varies, so that opioid peptide effects are dependent upon loci of administration.

Other studies have revealed that the opioid peptides participate in a descending system of pain modulation organized at the levels of the midbrain and medulla, and act to inhibit nociceptive input to the dorsal horn of the spinal cord (see review: Basbaum and Fields 1978; Fields and Basbaum 1978). In spite of these findings, administration of opioid peptides into the central nervous system often produces disappointing levels, and/or durations, of antinociception. The absence, weakness or short duration of these effects may be attributable to the rapid hydrolysis of the highly susceptible opioids by proteolytic enzyme peptidases.

While aminopeptidase and carboxypeptidase are capable of enkephalin inactivation, the major pathway of endogenous opioid peptide inactivation is thought to be mediated by endopeptidase 24.11 ("enkephalinase") (Schwartz et al. 1981; Malfroy et al. 1978; Turner et al. 1985). Recently, endopeptidase 24.15 has been shown to rapidly convert pro-dynorphin fragments and met-enkephalin-Arg^6-Gly^7-Leu^8 into their corresponding enkephalins (Chu and Orlowski 1985). Inhibitors of aminopeptidase, angiotensin converting enzyme (ACE), endopeptidase 24.11, and 24.15 have been developed that are effective in vitro and in vivo (Ondetti et al. 1977: Umezawa and Aoyagi 1977; Schwartz et

al. 1981; Zhang et al. 1982; Murthy et al. 1984; Acker et al. 1987; Orlowski and Chu 1985; Molineaux and Ayala 1990).

The specific goal of this dissertation research are to examine the modulatory role of endopeptidase 24.15 upon pain inhibition. Conclusive demonstration of the involvement of an enzyme in the metabolism of bioactive peptides requires the demonstration that its inhibition in vivo leads to changes in those functions dependent upon those peptides. The goals of the dissertation research were addressed through the central administration and co-administration of selective inhibitors of endopeptidase -24.15 and -24.11 and the endogenous opioids that serve as their substrates in vivo.

A series of four experiments compared the endopeptidase 24.15 inhibitors cFP-AAF-pAB (N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-Alanine-Phenylalanine-p-aminobenzoate) and cFP-A(D)AF-pAB (N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-(D)Alanine-Phenylalanine-p-aminobenzoate) to the endopeptidase-24.11 inhibitor cFP-F-pAB (N-[1(RS)-Carboxy-3-phenylpropyl]-Phenylalanine-p-aminobenzoate) for antinociceptive actions on the spinally-mediated tail-flick and the supraspinally-mediated jump tests. The first experiment examined time- and dose- dependent effects upon basal nociceptive thresholds after intracerebroventricular administration. Opioid involvement in any observed effects will be verified by subcutaneous administration of the general opioid antagonist naloxone. Since motor impairment may contribute to increases in nociceptive thresholds, a subset of naive animals will also be assessed for motor performance and activity levels following the largest dose of cFP-AAF-pAB.

Since dynorphin A_{1-8} and met-enkephalin-Arg^6-Gly^7-Leu^8 are rapidly hydrolyzed by endopeptidase 24.15 (Chu and Orlowski 1985), the second and third experiments will evaluate changes in their antinociceptive efficacy after co-administration with endopeptidase -24.15 (cFP-AAF-pAB) and -24.11 (cFP-F-pAB) inhibitors. The involvement of opioids and motor dysfunction in any observed effects will be assessed as in experiment 1. In the last study, inhibitors of endopeptidase 24.11 and 24.15 will be compared in their ability to potentiate the opioid-mediated antinociception following exposure to a natural-stressor, a swim in cold-water. These studies will evaluate the hypothesis that the magnitude and duration of antinociception exhibited by these peptides are under the regulation of endopeptidase 24.15, and that this enzyme plays a modulatory role in opioid peptide-mediated pain inhibition.

To provide the underlying conceptual basis for these experiments, the following background sections cover: a) a classification/description of the multiple opioid receptor subtypes and endogenous opioid peptide families, as well as their mutual selectivity, b) characterization of the endogenous opioid pain-inhibitory system including the relative

contributions of the multiple endogenous opioid ligands and opiate receptor subtypes, c) opioid regulation by neuropeptidases, the development of peptidase inhibitors and their effects upon nociceptive thresholds, and d) a rationale for the present experiments.

II. BACKGROUND

Part 1. Opioid Antinociception

A. Multiplicity of Opioid Receptors.

Initial structure-activity relationship investigations with literally thousands of synthetic and semisynthetic opiate compounds (Jacobson et al. 1970; Janssen et al. 1960, 1966) provided strong early pharmacological evidence in favor of a receptor-mediated mechanism of opioid action. It was revealed that such a site exhibited strict stereospecificity (Portoghese 1966, 1970), while in vivo testing and bioassays demonstrated cross-tolerance and dependence. Prior to 1973 however, attempts to label an opioid receptor biochemically were unsuccessful. Three independent laboratories then demonstrated opioid binding sites using radiolabeled naloxone (Pert and Snyder 1973), dihydromorphine (Terenius 1973), and etorphine (Simon et al. 1973). The work of Martin and co-workers (1976) on spinally prepared dogs led to the proposal of three classes of opioid receptors- mu for morphine-like compounds, kappa for ketocyclazocine, and sigma for SKF 10,047. They reported a lack of cross-tolerance across drugs of different classes. It should be noted that because the sigma agonist SKF 10,047 is in many ways uncharacteristic of opioids, i.e. it produces non-naloxone reversible behavioral and neurochemical actions, and potently displaces 3H-phencyclidine, it is not considered a true opiate receptor (Wood 1982).

The observed differential potencies and binding profiles of ß-endorphin, leu-enkephalin, and met-enkephalin on the guinea pig ileum and mouse vas deferens bioassay (Lord et al. 1977) led to the suggestion of three potential receptor sites interacting with opioids: mu, kappa, and delta. Additional differentiation of receptor sites occurred with the discovery of distinct high- and low- affinity mu binding sites, termed mu_1 and mu_2 respectively (Pasternak and Snyder 1975). The mu_1 receptor is the common high affinity site for opioids and most enkephalins, while mu_2 is morphine preferring (Pasternak et al. 1980).

Isolated tissue preparations and specific radioligand binding studies indicate that mu, delta, and kappa opioid receptors are heterogeneous with respect to anatomical distribution, affinity for endogenous opioid substrates, and physiological and behavioral

effects (Herz 1987). All have similar patterns of distribution, though there are overall density differences between subtypes (Millan 1986).

Mu Receptors. These receptors are widely distributed throughout the forebrain, midbrain, and hindbrain. Areas of great mu receptor density include the neocortex (laminae III and IV), caudate-putamen, nucleus accumbens, the thalamus, hippocampus, amygdala, the colliculi, nucleus tractus solitarius, the spinal trigeminal nucleus, and the dorsal horn (Mansour et al. 1988). The latter two sites are particularly important in demonstrating a role for mu receptors in sensory integration. Areas important to descending pain inhibitory systems, such as the PAG and the raphe nuclei, exhibit a moderate density of mu receptors, while relatively little binding is seen in the hypothalamus, preoptic area and globus pallidus.

Autoradiographic studies demonstrate that mu_1 and mu_2 binding sites have similar, but not identical distributions (Moskowitz and Goodman 1985; Goodman and Pasternak 1985). Cortical mu_1 binding is denser in the frontal lobe, whereas mu_2 binding predominates in the parietal, occipital, and temporal cortices. mu_1 binding is found in striatum, ventral pallidum, caudal nucleus accumbens, medial thalamus, interpeduncular nucleus and median raphe, whereas mu_2 binding is found in hippocampus and amygdaloid region. Additionally, mu_1 receptors are found in abundance in the ventral periaqueductal grey, while mu_2 but not mu_1 receptor binding is quite dense in the dorsal motor nucleus of the vagus nerve and the nucleus tractus solitarius. The differential distribution between mu_1 and mu_2 receptor subtypes provides anatomical correlates for the respective roles of these binding sites in antinociceptive and respiratory depressant effects following opioid administration (Pasternak and Snyder 1980).

Delta Receptors. These receptors predominate in forebrain structures, and appear densest in olfactory-related neural areas, neocortex, caudate-putamen, as well as nucleus accumbens, parts of the amygdala, and pontine nucleus. The distribution of these receptors are more restricted, with little or no delta binding observed in the thalamus, hypothalamus, PAG, brainstem, and spinal cord (Mansour et al. 1988).

Kappa Receptors. Kappa receptors are localized in an intermediate number of brain areas, with densest binding observed in the preoptic area, hypothalamus, caudate-putamen, nucleus accumbens, amygdala, hypothalamus, neural lobe of the pituitary, the median eminence, and nucleus tractus solitarius. Fairly dense binding is also seen in the PAG, raphe nuclei, spinal trigeminal nucleus, and the dorsal horn of the spinal cord (Mansour et al. 1988).

B. Multiplicity of Opioid Peptides.

Opioid peptides are characterized by the core amino acid sequence of Tyr-Gly-Gly-Phe- Met or Leu (yielding met- and leu- ENK, or Enkephalin, respectively). Their multiplicity may be distinguished however on the basis of their derivation from three distinct precursor molecules or prohormones, corresponding to three distinct genes (see reviews: Basbaum and Fields 1984; Akil et al. 1984).

ß-endorphin. Pro-opiomelanocortin is the common precursor for ß-endorphin, adrenocorticotropic hormone, Alpha-melanocyte-stimulating hormone, and related bioactive peptides. ß-endorphin, the 31-residue C-terminal component, is generated from the post-translational processing of ß-lipotropin from pro-opiomelanocortin. Certain C-terminal abbreviated fragments of beta-endorphin such as ß-endorphin $_{1\text{-}27}$ may also be produced. Although the met-enkephalin opiate core is contained within ß-endorphin, the post-translational processing of ß-endorphin does not yield a met-enkephalin end product. Processing preferences and peptide storage have been shown to be tissue-site dependent (Civelli et al, 1984; Holt 1986). In the brain, ß-endorphin synthesizing cells are localized mainly in the hypothalamic arcuate nucleus (Bloom et al. 1978; Watson et al. 1978). These neurons have short, local projections to other hypothalamic structures such as the supraoptic n., the paraventricular n., preoptic area, and the suprachiasmatic n.. There are also long-projecting pathways innervating limbic structures, including the amygdala, septum, bed nucleus of the stria terminalis, and the median eminence. ß-endorphin terminals also innervate the nucleus tractus solitarius, periaqueductal grey (PAG), and areas of the reticular formation such as the raphe magnus, nucleus reticularis gigantocellularis, and reticularis lateralis (Khachaturian et al. 1985). ß-endorphin immunoreactive material has also been reported in small neurons of the nucleus commisuralis and tractus solitarius which exhibit lateral projections that may innervate the lateral reticular nucleus (Schwartzberg and Nakane 1983).

The receptor selectivity characteristics for ß-endorphin have not yet been firmly established (Millan 1989). In guinea pig brain, ß-endorphin shows a slight binding preference for mu over delta receptor sites (Kosterlitz and Paterson 1985). ß-endorphin also demonstrates negligible affinity for kappa sites, as evidenced by its lack of activity on the rabbit vas deferens (Paterson et al. 1984). In mouse vas deferens preparations, the absence of substantial ß-endorphin mu receptor binding is thought to reflect ß-endorphin preference for the epsilon receptor type (Law et al. 1979). C-terminally shortened cleavage products of ß-endorphin, such as gamma-endorphin (1-17), alpha-endorphin (1-16), and met-enkephalin (1-5) are considerably less potent than the larger 31 (1-31) peptide fragment on rat vas deferens (Schulz et al. 1981). This finding may be attributable

to the preferential inactivation of the shorter peptides to enzymatic degradation (McKnight et al. 1983).

Pro-enkephalin. This is the source of four copies of met-enkephalin, and one each of leucine-enkephalin, met-enkephalin-Arg^6-Phe^7, and met-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL). Larger peptides bearing the met-enkephalin sequence at the N-terminus may also be generated (peptide E and peptide F) (Hollt 1986). The only source of met-enkephalin therefore is pro-enkephalin, while leucine-enkephalin may be cleaved off of dynorphin-like peptides (see below). Met-enkephalin and leucine-enkephalin appear to be present in all pituitary lobes, whereas the larger pro-enkephalin derived peptides are found in trace amounts only (Hollt 1986).

Enkephalinergic neurons are found along most of the neuraxis, including the cerebral cortex, olfactory tubercle, amygdala, hippocampus, bed nucleus of the stria terminalis, and preoptic area. In the diencephalon, enkephalinergic perikarya are found in most hypothalamic nuclei, as well as the periventricular and lateral geniculate nuclei of the thalamus. Midbrain structures include the colliculi, PAG, and interpeduncular nucleus. In the pons and medulla, perikarya are found in the raphe nuclei, nuclei reticularis gigantocellularis and paragigantocellularis, nucleus tractus solitarius, the lateral reticular nuclei, spinal trigeminal nucleus, and the spinal cord dorsal gray (Khachaturian et al. 1985).

As revealed by binding studies, met-enkephalin and leu-enkephalin possess a high selectivity for delta binding sites in guinea pig (Kosterlitz and Paterson 1985) and mouse brain (Holt et al. 1983). Met-enkephalin-Arg^6-Phe^7 and MERGL have comparable affinities for mu and delta receptors with lower affinities for kappa sites (Paterson et al. 1984). The larger opioid peptides derived from the peptide E domain of the precursor molecule have higher affinities for mu over kappa, with a low affinity for delta sites (Holt et al. 1983). The same pattern of selectivity is seen for metorphamide, also processed from the peptide E fragment (Weber et al. 1983). So as pro-enkephalin A processing proceeds, there is a shift in receptor selectivity towards delta-opioid receptors.

Pro-dynorphin. Opioid peptides derived from this prohormone include dynorphin A (the full 17 amino acid residue molecule), dynorphin A $_{1-8}$, rimorphin (DYN B), and alpha- and ß-neo-endorphin. Each bears an N-terminal-located leucine-enkephalin sequence (Hollt 1986). These peptides appear to be co-localized (Hollt 1986; Akil et al. 1984). The generation of metorphamide involves proteolytic cleavage at a single arginine amino acid - a cleavage site that exists in several peptide precursors (Weber et al. 1983). By analogy with pro-opiomelanocortin-derived peptides, relative concentrations of pro-dynorphin-derived peptides differ between tissue. (Hollt 1986). Immunoreactive

dynorphin perikarya are distributed in several cerebral cortical areas, striatum, amygdala, and hippocampus (Khachaturian et al. 1985). Brain dynorphin-related products are also found in hypothalamic suprachiasmatic, paraventricular, supraoptic, and arcuate nuclei,the midbrain PAG, and brainstem areas such as the spinal trigeminal nucleus, nucleus tractus solitarius, lateral reticular nucleus, and in the spinal cord dorsal horn (Robson et al. 1983).

With the exception of leu-enkephalin (see above), all opioid peptides derived from this gene-related family exhibit a pronounced selectivity for kappa receptors (Kosterlitz and Paterson 1985; Hollt et al. 1983; Goldstein and James 1984; Hollt et al. 1985). As revealed by binding experiments, dynorphin A has the highest affinity and selectivity for kappa binding sites, in contrast to its fragment dynorphin A $_{1-8}$, which shows a lesser affinity in guinea pig membranes (Kosterlitz and Paterson 1985). All of these peptides are also active in rabbit vas deferens, which is thought to contain exclusively kappa receptors (Corbett et al. 1982; Oka et al. 1982).

The structural determinants for kappa selectivity appear to be an arginine residue at position 7 and another basic amino acid at position 10 or 11 (Goldstein 1984). Dynorphin A, dynorphin B, and alpha-neo-endorphin fulfill these criteria, but dynorphin A $_{1-8}$ and ß-neo-endorphin do not, explaining their lower kappa activity. Thus, if dynorphin A or alpha-neo-endorphin were processed into dynorphin A $_{1-8}$ or ß-neo-endorphin, and further into leu-enkephalin, there would be a concomitant increase in selectivity for delta opioid receptors.

Some investigators have attempted to simplify the selectivity of gene-related opioid peptides by correlating them with the three receptor subtypes. Based on the differential pattern of binding affinities between receptors and opioid families, the probable endogenous ligand for delta receptors would be pro-enkephalin-derived peptides, while the ligand for kappa receptors would be pro-dynorphin-derived peptides. Since all ligands bind with the highest affinity to mu_1, this would be a common high affinity site for all endogenous opioids, including ß-endorphin. mu_2 would correspond to an endogenous morphine-like substrate not yet identified. A number of problems arise however, most notable among them being the inconsistent localization between a given receptor and its preferred substrate. Thus, although in the medulla and spinal cord the anatomy of opioid receptors and peptides generally conform to expectations, elsewhere in the brain, and especially in the forebrain, very little ligand-receptor relationship is observed. For example, mu and delta receptors are strikingly sparse in the areas that contain the densest levels of peptides, namely the medial hypothalamus and central amygdaloid nucleus. Conversely, mu receptors are densest in thalamic nuclei that contain no visible

immunoreactivity, and they show cortical lamination patterns that have no known correlate in peptide distribution patterns (Herkenham 1987). In the globus pallidus, thalamus, and substantia nigra, there is a lack of correlation between the distribution of pro-dynorphin and kappa receptors (Mansour et al. 1988). A number of explanations have been advanced to account for these differences. Some of these consider possible technical (limitations of immunocytochemistry, receptor autoradiography artifacts) and biochemical (receptors are occupied, existence of low-affinity, non-functional, ultra-structural, or occupied receptors) factors (Herkenham 1987). Although these limitations will undoubtedly be overcome with the advent of finer localization techniques, the correspondence between a particular opioid receptor subtype with a particular peptide family is not supported by the anatomical data.

C. Opioid Peptide Subtypes and Antinociception.

In discussing the role of opioid peptides in antinociception, consideration will be given to differentiating between the peptides themselves (i.e. ß-endorphin vs. dynorphin), but also among the various loci (i.e. dynorphin in the spinal cord vs. dynorphin in the midbrain).

ß-endorphin. ß-endorphin produces the most potent antinociception in mice after central administration (Hollt et al. 1982). The complete carboxyterminal end appears necessary for full potency. Therefore, ß-endorphin $_{1\text{-}27}$ is less effective than the longer $_{1\text{-}31}$ fragment of the peptide, and may even antagonize its effects (Hammonds et al. 1984). The loss of the pain inhibitory potency of the shorter fragment is not accompanied by changes in affinity to either mu or delta receptors (Schulz et al. 1981) in isolated tissues or in its binding properties to rat membranes (Hammonds et al. 1982).

It is thought that the ß-endorphin containing neurons of the arcuate nucleus of the hypothalamus may mediate stimulation-produced antinociception of the midbrain, particularly given the course of their axonal projections (Reichling et al. 1988). Indeed, rats sustaining lesions of the arcuate display a pronounced blockade of antinociception generated by midbrain electrical stimulation or acute stress without altering basal nociceptive thresholds (Millan et al. 1980; Millan et al. 1986). Furthermore, microinjections of antibodies raised against ß-endorphin into the midbrain PAG also attenuates electroacupuncture antinociception without accompanying basal threshold alterations (Han and Terenius 1982; Xie et al. 1983; Bodnar et al. 1984; Han 1984; Han et al. 1984). These data suggest that ß-endorphin plays no major role upon basal nociceptive thresholds, but participates in the antinociception induced by acute stress, electroacupuncture, and electrical stimulation of the PAG.

Other studies further suggest that central (Millan 1981; Millan et al. 1981; Przewlocki et al. 1982; Millan et al. 1982), but not circulating systemic ß-endorphin released from hypophyseal pools (Lewis et al. 1980; Millan et al. 1981; Bodnar et al. 1982; MacLennan et al. 1982; Birnberg et al. 1983; Bodnar 1984) mediate antinociception following stress or PAG electrical stimulation. This is based on studies which examined antinociception after either hypophysectomy (or selective ablation of individual pituitary lobes), adrenalectomy, or pharmacological manipulation of circulating corticosteroid levels.

Enkephalins. There are data from studies with antibodies raised against met-enkephalin which indicate at least a partial mediation of high intensity / low frequency electroacupuncture antinociception both at the level of the midbrain PAG and spinal cord (Han et al. 1982; 1984).

The data from central administration of enkephalins has been disappointing, producing few notable opiate effects. This is assumed to be due, at least in part, to their rapid breakdown upon exposure to brain membranes (Belluzi et al. 1976; Meek et al. 1977). However, the ability of inhibitors of variable specificity against met- and leu-enkephalin degradation (in particular against aminopeptidase and/or the metalloendopeptidase 'enkephalinase') to produce opiate antinociception suggests some role for met- and leu- enkephalin in pain inhibitory processes (Chaillet et al. 1983; Fournie-Zaluskie et al. 1984). Such inhibitors have also potentiated stress-induced antinociception when administered intracerebroventricularly to the mouse and increase the efficacy of electroacupuncture antinociception after intrathecal delivery to the rat (Chipkin et al. 1983; Costa 1984; Greenberg and O'keefe 1982).

The C-terminal extended enkephalins, MERGL and met-enkephalin-Arg^6-Phe^7, have however been shown to possess potent opioid activity (Zajac et al. 1983; Giraud et al. 1983) and they produce greater antinociception than that of leu- or met- enkephalin (Inturrisi et al. 1980; Iadorola et al. 1986). Like met- and leu- enkephalin, they are rapidly inactivated (Patey et al. 1983), and their antinociceptive effects are potentiated by peptidase inhibitors (Mellstrom et al. 1987).

Met-enkephalin is released from the adrenal medulla into systemic circulation (Clement-Jones et al. 1980), and may be able to modulate nociception based on the ability of adrenalectomy or adrenal demedullation to modulate stress-induced analgesia in the rat (Bodnar 1984; Bodnar et al. 1982, Lewis et al. 1982; Watkins and Mayer 1982).

Dynorphin. The striking feature of this opioid peptide is its lack of antinociceptive activity when injected in the mammalian brain, an observation confirmed by many laboratories (Freidman et al. 1981; Chavkin et al. 1982; Walker et al. 1982). In contrast to supraspinal administration, several investigators report that dynorphin B, dynorphin A_{1-}

$_{13}$, and dynorphin $A_{1\text{-}9}$ are analgesic when injected intrathecally (Han and Xie 1982; Porecca et al. 1983; Han et al. 1984; Herman and Goldstein 1985; Jhamandas et al. 1986), with tolerance developing after chronic infusion (Lee and Smith 1984). So, while dynorphin antiserum applied to the midbrain had no notable effects upon antinociception, antiserum applied to the spinal cord did block electroacupuncture antinociception without altering basal nociceptive thresholds (Han 1984; Han and Xie 1984; Han et al. 1984). Furthermore, both dynorphin $A_{1\text{-}8}$ and dynorphin $A_{1\text{-}13}$ induced antinociception that was present 24 hrs after administration into the cord (Jhamandas et al. 1986). This suggests that dynorphin action is predominantly spinal, consistent with the pharmacological profile of other kappa-preferring agonists.

Others argue that the antinociceptive effects seen after intrathecal dynorphin administration actually reflects the activity of the non-opiate metabolites of dynorphin. For example, [^{3}H-dynorphin] is rapidly converted to ^{3}H-Tyrosine and des-Tyr-dynorphin after central administration (Walker et al. 1982; Young et al. 1986). The dysfunctional effects these non-opioid metabolites have upon motor performance after icv (Herman et al. 1980; Long et al. 1988) and intrathecal (Stevens and Yaksh 1986) administration are well documented. Indeed, the behavioral and physiological effects of dynorphin have been previously reported not to be reversed by high doses of naloxone (10 mg/kg) or other opiate antagonists, while dynorphin A effects can be produced by the non-opioid dynorphin $A_{2\text{-}13}$ or dynorphin $A_{2\text{-}17}$ (Faden and Jacobs 1984; Przewlocki et al. 1983; Walker et al. 1982). However, in a recent study in which low doses of dynorphin was co-administered with the aminopeptidase inhibitor bestatin, which blocks the enzymatic degradation of the opioid fraction of dynorphin only, the naloxone-reversible increase in nociceptive thresholds was not accompanied by a detectable motor impairment. In addition, bestatin had no effect after co-administration with des-Tyr-dynorphin, which did produce naloxone-irreversible motor impairment (Nakazawa et al. 1989).

The very rapid degradation of dynorphin in vitro and in vivo has also been offered as an explanation for its lack of antinociceptive efficacy (Young et al. 1986). One approach to the problem of investigating the characteristics of a particular peptide system has been to synthesize stable analogues. Although dynorphin A analogues have indeed been shown to possess good antinociceptive potency, some argue that they also demonstrate an altered pharmacological profile, differentially increasing mu binding compared to kappa or delta (Chavkin and Goldstein 1981). However, analogues displaying a greater dynorphin-like pharmacological profile have recently been synthesized (Walker et al. 1987).

D. Opioid Receptor Subtype and Antinociception.

Another distinction that can be drawn regarding the opioid receptors in addition to their anatomical distribution, physiological effects and affinity for various ligands, is their ability to mediate antinociception along the neuraxis and the potency of effect. As with the endogenous opioid ligands themselves, different receptor subtypes are most active at either spinal or supraspinal locations (Wood and Iyengar 1988), depending on the quality of the noxious test stimuli (Millan 1986). Caution must thus be employed when directly comparing the antinociceptive actions of receptors across studies, given the variability of the test procedures employed, and the loci of administration.

Mu_1 and Mu_2 Agonists and Antagonists. There is good agreement that mu receptor activation yields the most consistent and efficacious antinociception. But mu antinociception is mediated predominantly supraspinally, with the PAG being a key site of action (Yaksh And Rudy 1978). Microinjection of morphine quantities as minute as 1-5 ug elicits profound analgesia, whereas intrathecal morphine in rats display weaker effects (Wood et al. 1981a). At both spinal and supraspinal sites however, mu agonists are effective against all modes of stimuli such as chemical, thermal, and pressure (Millan 1986).

While there is no clear answer as to which type of opioid receptor (or ligand for that matter) mediates the antinociceptive effects of PAG electrical stimulation (Millan 1986), there is circumstantial evidence to suggest that the mu receptor is involved here too. For example, electrical stimulation of the midbrain to induce antinociception lowered the concentrations of ß-endorphin (but not of dynorphin or met-enkephalin) in the PAG area. After repeated stimulation, the antinociceptive effect of stimulation was decreased, and morphine was less effective (Millan et al. 1987). The authors concluded that the demonstration of cross-tolerance between morphine and ß-endorphin indicates that release of ß-endorphin and the activation of mu receptors are important in PAG stimulation produced analgesia. This is supported by data showing that the disruption of the hypothalamic arcuate nucleus significantly reduces stimulation-produced analgesia from the PAG (Millan et al. 1986).

As the mu subtype can be further subdivided into mu_1 and mu_2 subtypes, the relative contribution of each to antinociception must be assessed. Much evidence suggests that mu_1 sites mediate morphine antinociception (Pasternak and Wood 1986). Naloxazone and naloxonazine, two long-acting, mu_1-selective antagonists, dramatically decrease morphine antinociception in rats and mice (Zhang and Pasternak 1981a), and attenuate the antinociceptive effects of other opiates in a manner consistent with their affinity for the receptor (Ling et al. 1983). Heyman and colleagues (1988) found that naloxonazine

attenuated the antinociceptive effects of intracerebroventricularly administered mu receptor agonists morphine and [D-Ala^2, Met-Phe^4, Gly(ol)^5-enkephalin (DAMGO). These findings have been extended, and also include antinociception induced by the mixed mu_1/delta agonist [D-Ser^2, Leu^5]-enkephalin-Thr^6 (DSLET) (Paul et al. 1989). Additionally, investigators using naloxonazine to antagonize morphine, the delta selective peptide [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), and DSLET demonstrated that the antinociceptive effects of opiates into either the PAG, locus coeruleus, n. raphe magnus, and the n. reticularis gigantocellularis are mediated by the mu_1 receptor subtype (Bodnar et al. 1988).

There is also a good correspondence between the autoradiographic distribution of mu_1 receptors and supraspinal sites implicated in antinociception as revealed by pharmacological studies (Goodman and Pasternak 1985; Moskowitz and Goodman 1985).

In contrast to supraspinal antinociception, spinally-mediated antinociception seems to be mediated in part by the mu_2 receptor subtype. Naloxonazine pretreatment, while shifting supraspinal DAMGO and DSLET dose-response curves to the right 4-10 fold, failed to concomitantly affect their spinal dose-response curves (Heyman et al. 1988, Paul et al. 1989). DAMGO antinociception was however blocked by the irreversible mu antagonist ß-funaltrexamine (ß-FNA). Thus, different mu receptor subtypes may mediate spinal and supraspinal antinociception.

Delta Agonists and Antagonists. It is by no means clear that the enkephalins act primarily at delta receptors, and they have weak antinociceptive potency due to their high susceptibility to enzymatic degradation (Schwartz et al. 1976). Stabilized analogues of enkephalins with a preference for the delta receptor induce antinociception against thermal and and non-thermal stimuli after introduction into the brain (Millan 1986). Although cross-tolerance studies with mu agonists indicate that delta agonists might partially exert their effects via mu receptor activation, the analgesic effects of the delta selective agonist DPDPE does suggest a role for delta receptors in brain (Galligan et al. 1984; Jensen and Yaksh 1986).

In contrast to mu-mediated antinociception, delta receptor activation is most effective in mediating spinal antinociception in animals against thermal stimuli (Hylden 1983; Yaksh 1985) and in humans (Moulin et al. 1984). Although delta agonists have also been reported to be active via delta receptors against pressure, both positive and negative findings have been reported when chemical stimuli are used (Schmauss and Yaksh 1984; Schmauss et al. 1985).

While central ventricular administration is not nearly as efficacious as application directly into the spinal cord (Wood 1982a), delta receptor activation supraspinally is not without antinociceptive effects. Porreca and colleagues (1987) reported that only the morphine, but not DPDPE dose-response curve was shifted to the right in mice after being made acutely mu tolerant by large systemic doses of morphine. Furthermore, the analgesia produced by DPDPE, but not morphine, was blocked by the delta antagonist ICI 174, 864. Delta ligands also elicit an antinociceptive effect in 'jimpy' mice, a mutant strain that is deficient in mu receptors. Mu ligands, in contrast to delta ligands, were without antinociceptive potency (Mathiasen and Vaught 1987).

Kappa Agonists and Antagonists. Kappa receptor agonists exert potent antinociception in a large number of experimental pain paradigms (Wood and Iyengar 1988). The spinal actions of kappa agonists are potent, as has been demonstrated by intrathecal injections of both kappa opiates (Wood et al. 1981a) and opioid peptides (Han and Xie 1982). In spinal animals, only kappa opiate agonists demonstrate no loss of antinociceptive potency as assessed by the tail-flick test (Wood et al. 1981). Presumably, these actions are mediated by the dense number of kappa receptors localized in the dorsal spinal cord of many species (Khachaturian et al. 1985).

At more rostral sites within the central nervous system, kappa receptors are not traditionally considered to have antinociceptive abilities. For example, icv kappa opiates (Wood et al. 1981) and kappa opioid peptides (Hollt et al. 1982) are reportedly without effect. Similarly, local injections into the PAG do not elevate nociceptive thresholds, in stark contrast to the potency of mu agonists (Wood et al. 1981).

However, the recent advent of more selective kappa-receptor agonists and antagonists is beginning to reveal a more active role for kappa receptors in supraspinal antinociception than what was previously thought. The arylacetamides such as U50488H and PD117302 are much more selective than their predecessor kappa agonists, the benzomorphans (i.e., keto- and ethyl- cyclazocine, bremazocine, and MR2266, while the naltrexone derivative nor-binaltorphamine (nor-BNI) has been characterized as a kappa- selective anatagonist (Takemori et al. 1988). Furthermore, the ability of spinally-applied kappa antagonists to attenuate, but not block, systemically administered kappa agonists suggest that there may be an additional site of action. Indeed, icv administration of nor-BNI into the brain also diminishes the antinociceptive action of systemically applied U50488H (Millan 1990). Correspondingly, kappa agonists elicit an antinociceptive action against a wide variety of stimuli (Leighton et al. 1988; Millan et al. 1989; Tiseo et al. 1988; Calcagnetti et al. 1988).

In contrast to mu-receptor agonists, which produce very potent antinociception against all noxious modes, kappa-receptors are thought to act exclusively against non-thermal stimuli (Tyers 1980; Schmauss and Yaksh 1984), and that this may also explain the apparent lack of potency of kappa agonists on some tests. However, the kappa opiate U50488H was found to be active on the tail-flick test to thermal stimuli (Von Voigtlander et al. 1983) while a kappa-selective congener of U50488H, spiradoline, was effective against warm plate, hot plate, tail-flick, and tail immersion tests of thermal reactivity (Von Voigtlander and Lewis 1988). Other investigators claim that the relevant factor is not so much stimulus quality as much as stimulus intensity. Stimulus intensity has been shown to be of general significance in determining the potency of agonists at all opioid receptor types (Shaw et al. 1988). Indeed, if the intensity of noxious heat and pressure stimuli applied to the tail were adjusted to yield identical latencies to withdrawal, both mu and kappa agonists were equipotent against heat and pressure stimuli in rats (Millan 1989). Furthermore, increasing the intensity of the heat, but not pressure, diminished the efficacy of kappa, but not mu, agonists (Millan 1989). In addition, investigators using a cold-water-ethylene glycol mixture (-10 degrees C) as a noxious stimulus were able to demonstrate a dose-related, naloxone-reversible antinociceptive effect after dynorphin A $_{1-17}$ and U50488H central administration (Tiseo et al. 1988). These results suggest that the action of kappa agonists against noxious heat is intensity dependent, and that previously reported failures of these drugs to produce antinociception against noxious thermal stimuli may reflect the use of high intensity heat stimuli.

E. Organization of Pain Inhibitory Systems.

Microinjection of opiates into, or the electrical stimulation of the PAG generates analgesia, and inhibits firing of dorsal horn neurons (Murfin et al. 1976; Basbaum et al. 1977; Liebeskind et al.. 1973). Furthermore, the analgesia is elicited following either electrical stimulation of, or glutamate injection into the PAG (Reichling et al. 1988; Behbehani and Fields 1979). Since electrolytic lesions of the PAG do not produce analgesia (Dostrovsky and Deakin 1977), it has been concluded that descending antinociceptive controls are initiated at the PAG and that the output neuron is excitatory. Although some recent studies suggest that the PAG is largely homogenous with respect to cytoarchitecture and connectivity (Gioia 1984), more recent studies support the division of the PAG into distinct areas (Hamilton 1973; Beitz 1985). Whether the PAG is functionally homogeneous is controversial. Although antinociception can be generated from all areas of the PAG, the ventrolateral region is regarded by many workers to be the most effective site (Gebhart & Toleikis 1978).

The PAG is pivotally situated to initiate descending centrifugal control of spinal neurons given its cortical (frontal and insular cortex), diencephalic (medial basal hypothalamus) and limbic (amygdala) inputs (Beitz 1982; Mantyh 1983). The PAG also receives input from the brainstem. These derive from the n. cuneiformis, the pontine reticular formation, and from the locus coeruleus. When considered collectively with a known direct spinal input (Mehler 1962), these regions are thought to provide a probable relay for nociceptive input that activated PAG neurons (Gebhart 1982).

Although there are extensive direct PAG-spinal projections, as revealed by retrograde tracer studies (Mantyh and Peschanski 1982), medullary structures still play an important role in spinal inhibition originating in the PAG. Raphe and adjacent reticular nuclei neurons project via the dorsal part of the lateral funiculus to regions of the dorsal horn of the spinal cord and its trigeminal equivalent, the nucleus caudalis. These areas are primary sensory information pathways, and they contain nociceptors (Basbaum et al. 1978; Basbaum and Fields 1979). These raphe-spinal neurons thus act to inhibit the firing of dorsal horn sensory-nociceptive neurons, the interneurons mediating connections between them (Fields et al. 1978), as well as a population of rostrally projecting spinothalamic and spinoreticular neurons (Willis et al. 1977).

The medullary cells of origin that have axons projecting to the cord are found in the nucleus raphe magnus, nucleus reticularis gigantocellularis (NRGC), nucleus reticularis paragigantocellularis (NRPGC), and the n. reticularis paragigantocellularis lateralis (NRPGCL) (Basbaum and Fields 1984). All receive projections from the PAG (Mantyh 1983), which has predominantly excitatory effects upon these structures after electrical stimulation (Fields and Anderson 1978), opiate microinjection (Behbehani and Pomeroy 1978), or glutamate injection (Behbehani and Fields 1979). In addition, these nuclei send axons to the spinal cord via the dorsal-lateral funiculus, and produce antinociception when stimulated electrically (Zorman et al. 1981). In order to block completely antinociception induced by midbrain stimulation, all must be simultaneously interrupted, either by lesions (Prieto et al. 1983) or by local anaesthetic (Sandkuhler et al. 1982). Interruption of the n. raphe magnus and the NRPGCL counteracts the effects of PAG stimulation (Basbaum and Fields 1984).

The end point for the descending bulbar inhibition of pain perception is the spinal cord, upon neurons thought to be located in laminae II (after the general laminar organization of Rexed), or the substantia gelatinosa of the dorsal horn. The substantia gelatinosa can be divided into an outer and inner layer. Neurons of the outer layer receive inputs from small diameter high threshold primary afferent fibers (Light & Perl 1979), and contains neurons physiologically similar to neurons in lamina I (Marginal layer) that

respond to both noxious and non-noxious inputs (Light et al. 1979, Bennet et al. 1980). There is evidence that outer layer neurons in the substantia gelatinosa relay nociceptive inputs from primary afferents to marginal neurons (Price et al. 1979). It has been hypothesized that the bulbospinal inhibition of spinal neurons are indirect, i.e. by inhibition of these putative relay interneurons in the outer layer (Basbaum and Fields 1978). In contrast, the inner layer receives input from small diameter, low threshold mechanoreceptors and contains neurons predominantly non-responsive to noxious stimuli.

Relevance for the PAG in opioid mediated antinociception comes from studies showing that the PAG contains both high affinity opiate binding sites as well as significant levels of endogenous opioid peptides (Atweh and Kuhar 1977; Moss et al. 1973). The antinociception produced by opiates administered systemically or via microinjection into the PAG has also been shown to result from activation of raphe-projecting PAG neurons (Fields and Anderson 1978; Behbehani and Pomeroy 1978). Injection of the opiate antagonist naloxone directly into the PAG blocks antinociception by systemic opiates (Tsou and Jang 1964).

Opioidergic synapses however are believed to be exclusively inhibitory (Nicoll and Alger 1979). Thus, opiates would be expected to inhibit the postsynaptic neuron they contact in the PAG. Since excitation of the output neuron is required to initiate a descending control, it has been proposed that the opioid peptides act to disinhibit, i.e. inhibit an interneuron that tonically inhibits the output neuron (Yaksh et al. 1976). Pharmacological, physiological, and anatomical evidence suggest that the tonically active inhibitory interneuron is GABAergic (Basbaum and Fields 1984). Immunoreactive GABA cell bodies are very common in the PAG, particularly in regions where opioid terminals are most dense, the ventrolateral extension of the nucleus medialis (Reichling and Basbaum 1987). PAG neurons receiving GABA input, and that project to the n. raphe magnus, have also been reported (Basbaum and Menetrey 1987). The excitatory output neuron of the PAG to the ventral rostral medulla is thought to be neurotensinergic. Intracisternal neurotensin produces profound analgesia (Kalivas et al. 1982), and neurotensin immunoreactive cells of the PAG project to the Rostral ventral medulla (Beitz 1982).

F. Stress-Induced Analgesia (SIA).

Stressors. The functional significance of the endogenous opioid pain-inhibitory system is best appreciated after an organism has been exposed to a stressful stimulus. Several laboratories, working independently, made the observation that one consequence

of such exposure is a transient analgesic response (Chance et al. 1977; Hayes et al. 1988; Madden et al. 1977). The stressful consequences of the stimulus, and not the stimulus per se, was determined to be responsible for the analgesic response since repeated exposure to the stimuli resulted in adaptation (Madden et al. 1977; Bodnar et al. 1980; Lewis et al. 1981). It therefore seems that one function of the endogenous opioid system is to phasically inhibit pain after exposure to fear or harm, thereby allowing adaptive responses such as fight or flight to be executed unimpeded. Some stressors known to elicit antinociception include electric footshock, immobilization, centrifugal rotation, cold-water swims, and glucoprivation in response to either 2-deoxy-d-glucose (2-DG, a non-metabolic glucose analogue) or insulin injections (Hayes et al. 1978; Chance 1980; Amir et al. 1980; Bodnar et al. 1980).

Initial studies with the general opioid receptor anatagonist naloxone showed that some forms of stress-induced antinociception are mediated by endogenous opioids. So, while footshock analgesia was partially antagonized by large (10 mg/kg) but not small (1 mg/kg) doses of naloxone, antinociception elicited by a continuous 3.5 minute cold-water swim failed to be reversed at a broad range of naloxone doses (1-20 mg/kg), or to show cross tolerance with morphine (Madden et al. 1977; Chesher and Chan 1977; Jackson et al. 1979; Bodnar et al. 1980). However, naloxone clearly did block antinociception induced by immobilization (Amir et al. 1980) and food deprivation (Bodnar et al. 1980). These initial studies suggested that multiple endogenous opioid and nonopioid mediated pain inhibitory systems exist, and that they are differentially activated depending on the nature of the stressor. While later studies (see below) rectified the apparent opioid-nonopioid discrepancy by demonstrating that parametric variations of even a single stressor differentially alters the "substrate" mediating the subsequent SIA, they also confirmed the existence of these multiple antinociceptive systems.

Parametric Variations - Opioid and Nonopioid Forms of SIA. Opioid and nonopioid forms of pain inhibition can be activated differentially not only by the type of stressful situation, but also by different parameters of the same stressful situation. Lewis and co-workers (1980) were able to distinguish between naloxone sensitive and naloxone insensitive footshock antinociception on the basis of intermitency vs. continuity respectively. Accordingly, animals exposed to prolonged intermittent footshock produced antinociception blocked by naloxone, while exposure to brief continuous footshock did not. Parametric variations in the number (i.e., 80 shocks vs 20 shocks) and placement (i.e., forepaw vs. hindpaw) of the shocks induced differential opioid and nonopioid antinociceptive responses (Grau et al. 1981; Watkins and Mayer 1982).

Footshock was not the only stressor whose opioid and nonopioid form was subject to parametric variation. Variations in the temperature, duration, and patterning of swims all contribute to producing a differential antinociceptive response. For example, continuous cold-water swims (CCWS) produce nonopioid antinociceptive responses (Bodnar et al. 1980) except at warmer temperatures (Bodnar and Sikorszky 1983; Christie et al. 1981; Christie et al. 1982 a, b; O'Connor and Chipkin 1984), while intermittent cold-water swims elicit an antinociception that is blocked by morphine tolerance and opioid receptor antagonism (Girardot and Holloway 1984 a, b; Girardot and Holloway 1985; Romero et al. 1988).

Later studies revealed that the antinociceptive responses to stress can be delineated along still other dimensions. This formed the basis of a proposal by Watkins and Mayers (1982) which argued for four categories of antinociceptive responses which would take neural vs. hormonal determinants into consideration along with the opioid vs. nonopioid distinction (i.e. opioid-neurohormonal, opioid-neural, nonopioid-neurohormonal, and nonopioid-neural).

Part 2. Opioid Regulation

G. Endogenous Opioid Peptidases

The biosysnthesis and release of biologically active neuropeptides are due to the limited and selective proteolysis of larger prohormones by enzymes. Sometimes the products of this process are further modified by enzymatic acetylation, or by secondary proteolysis. Transformations, especially by proteolysis, may lead to inactivation. It is commonly accepted that extracellular proteolysis is the primary method of opioid peptide inactivation in the central nervous system (Terenius 1988).

Peptidases are generally classified as either exopeptidases, where the mechanism of proteolysis is by the removal of one or two residues from either the N- or C- terminus, or endopeptidases, where hydrolysis occurs at internal sites within peptide chains (Turner et al. 1985). Those peptidases thought to be most active in opioid degradation are the two metalloendopeptidases found in brain, endopeptidase -24.11 and -24.15, angiotensin converting enzyme, (ACE), and aminopeptidase. Since this dissertation examined the actions of endopeptidase-24.15 and compared it with endopeptidase-24.11, they will now be characterized and followed by a brief consideration of ACE and aminopeptidase.

Endopeptidase E.C. 3.4.24.11 (endopeptidase-24.11). Membrane metalloendopeptidase was first identified in kidney brush border membranes (Kerr and Kenny 1974) and has been given the trivial name 'enkephalinase', suggestive of a specific

function. However, this enzyme is neither specific to the brain, to a particular neuropeptide, nor to the enkephalins per se. In brain it can degrade some peptides with an even higher affinity than enkephalins, such as substance P and neurotensin (Terenius 1988). 'Enkephalinase' is immunologically identical with endopeptidase-24.11 (Matsas et al. 1983; Almenoff and Orlowski 1984).

Endopeptidase-24.11 is an integral membrane glycoprotein containing a Zn^{2+} atom at the active site (Turner et al. 1985). Endopeptidase-24.11 is situated on the surface of the cell membrane, ideal for peptide inactivation (Matsas et al. 1983). It is associated with brain membrane fractions (Matsas et al. 1984), but is also reported to be present on glial cell membranes (Lentzen and Palenker 1983). The enzyme exhibits thermolysin-like specificity in that it preferentially cleaves bonds on the amino side of hydrophobic amino acids, the Gly^3-Phe^4 bond of enkephalins.

Although it has been suggested that the enzyme be designated a peptidyl dipeptidase because it releases the dipeptide Phe-Leu from the C-terminus of leu-enkephalin (Malfroy and Schwartz 1982a), a critical examination of the nature of the susceptible bonds reveals that the enzyme is a genuine endopeptidase. For example, enkephalins with a C-terminal extension, such as leu-enkephalin-Arg^6, met-enkephalin-Arg^6, and met-enkephalin-Arg^6-Phe^7, are metabolized at least as efficiently as the parent peptide with hydrolysis still occurring at the Gly^3-Phe^4 bond (Turner et al. 1985).

Endopeptidase-24.11 is relatively abundant in striatum and the spinal cord, and its overall distribution in brain matches with enkephalin in a way not inconsistent with a role in its metabolism (Matsas et al. 1986).

Endopeptidase E. C. 3.4.24.15 (endopeptidase-24.15). When this enzyme was initially identified and partially purified from rat brain, it was predominantly associated with the soluble fraction of homogenates (Orlowski et al. 1983). A membrane bound form of the enzyme having similar properties to the soluble form was found to be associated with with brain particulate fractions, including synaptosomes. The membrane bound form was determined to constitute approximately 20% of the total activity (Acker et al. 1987). Although the isolation of a homogeneous preparation of endopeptidase-24.15 had to be obtained from testes in order to overcome difficulties encountered with conventional enzyme purification methods utilizing brain, there is evidence demonstrating that the testis and brain enzymes are immunologically and catalytically closely related, if not identical (Orlowski et al. 1989). It has been determined that endopeptidase-24.15 is highly active in brain, anterior and posterior pituitary, and testis (Acker et al. 1987). Unlike endopeptidase-24.11, which is widely distributed in peripheral tissues and is several hundred times more active in the kidney than in the brain, endopeptidase-24.15 is

a more brain-specific enzyme since its activity in most peripheral tissues including kidney and liver is only 10-20% of that found in brain (Chu and Orlowski 1985).

Specificity studies with model synthetic substrates indicate that endopeptidase-24.15 contains an extended substrate binding site accommodating a minimum of five amino acid residues, with two of the residues binding on the amino side and three on the carboxyl side of the bond undergoing hydrolysis (Orlowski et al. 1983). The enzyme preferentially cleaves bonds in peptides in which the P_1 and P_2 positions are represented by hydrophobic amino acid residues (amino acid residues in the substrate are designated as P_1, P_2, P_3 etc. in the N-terminal direction and P_1', P_2', P_3' etc. in the C-terminal direction from the bond undergoing cleavage after the nomenclature of Schechter and Berger (1967)). In addition, the amino acid residue in the P_3' position is an important factor determining the binding affinity of the peptide to the substrate recognition site.

Since endopeptidase-24.15 does not cleave proteins and large peptides containing more than 20 amino acid residues, it has been suggested that the enzyme is involved in the metabolism of bioactive peptides. Indeed, experiments with purified preparations of the enzyme from rat brain as well as with synaptosomal membranes have shown that the enzyme converts the opioid peptides dynorphin A_{1-8}, alpha- and ß-neo-endorphin, metorphamide, and MERGL into leu- and met-enkephalin respectively in vitro with binding affinities in the micromolar range (Chu and Orlowski 1985; Acker et al. 1987). These findings are consistent with work from other laboratories that show a), that leu-enkephalin can indeed be derived from some of these precursor molecules (Kilpatrick et al. 1983; Zamir et al. 1984) and b), immunoreactive alpha-neo-endorphin is found in much higher concentrations than ß-neo-endorphin (Weber et al. 1983; Seizinger et al. 1984). The latter point agrees with the reported affinity values of endopeptidase-24.15 for ß- over alpha-neo-endorphin (Chu and Orlowski 1985). Both leu- and met- enkephalin pentapeptides are resistant to endopeptidase-24.15 hydrolysis.

Angiotensin Converting Enzyme (ACE). Brain tissue contains the components of the renin-angiotensin system, including ACE (E. C. 3.4.15.1), which is enriched in synaptosomes (Benuck and Marks 1978). Although ACE catalyses the hydrolysis of angiotensin I to yield the vasoactive peptide angiotensin II (Soffer 1976), it is likely that the enzyme plays a far greater role in the metabolism of biologically active peptides in brain than is implied by its name. Although the ACE is a Zn^{2+} containing glycoprotein like endopeptidase-24.11, it is far less selective (Bunning et al. 1983). The primary specificity of the enzyme is to release dipeptide fragments sequentially from the C-terminus of oligopeptides (Turner et al. 1985). It has been shown to release the Phe^4-Leu^5 from the C-terminus of leu-enkephalin (Erdos et al. 1978) and to degrade met-

enkephalin-Arg^6-Phe^7 at the Met^5-Arg^6 bond (Yang et al. 1981; Benuck et al. 1981). Although the hydrolysis of enkephalins is primarily carried out by endopeptidase-24.11 at the Gly^3-Phe^4 bond, it is possible that peptidyl dipeptidase plays a role in the metabolism of opioid precursors (Demmer and Brand 1983).

Immunocytochemical studies of ACE in the brain show localization in capillary endothelial cells throughout the brain, and particularly in the choroid plexus (Rix et al. 1981; Brownfield et al. 1982). There also appears to be some detectable ACE activity in the neuronal striatonigral pathway (Arregui et al. 1978).

Aminopeptidase. Initial studies on the inactivation of enkephalin by brain homogenates revealed that a major site of hydrolysis was the Tyr^1-Gly^2 bond (Hambrook et al. 1976; Meek et al. 1977). Much of this activity proved to be mediated by cytosolic and some membrane-bound aminopeptidase (Lane et al. 1977; Knight and Klee 1978). Regulatory peptides that are unprotected at the N-terminus, like the enkephalins, will be susceptible to the actions of aminopeptidases (Turner et al. 1985). Membrane aminopeptidase activity exceeds that of endopeptidase-24.11 in all brain regions examined (Hudgin et al. 1981). Some have proposed that a combination of both enzymes is involved in the inactivation of the enkephalins (DeLabaume et al. 1983). Indeed, inhibiting both endopeptidase-24.11 with thiorphan and aminopeptidase with bestatin provides greater potentiation of enkephalin-induced antinociception than is produced by either inhibitor alone (De Labaume et al. 1982).

A number of recent studies have attempted to characterize the aminopeptidases that hydrolyze the Tyr^1-Gly^2 bond of enkephalin. Most of these studies have concluded that the cytosolic enzymes purified from monkey, rat, human, and bovine brain closely resemble cytosolic aminopeptidase present in other tissue (Geary et al. 1982; Hayashi and Oshima 1977; Schnebli et al. 1979; Traficante et al. 1980; Hersh and McKelvy 1981). Of greater physiological interest in terms of enkephalin metabolism are the membrane aminopeptidases. Hersh (1981) has reported on two membrane enzymes from bovine brain able to cleave the Tyr^1-Gly^2 bond of enkephalin. One of these, designated M2, had many properties in common with cytosolic aminopeptidases (similar K_m for enkephalin and sensitivity to puromycin). The other enzyme (M1) was distinct from cytosolic aminopeptidases, but had some characteristics similar to aminopeptidase N. A purified metallo-protein "enkephalin aminopeptidase" from rat brain, sensitive to inhibition by bestatin, has also been reported. It exhibited a K_m for enkephalin in the millimolar range (Hui et al. 1983). Whether the membrane form of "enkephalin aminopeptidase" is identical with aminopeptidase N is uncertain. However, data obtained in other laboratories indicate that approximately 10% of brain aminopeptidase activity is indeed

immunologically similar to kidney microvillar aminopeptidase N (Gros et al. 1985). Like endopeptidase-24.11 and peptidyl dipeptidase A, aminopeptidase N is a membrane glycoprotein, and like the others, it is a metallo-enzyme containing a Zn^{2+} atom at the active site (Turner et al. 1985).

H. Peptidase Inhibitors and Antinociception.

ACE Inhibitors. The active site for ACE contains a basic group which forms an ionic bond with the terminal carboxyl group of the substrate (Quiocho and Lipscomb 1971). In contrast to the ACE model enzyme carboxypeptidase A, ACE does not require an adjacent hydrophobic pocket responsible for the specificity of carboxypeptidase A towards substrates containing a C-terminal aromatic amino acid. The $zinc^{2+}$ atom participates directly in the catalytic actions of both enzymes (Turner et al. 1985).

Captopril, an orally active inhibitor stable to hydrolysis by peptidases (Ondetti et al. 1977) was designed along these criteria. The essential features of captopril that produce potent inhibition ($K_i = 2$ nM) are the presence of a thiol group as a $zinc^{2+}$-ion ligand and a prolyl residue at the C-terminus (Turner et al. 1985). Release experiments using depolarized brain slices reveal that the recovery of C-terminal extended enkephalins, such as met-enkephalin-Arg^6-Phe^7, can be increased after co-administering captopril and bestatin, an aminopeptidase inhibitor (Patey et al. 1983). Intrathecal co-administration of the two inhibitors also potentiates antinociception following electroacupuncture (Chou et al. 1984). There were similar increases in met-enkephalin-Arg^6-Phe^7 recovery from brain slices and antinociception when substituting captopril with Hoe 498 diacid, an ACE inhibitor capable of crossing the blood-brain barrier (Mellstrom et al. 1987). However, co-administration of Hoe 498 diacid with bestatin did not increase recovery of MERGL from depolarized brain slices or potentiate MERGL antinociception above those levels obtained with bestatin alone (Mellstrom et al. 1987). Kelatorphan, a highly efficient inhibitor of endopeptidase-24.11, aminopeptidase, and ACE (Fournie-Zaluski et al. 1984; Fournie-Zaluski et al. 1985; Roques and Fournie-Zaluski 1987), also produced increases in nociceptive thresholds that are higher than those seen after thiorphan and bestatin central co-administration in mice (Fournie-Zaluski et al. 1984). It has been shown to inhibit enkephalin metabolism in vitro (Waksman et al. 1985) and in vivo (Bourgoin et al. 1986). Thus, the added inhibition of ACE by captopril and Hoe 498 diacid in studies with bestatin and kelatorphan produces and potentiates opioid-mediated pain inhibition.

Other sources of ACE inhibitors are some naturally occurring compounds. For example, the snake venom peptide, Gly-Lys-Trp-Ala-Pro (bradykinin potentiating factor 5a), is the most potent of a series of such peptides that inhibit ACE, although it itself is

susceptible to the enzymes hydrolytic actions (Cheung and Cushman 1973). More stable venom peptides exist but they are not as potent or orally active (Engel et al. 1972; Gavras et al. 1974).

Aminopeptidase Inhibitors. Although neuropeptides such as enkephalins and CCK are susceptible to the actions of aminopeptidases, the development of novel inhibitors of these enzymes have attracted less interest than the others. Although puromycin is commonly used (Schwartz et al. 1981; Vogel and Altstein 1979), its actions as an inhibitor of aminopeptidase activity in studies of neuropeptide metabolism is rather general given its relative lack of potency and specificity (Turner et al. 1985).

Bestatin and amastatin, two natural products, are more potent aminopeptidase inhibitors. Although bestatin and amastatin were initially characterized as inhibitors of aminopeptidase B and A respectively, they both exhibit an overlapping specificity against a variety of aminopeptidases. Bestatin is also an effective inhibitor of kidney cytosolic leucine aminopeptidase (Umezawa and Aoyagi 1977; Aoyagi et al. 1978; Wilkes et al. 1984) and, in combination with thiorphan, has been shown to protect endogenous enkephalins released from brain slices against metabolism by extracellular peptidases (De Labaume et al. 1982; Chou et al. 1984). However, there is still no specific inhibitor of membrane aminopeptidase that approaches the selectivity or the potency that phosphoramidon (K_i = 2 nM) or thiorphan exhibit towards endopeptidase-24.11 (Turner et al. 1985).

Studies of inhibitor effects upon antinociception also support a role for aminopeptidase in opioid-mediated pain inhibitory processes. For example, bestatin and amastatin have been shown to increase basal nociceptive thresholds (Chaillet et al. 1983), and to potentiate the effects of intrathecal met-enkephalin (Noueihed and Yaksh 1985), central met-enkephalin -Arg^6-Phe^7 and -Arg^6-Gly^7-Leu^8 (Mellstrom et al. 1987), and intrathecal (Satoh et al. 1983) and intracerebral (Nakazawa et al. 1989) dynorphin after co-administration. Finally, administering bestatin with the endopeptidase-24.11 inhibitor thiorphan provides greater potentiation of enkephalin-induced antinociception than is produced by thiorphan alone (De Labaume et al. 1982).

Endopeptidase-24.11 Inhibitors. The design of potent and selective inhibitors of endopeptidase-24.11 are based on the $zinc^{2+}$-containing enzyme thermolysin, which serves as a model. All effective endopeptidase-24.11 inhibitors have a group capable of coordinating with the $zinc^{2+}$ ion active-site (Turner et al. 1985). Phosphoramidon, a natural product from *Streptomyces tanashiensis*, inhibits thermolysin and endopeptidase-24.11 (Kenny 1977). Although it remains one of the most potent inhibitors of endopeptidase-24.11, it is not wholly specific (Matsas et al. 1984). Thiorphan, a thiol

compound, has been shown to enhance the recovery of enkephalins from depolarized brain slices (Roques et al. 1980; Schwartz et al. 1981), and to increase the level of striatal immunoreactive met-enkephalin by 30% after icv administration (Zhang et al. 1982). Thiorphan also increases nociceptive thresholds on the hot plate test (Roques et al. 1980), and potentiates antinociception following exposure to stress (Greenberg and O'Keefe 1982), and administration of [D-Ala^2, Met^5]-enkephalin, an analog resistant to aminopeptidase degradation (Yaksh and Harty 1982). Administering thiorphan with bestatin provides greater potentiation of enkephalin-induced antinociception than is produced by bestatin alone (De Labaume et al. 1982). However, negative results on the rat tail-flick test were reported after large doses were administered subcutaneously (Chipkin et al. 1982), and in one study thiorphan failed to produce the narcotic cue (Buxton et al. 1982). The utilization of thiorphan in pharmacological studies has been limited by its poor penetration into the brain after parenteral administration. Acetorphan, a parenterally active derivative of thiorphan, produces a naloxone-reversible long-lasting potentiation of [D-Ala^2, Met^5]-enkephalin antinociception on some tests (hot plate and irritant-induced writhing) but not others (hot plate licking and tail withdrawal) (Lecomte et al. 1986). The more recently synthesized SCH 34826 has been shown to be an orally active enkephalinase inhibitor (Chipkin et al. 1988). cFP-F-pAB, a reversible and potent inhibitor of endopeptidase-24.11, also increases striatal met- and leu- enkephalin levels and nociceptive thresholds following intraperitoneal administration (Murthy et al. 1984).

Endopeptidase-24.15 Inhibitors. Like endopeptidase-24.11, endopeptidase-24.15 is a metallo-enzyme with a Zn^{2+} atom at the active site. As is characteristic of Zn^{2+} peptidases, active-site directed N-carboxymethyl peptides act as specific and potent enzyme inhibitors (Chu and Orlowski 1984; Orlowski et al. 1988). Potent inhibitors are substrate-related peptides containing a free N-(carboxymethyl) group, capable of coordinating with the active site Zn^{2+} atom, and which have sequences that fulfill the binding requirements of the substrate recognition site. This is in accord with inhibitors of other metalloendopeptidases (Cushman et al. 1977; Holmquist and Vallee 1979; Kam et al. 1979).

The potency of inhibition is dependent on the nature and position of specific residues (Orlowski et al. 1988). For example, changing from an N-(carboxymethyl) to an N-(carboxyphenylpropyl) group at one specific point of the inhibitor lowers the K_i values by more than three orders of magnitude. This indicates that the main determinants of primary specificity are the residues binding to this subsite of the enzyme (Orlowski et al. 1988).

The inhibitors of endopeptidase-24.15 synthesized and evaluated to date are resistant to degradation by the enzyme. Experiments show that the same degree of inhibition is obtained even after preincubation with the enzyme 15 minutes before substrate introduction (Orlowski et al. 1988). This indicates that the dissociation of the enzyme-inhibitor complex is a rapid equilibrium process.

Inhibitors fulfilling the requirements necessary for effective endopeptidase-24.15 inhibition are themselves rendered susceptible to endopeptidase-24.11 hydrolysis. Indeed, experiments have shown that the Phe-pAB and Tyr-pAB moieties of endopeptidase-24.15 inhibitors are slowly cleaved by the endopeptidase-24.11 enzyme. This can be overcome by stabilizing the inhibitor by inclusion of a (D)-Ala residue, i.e. N-[1(RS)-carboxy-3-phenylpropyl]-Ala-(D)Ala-Phe-p-aminobenzoate (cFP-A(D)AF-pAB). Conversely, some endopeptidase-24.15 inhibitors having an aromatic residue at specific subsites inhibit endopeptidase-24.11 (Orlowski et al. 1988). The potency of this inhibition is however much less than for endopeptidase-24.15. Thus, for example N-[1(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate ($K_i = 0.02$ uM) inhibited endopeptidase-24.11 with a K_i more than three orders of magnitude higher than for endopeptidase-24.15. Similar results are obtained when -Tyr residue was replaced by Phe (cFP-AAF-pAB, ($K_i = 0.03$ uM). The latter inhibitor has also been shown to completely inhibit membrane-bound (Acker et al. 1987) and soluble forms (Orlowski and Chu 1985) of endopeptidase-24.15 from converting dynorphin A $_{1-8}$, alpha- and ß- neo-endorphin into leu-enkephalin, and met-enkephalin-Arg^6-Gly^7-Leu^8 to met-enkephalin in vitro, and in vivo (Molineaux and Ayala 1990). In contrast, inhibitors of endopeptidase-24.11 have no effect upon endopeptidase-24.15 (Orlowski et al. 1988).

Despite strong evidence of a physiological role for endopeptidase-24.15 in opioid peptide metabolism, there are currently no data on the in vivo effects of endopeptidase-24.15 inhibition upon opioid-mediated antinociception.

III. Rationale

It is well established that the endogenous opiate peptides act to inhibit pain in the central nervous system by acting within a descending system involving the midbrain PAG, and medullary structures that course via the dorsolateral funiculus and terminate in the the dorsal horn of the spinal cord. There, incoming nociceptive information is presumably inhibited either by opiates and/or other neurotransmitter and/or peptidergic systems. Exogenously applied endogenous opioids however have been singularly

disappointing in their pain inhibiting efficacy, none showing the potency of morphine. This has been attributed to the rapid degradation of opioids by peptidases.

Although the enkephalins are substrates for a variety of peptidases, it appears that two enzymes, aminopeptidase N and particularly "enkephalinase", are responsible for their degradatory inactivation. "Enkephalinase", a zinc-metalloendopeptidase associated with synaptic membranes, cleaves Gly^3-Phe^4 bonds on the amino side of hydrophobic amino acid residues and has been designated as endopeptidase-24.11. The role of endopeptidase-24.11 in opioid peptide function has been extensively studied through the development of highly-selective inhibitors of the enzyme, most notably thiorphan, acetorphan and SCH 34826. The inhibitors have been shown to protect endogenous enkephalins released by depolarization of brain slices from degradation, and to produce biological responses similar to those elicited by opioids, such as antinociception, in vivo.

Another brain zinc-metalloendopeptidase, endopeptidase-24.15, has been shown to be associated with both the soluble and membrane fraction of brain homogenates with an active site capable of accommodating five amino acid residues. This enzyme cleaves preferentially peptide bonds on the carboxyl side of hydrophobic residues, and prefers substrates with an additional hydrophobic residue in the P_3 position. Unlike endopeptidase-24.11, which is widely distributed in peripheral tissues and is several hundred times more active in kidney than in brain, endopeptidase-24.15 is more brain-specific since its activity in most peripheral tissues is only 10-20 % of that in brain.

Both the synaptosomal membrane-bound and soluble forms of endopeptidase-24.15 generate leu-enkephalin from dynorphin A $_{1-8}$, alpha-neo-endorphin, and met-enkephalin from met-enkephalin-Arg^6-Gly^7-Leu^8. The formation of leu- and met-enkephalin from these substrates can be almost completely halted in vivo by an active-site directed inhibitor. However, conclusive demonstration of the involvement of an enzyme in the metabolism of bioactive peptides like the opioids requires the demonstration that its inhibition in vivo leads to changes in those functions which are dependent upon opioid peptide function. In addition, inhibitors of peptidases are of interest because of their potential pharmacological properties related to the function of the enzymes. Thus, inhibitors of angiotensin converting enzyme (ACE) have found application in the treatment of hypertension and congestive heart failure, while inhibitors of endopeptidase-24.11 have antinociceptive properties.

Unlike endopeptidase-24.11 however, nothing is presently known about what effects inhibitors of endopeptidase-24.15 might have in vivo upon the pain inhibitory processes mediated by endogenous opioids.

There were four specific aims of the dissertation research:

1) to assess the effects of endopeptidase-24.15 inhibition upon basal nociceptive thresholds after intracerebroventricular administration, and to compare these effects to those produced by an inhibitor of endopeptidase-24.11. This allowed for an evaluation of the modulatory role of endopeptidase-24.15 in antinociceptive processes;

2) to examine what, if any, role endopeptidase-24.15 may play in dynorphin A $_{1-8}$ antinociception by administering selective endopeptidase-24.15 inhibitors centrally;

3) to evaluate whether endopeptidase-24.15 modulates the efficacy of antinociception induced by MERGL, and to compare the specificity of these effects by comparison with met-enkephalin through the use of centrally administered inhibitors;

4) to evaluate the role endopeptidase-24.15 has in the expression of antinociception after exposure to an opioid-mediated stressor, intermittent cold-water swims following central administration of endopeptidase inhibitors.

The following experiments were designed to test these aims.

In experiment 1a, endopeptidase-24.15 inhibitors cFP-AAF-pAB and cFP-A(D)AF-pAB were assessed for their ability to alter basal nociceptive thresholds after intracerebroventricular administration in rats. cFP-AAF-pAB was selected among other available endopeptidase-24.15 inhibitors because of its high affinity and specificity for this enzyme, and because of its previously established efficacy to protect pro-dynorphin-derived peptides in vivo. cFP-A(D)AF-pAB was selected in order to assess the relative value of an inhibitor exhibiting lower affinity but greater resistance to enzymatic deactivation by endopeptidase-24.11. Since there are no previous data as to an ED_{50} value, an initial dose of 25 nmol was administered, followed by increases or decreases in increments of one log unit until a physiologically relevant range was determined. The data were used to construct a dose-response curve. Nociceptive threshold alterations were plotted as a function of time in order to determine the existence of time-dependent effects. The efficacy of the inhibitors were also compared to equivalent doses of cFP-F-pAB, a structurally-similar inhibitor of endopeptidase E.C. 3.4.24.11, an enzyme known to metabolize pro-enkephalin derived products. This inhibitor has already been shown to produce antinociception after peripheral administration (Murthy et al. 1984).

In this and all subsequent experiments, antinociceptive evaluations were made on two standard measures. The tail-flick test measures reactivity to a thermal stimulus by way of radiant heat directed at the tail. The response (tail-withdrawal) is organized at the level of the spinal cord (D'Amour and Smith 1941). The jump test measures reactivity to electric shock applied to the paws via cage grids. This response (hind-paw "jump") is organized supraspinally (Evans 1961). Tail-flick latencies were assessed first to minimize carry over effects within an experimental trial (Kelly 1982).

1b) Opioid involvement in any observed effects upon basal nociceptive thresholds were assessed by the general opiate receptor antagonist naloxone. This drug was given to naive rats subcutaneously 15 minutes prior to the peak antinociceptive effect determined in experiment 1a. An attenuation of increases in nociceptive thresholds following administration of this drug indicated that an opioid synapse mediates increases in antinociception caused by the inhibitors.

1c) To address the possibility that increases on nociceptive tests may result from alterations in motor rather than sensory-perceptual systems, rat motor abilities were examined across the time course used to assess antinociceptive effects. Locomotor counts, righting reflexes and performance on an inclined plane were assessed following the largest dose of cFP-AAF-pAB, and were compared to saline treated rats.

Experiment 2 evaluated the ability of dynorphin A $_{1-8}$ to produce antinociception either alone or after co-administration with either the endopeptidase-24.15 inhibitor cFP-AAF-pAB or the endopeptidase-24.11 inhibitor, cFP-F-pAB. This evaluation was conducted across a time course of 15-90 minutes post-injection, and utilized dynorphin doses ranging from 1-20 ug. Proper inhibitor doses were determined based on results from experiment 1a. If indeed dynorphin antinociception is limited by rapid endopeptidase-24.15 degradation, co-administration of the endopeptidase-24.15 inhibitor cFP-AAF-pAB with dynorphin should lead to increases in nociceptive thresholds. In contrast, negative effects are expected for the endopeptidase-24.11 inhibitor cFP-F-pAB.

Experiment 2b examined opioid involvement in any observed changes after co-administration of dynorphin with CFP-AAF-PAB. Animals were pretreated 15 minutes prior to co-administration with vehicle and the general opiate receptor antagonist naloxone. It was expected that naloxone pretreatment will attenuate any observed increases in threshold following co-administration.

In order to determine if dynorphin A $_{1-8}$ / endopeptidase 24.15 inhibitor induced antinociception is not mediated by the dynorphin-processing product leu-enkephalin, experiment 2c compared a separate group of rats pretreated with one of three opioid receptor antagonists: ß-FNA (mu-selective), nor-BNI (kappa-selective), and the general opioid antagonist naltrexone before co-administration of cFP-AAF-pAB with dynorphin A $_{1-8}$. Dynorphin A $_{1-8}$ has a greater affinity for kappa receptors than leu-enkephalin, and dynorphin peptide effects are thought to be kappaergic. An attenuation of nociceptive threshold increases after nor-BNI, but not ß-FNA indicates kappaergic involvement.

Experiment 2d controled for the possibility that previously reported motor impairments following dynorphin administration (Caudle and Isaac 1987; Herman et al. 1980; Long et al. 1988) would be responsible for possible antinociceptive effects. To

evaluate this, locomotor counts, righting reflexes and performance on an inclined plane were assessed following co-administration of cFP-AAF-pAB and dynorphin A $_{1-8}$ at a dose determined from experiment 2a to be effective for antinociception.

Experiment 3 evaluated the pro-enkephalin-derived octapeptide met-Enkephalin-Arg^6-Gly^7-Leu^8 (MERGL, 3a) and pentapeptide met-enkephalin (3b) upon nociceptive thresholds before and after co-administration with cFP-AAF-pAB and cFP-F-pAB. Whereas endopeptidase-24.15 degrades MERGL, endopeptidase-24.11 degrades met-enkephalin. Although the pro-enkephalin-derived opioid peptide MERGL has been shown to possess antinociceptive potency (Iadarola et al. 1985), its actions are short-lived due to its rapid inactivation by peptidases (Patey et al. 1983), particularly endopeptidase-24.15 (Chu and Orlowski 1985; Acker et al. 1987). Inhibitors of endopeptidase-24.15 have been shown to increase recovery of MERGL in vitro (Acker et al. 1987; Orlowski and Chu 1985). Although MERGL contains the met-enkephalin sequence, and this sequence of amino acids is susceptible to hydrolysis by endopeptidase-24.11, endopeptidase-24.11 is less effective than endopeptidase-24.15 upon peptides with C-terminal extensions. It was therefore expected that co-administration of the endopeptidase-24.15 inhibitor cFP-AAF-pAB will potentiate the antinociception induced by MERGL to a greater extent than would the endopeptidase-24.11 inhibitor cFP-F-pAB. Conversely, it was expected that cFP-F-pAB, but not cFP-AAF-pAB, would potentiate met-enkephalin antinociception. Because MERGL and met-enkephalin are rapidly degraded, assessment of latencies and thresholds began 5 minutes following co-administration and lasted for 90 minutes at a dose range of 25-200 ug.

Experiment 4 evaluated the effects of endopeptidase inhibitors upon endogenous opioid antinociception activated in response to an environmental stressor, cold-water swims. Rats were placed in a 5 degree Celsius bath alternately for 5 seconds and removed for 5 seconds, ten times (total time = 1'40"). This procedure is known to increase nociceptive thresholds via an opioid mechanism (reversed by naloxone) that is not paralleled by hypothermia (Bodnar et al. 1980; Romero et al. 1988). Antinociception induced by the swim was compared to rats pretreated with either cFP-AAF-pAB (endopeptidase 24.15 inhibitor) or cFP-F-pAB (endopeptidase 24.11 inhibitor). Antinociception and core body temperature was measured 30-60-90 minutes post-swims. The results from this experiment addressed the possible modulatory role of endopeptidase 24.15 upon pre-existing opioid pools activated phasically in response to stress, but also upon core body temperature changes in response to stress.

Because parametric variations such as pattern and temperature determines the physiological substrates (opioid vs. nonopioid) mediating the antinociceptive response of

cold-water swims (Bodnar et al. 1980; Bodnar and Sikorszky 1983; Christie et al. 1981; Christie et al. 1982 a, b; O'Connor and Chipkin 1984; Girardot and Holloway 1984 a, b; Girardot and Holloway 1985; Romero et al. 1988), the general opioid antagonist naloxone (5 mg/kg) was administered sc 20 min prior to cold-water swims (experiment 4b). Attenuation or block of swim-induced antinociception would indicate that the physiological substrates mediating the antinociceptive effect are opioid in nature.

IV. GENERAL METHODS

The care, use and well-being of the subjects used in this dissertation, as well as all procedures and protocols, were under the approval of, and complied with, the rules and regulations set forth by the Queens College Institutional Animal Care and Use Committee (IACUC). Towards this end, between 6-12 rats were used for each experimental drug condition. This reflects a compromise between using large groups for statistical power and small groups that limit the number of animals exposed to stressful procedures and sacrifice.

A. Surgery.

Subjects. Male albino Sprague-Dawley rats weighing between 400-500 grams were employed as subjects for all tests in all experiments. Animals were housed individually in the Queens College Vivarium Facility and maintained on a 12 hour light-12 hour dark schedule at ambient temperatures between 22 and 25 degrees Celsius. Rat chow and water were accessible ad libitum.

ICV Cannulation. Before surgery, rats were pretreated with chlorpromazine (3 mg/kg, IP) 20 min prior to anesthesia with Ketamine HCl (100 mg/kg IM). One stainless steel guide cannula was stereotaxically (Kopf Instruments) aimed so that its tip was positioned 0.3 mm above the left lateral ventricle. With the incisor bar set at +5 mm, these coordinates were 0.5 mm anterior to the bregma suture and 3.6 mm from the top of the skull. The cannula was secured to three stainless steel anchor screws with dental acrylic. All animals were allowed to recover for 10 days before behavioral testing began.

Placement Verification. Following experimental testing, each animal was anesthetized with a barbiturate mixture (Euthanasia #5, H. Schein Co.), sacrificed, and its brain removed. The brains were cut through the lateral ventricle, and examined visually to verify cannula placement. Only animals with cannula placements confirmed to the lateral ventricles were included in the statistical analysis.

B. Tests.

Nociception.

Tail-Flick Test. This test measures reactivity to thermal heat directed at the tail (D'Amour and Smith 1941). The stimulus source (IITC Company, Woodland Hills, Ca.) was mounted 8 cm above the dorsum and 3-8 cm proximal to the tip of the tail of a lightly restrained rat. The intensity of the thermal stimulus was set to produce stable baseline tail-flick latencies between 2.5-3.5 sec. Each tail-flick session consisted of three latency determinations made at 10-sec. intervals. In order to avoid tissue damage, a trial was terminated if a response did not occur within 10 sec. The mean of the three determinations in sec constituted the final latency score for that trial.

Jump Test. This test measures reactivity to electric shock of the paws (Evans 1961). Rats were placed in a 30 cm x 24 cm chamber with a floor consisting of 16 grids. Electric shock was delivered to the grids by a shock generator (BRS/LVE) through a shock scrambler (Campden Instruments). Using an ascending method of limits procedure, the jump threshold was defined in mA as the lowest of two consecutive intensities in which the animal simultaneously removed both hindpaws from the grids. Each trial began with an animal receiving a 300 msec footshock at a current intensity of 0.10 mA. Subsequent shocks were increased in 0.05 mA increments at 10-sec intervals until the jump threshold was determined. After each trial, the current intensity was reset to 0.10 mA and the procedure repeated until six trials were completed. The mean of the six determinations became the threshold value in mA for that trial. Because of time constraints, in some experiments only three determinations contributed to the means.

The order of tail-flick latency determinations followed by jump threshold determinations was employed because this yields minimal carry-over effects in baseline testing (Kelly 1982). Baseline latencies and thresholds were determined over at least four days before any experimental conditions began in order to establish stability of nociceptive thresholds.

Motor tests.

Inclined Plane. Rats were placed in the middle of of a wire-mesh plane which was subsequently inclined to 45 and 90 degree angles, and their ability to maintain position was assessed.

Righting Latency. Righting reflexes were evaluated by lightly restraining each rat, placing them upside down and recording latency to righting. Latencies greater than 1 sec were considered to be indicative of impairment.

Activity Counts. Rats were assessed for locomotor activity by placing them in a 19" X 10.5" X 8" plastic cage situated on top of an Omicron Activity Meter (Omnitech) with an LED readout counter, which indicated the number of vertical and horizontal movements.

C. Drugs.

All centrally-administered drugs were dissolved in distilled water to desired concentration so that volume (5 ul) remained constant.

Endopeptidase Inhibitors.

cFP-AAF-pAB. N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-Alanine-Phenylalanine-p-aminobenzoate (K_i = 0.03 uM; Fig. 1) was prepared as described previously (Orlowski et al. 1988).

cFP-A(D)AF-pAB. N-[1(RS)-Carboxy-3-phenylpropyl]-Alanine-(D)Alanine-Phenylalanine-p-aminobenzoate (K_i = 1.5 uM; Fig. 1) was prepared as previously described (Orlowski et al. 1988).

cFP-F-pAB. N-[1(RS)-Carboxy-3-phenylpropyl]-Phenylalanine-p-aminobenzoate (K_i = 0.038 uM; Fig. 1) was prepared as previously described (Almenoff and Orlowski 1988). Acetic acid (0.2%) was added to distilled water to adjust the pH to 7.0 and facilitate solubility.

Opioid Peptides.

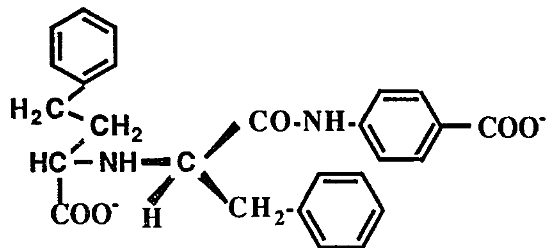
Dynorphin A_{1-8}, Met-enkephalin-Arg^6-Gly^7-Leu^8, and Met-enkephalin. Dynorphin A_{1-8}, met-enkephalin-Arg^6-Gly^7-Leu^8, and met-enkephalin were purchased from Peninsula Laboratories.

Opiate Receptor Antagonists.

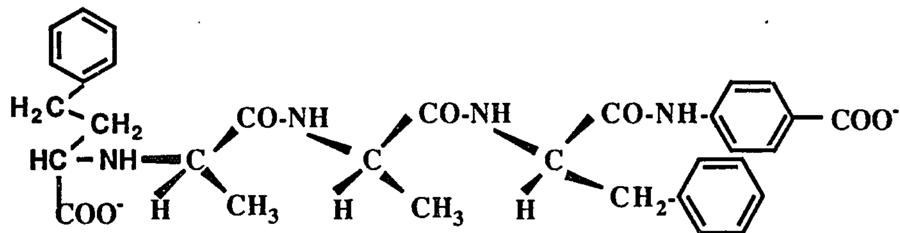
Naloxone, Naltrexone, Nor-BNI, and ß-FNA. Naloxone hydrochloride and naltrexone were purchased from Sigma Drugs. nor-BNI (nor-binaltorphimine) and ß-FNA (beta-funaltrexamine)were purchased from Research Biochemicals.

FIGURE 1. Structure, enzyme preference, and K_i values of the endopeptidase-24.11 inhibitor cFP-pAB, and the endopeptidase-24.15 inhibitors cFP-AAF-pAB, and cFP-A(D)AF)-pAB.

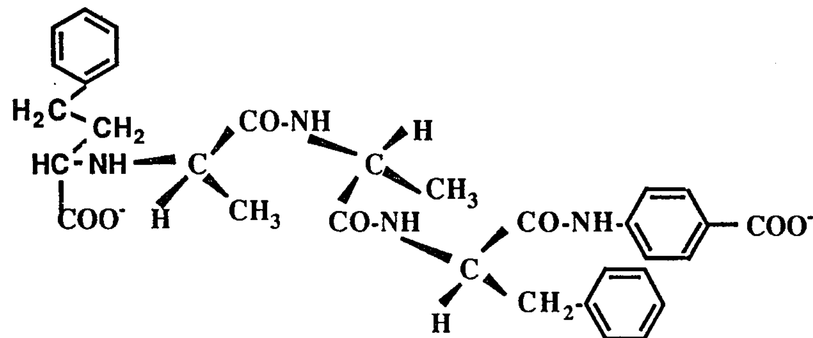
Inhibitor	Enzyme	K_I
1. N-[1(RS)-carboxy-3-phenylpropyl]-Phe-pAB	Endopeptidase 24.11	0.038 μM



Inhibitor	Enzyme	K_I
2. N-[1-[RS]-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB	Endopeptidase 24.15	0.03 μM



Inhibitor	Enzyme	K_I
3. N-[1-[RS]-carboxy-3-phenylpropyl]-Ala-D-Ala-Phe-pAB	Endopeptidase 24.15	1.5 μM



D. Drug Administration.

ICV Microinjection. All central injections were placed intracerebroventricularly (icv). All drugs and vehicle were infused in a 5 ul volume by a Hamilton syringe attached to polyethylene tubing at a rate of 1 ul every 10 sec through a 28 gauge stainless steel internal cannula (Plastic Products). Internal cannulaes protruded 0.5 mm beyond the tip of the guide cannula while rats were lightly restrained.

Every animal in every condition across all experiments first received an intracerebroventricular vehicle (saline) injection and then tested before receiving any experimental drug treatment. In co-administration paradigms in which animals received an inhibitor injection in addition to peptide, animals undergoing vehicle testing were co-administered physiological saline as a control (i.e. vehicle-vehicle). Except for naloxone in experiments 1, 2, and 4, all drugs were administered intracerebroventricularly according to the above method.

Co-administration. Individual injections in co-administration protocols were via icv microinjection procedures and separated by a latency of 10 minutes.

Sub-Cutaneous. Injection. Sub-cutaneous injections were administered using a 1cc plastic syringe (B & D Products) into the loose folds of the neck in lightly restrained rats. Only naloxone was administered in this way.

E. Statistical Analyses.

Difference Scores and ANOVA. Data obtained from nociceptive tests were converted to difference scores by subtracting the value of the experimental condition from that subject's corresponding vehicle value. This facilitates comparisons between groups with differing baseline values. All nociceptive statistical tests were performed upon the converted difference scores. These difference scores were then used to determine peak antinociception, which represents the largest mean increase relative to vehicle. Total antinociception scores, which were derived by summing all the difference scores across the entire testing time course for that condition, were similarly derived using difference scores.

Split-plot analyses of variance were used to assess significant drug effects upon nociceptive thresholds over corresponding vehicle values; in the case of significance, Dunnett and Dunn comparisons ($p < .05$) were employed to evaluate significant time and dose effects relative to vehicle values and other treatment conditions respectively.

Regression Analyses. To determine alterations in dose-response functions, linear regression analyses evaluated peak and total antinociceptive effects relative to the logarithmic transformation of inhibitor and peptide doses to determine significant differences among slopes, intercepts, and the ED_{50} using 95% confidence intervals.

Motor Tests. Paired Student's *t*- tests and Wilcoxon non-parametric analyses were used to evaluate the effects upon activity and motor performance respectively (experiments 1c and 2d).

V. EXPERIMENT 1: ENDOPEPTIDASE -24.15 AND -24.11 INHIBITORS AND BASAL ANTINOCICEPTION.

Endopeptidase-24.15 and -24.11 are two brain metalloendopeptidases active in opioid peptide degradation (Acker et al. 1987; Chu and Orlowski 1984; Almenoff et al. 1981; Altstein et al. 1981; Fulcher et al. 1982). Inhibitors of endopeptidase-24.11 have been shown to slow the in vivo hydrolysis of endogenous enkephalins (DeLabaume et al. 1983; Malfroy and Schwartz 1982, 1984) and to produce antinociception after central administration (Chipkin et al. 1988; Chipkin et al. 1982; Murthy et al. 1984). Although inhibitors of endopeptidase-24.15 have also been shown to slow the in vivo and in vitro degradation of several endogenous opioids (Acker et al. 1987; Chu and Orlowski 1984; Orlowski et al. 1988; Molineaux and Ayala 1990), it is not known what antinociceptive effects such inhibitors would have after central administration. In the present experiment, inhibitors of endopeptidase-24.15 were administered intracerebroventricularly and compared with an endopeptidase-24.11 inhibitor for antinociceptive ability. Opioid-participation and motoric dysfunction in any observed nociceptive threshold changes were also evaluated. Parts of the following experiment have been published (Kest et al. 1991).

Methods

Protocol 1a. Twenty-one naive rats were assigned to one of three inhibitor treatments based upon matched baseline latencies and thresholds following central vehicle injections. Each rat then received two to three icv injections of either cFP-A(D)AF-pAB, cFP-AAF-pAB, or cFP-F-pAB, within a dose range of 0.25-250 nmols utilizing one log unit increments (cFP-A(D)AF-pAB: 0.25 nmol, n= 3; 2.5 nmol, n= 5; 25 nmol, n= 5; 250 nmol, n= 4; cFP-AAF-pAB: 0.25 nmol, n= 3; 2.5 nmol, n= 7; 25 nmol, n= 7; 250 nmol, n= 4; cFP-F-pAB: 0.25 nmol, n= 3; 2.5 nmol, n= 6; 25 nmol, n= 7; 250 nmol, n= 4). Latencies were assessed 1, 3, 5, and 7 hours post-injection. A period of at least three days elapsed between injections.

Protocol 1b. To evaluate opioid involvement in nociceptive threshold changes, nine naive rats received the following four central injections at weekly intervals: vehicle, and a 25 nmol dose of cFP-A(D)AF-pAB, cFP-AAF-pAB, and cFP-F-pAB. Latencies and thresholds were assessed 5 and 7 hrs thereafter with naloxone (1 mg/kg, sc) administered 15 minutes prior to the 7 hr test.

Protocol 1c. To examine whether any inhibitor-induced changes in nociceptive thresholds are due to motor dysfunction, eight naive rats received two icv injections of vehicle and two of cFP-AAF-pAB (250 nmol). One vehicle and one inhibitor were followed by assessment of locomotor activity; the others were followed by assessments of righting reflexes and performance on an inclined plane. All assessments were conducted 1, 3, 5, and 7 hrs post-injection.

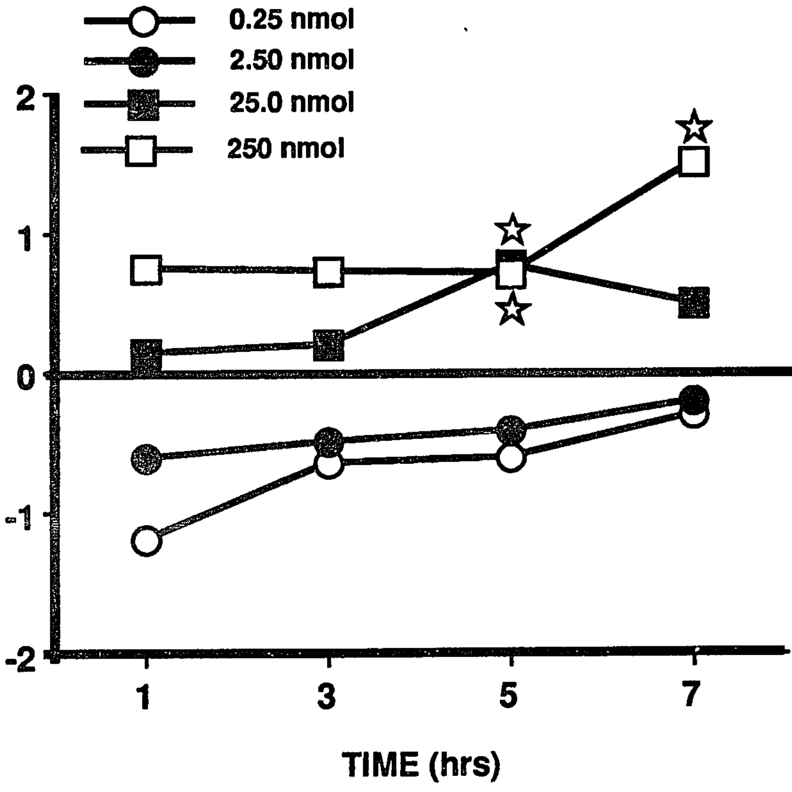
Results

Endopeptidase Inhibitors and Basal Antinociception. Figure 2 illustrates the respective alterations in tail-flick latencies (top panels) and jump thresholds (bottom panels) following administration of the endopeptidase-24.15 inhibitor cFP-A(D)AF-pAB. cFP-A(D)AF-pAB produced small, significant increases in tail-flick latencies at doses of 250 (F (1,3)= 12.1, $p < .01$; peak: 36%) and 25 (F (1,4)= 4.3, $p < .05$; peak: 25%) nmol, and small, significant peak increases in jump thresholds after doses of 250 (F (1,3)= 78.3, $p < .01$; peak: 59%) and 25 (F (1,4)= 13.8, $p < .05$; peak: 21%) nmol. In contrast, the 0.25 (flick: F (1,4)= 6.59; jump: F (1,4)= 6.10) and 2.5 (flick: F (1,2)= 3.60; jump: F (1,2)= 0.14) nmol doses were ineffective on both tests. No significant time effects for antinociception was observed following any dose on the tail-flick test, but significant time effects on the jump test were observed after the 250 nmol dose only (F (3,9)= 4.82, $p < .05$). Significant interactions between time and dose were observed on the tail-flick test after the 0.25 nmol dose (F (3,6)= 5.27, $p < .05$), and after the 50 (F (3,9)= 7.31, $p < .01$) nmol dose on the jump test.

cFP-AAF-pAB (Fig. 3) also produced significant tail-flick latency increases at doses of 250 (F (1,3)= 25.4, p = 01; peak: 36%) and 25 (F (1,6)= 5.42, p = .05; peak: 25%) nmol, as well as on jump thresholds after doses of 250 (F (1,3)= 101.9, $p < .01$; peak: 59%) and 25 (F (1,6)= 4.11, $p < .05$); peak: 21%) nmol. Significant time effects were not observed for the tail-flick test. Time effects were however observed on the jump test after the 250 (F (3,9)= 5.25, $p < .05$) 25 (F (3,18)= 11.7, $p < .001$) and the 2.5 (F (3,18)= 7.28, $p < .01$) nmol doses. Interactions between time and dose failed to occur on either the tail-flick or jump tests.

Figure 4 illustrates alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) after administration of cFP-F-pAB. Whereas the inhibitor failed to alter tail-flick latencies at any dose level (250 nmol: F (1,3)= 4.13); 25 nmol: F (1,6)= 2.7; 2.5 nmol: F (1,5)= 3.04; 0.25 nmol: F (1,2)= 0.01), significant increases in jump thresholds following the 250 (F (1,3)= 108.27, $p < .01$; peak: 41%), 25 (F (1,6)= 205.6, $p < .001$; peak: 37%), and 2.5 (F (1,5)= 23.96, $p < .01$; peak: 35%) nmol doses were observed.

FIGURE 2. Alterations in tail-flick latencies(top panel) and jump thresholds (bottom panel) following icv administration of cFP-A(D)AF-pAB. Significant differences relative to vehicle were observed after the 250 and 25 nmol doses on both tests, and are indicated by stars (Dunnett comparisons, $p < .05$).


LATENCY DIFFERENCE SCORES (sec)
0.25 nmol
2.50 nmol
25.0 nmol
250 nmol
2
1
0
-1
-2
1
3
5
7
TIME (hrs)

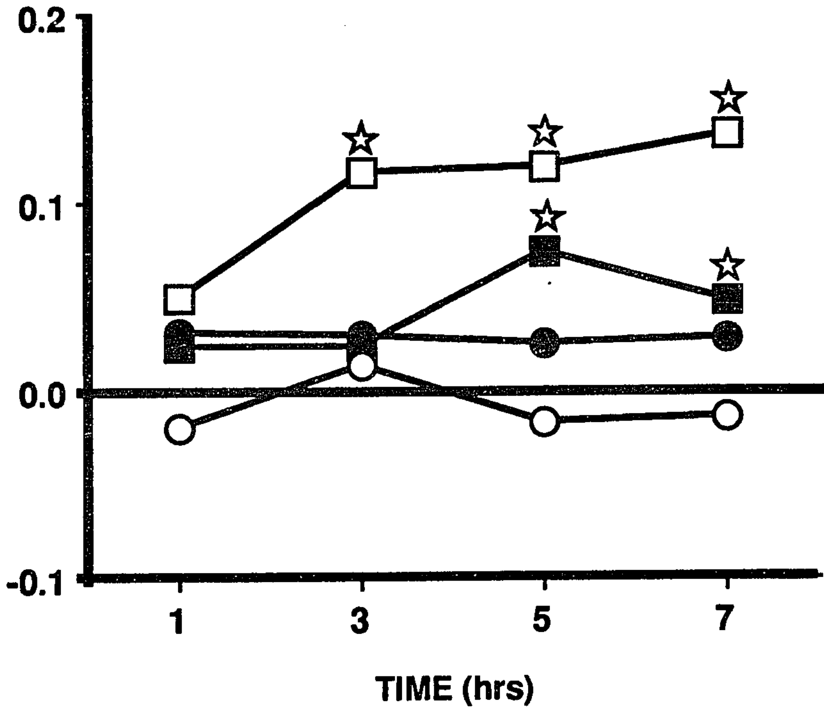
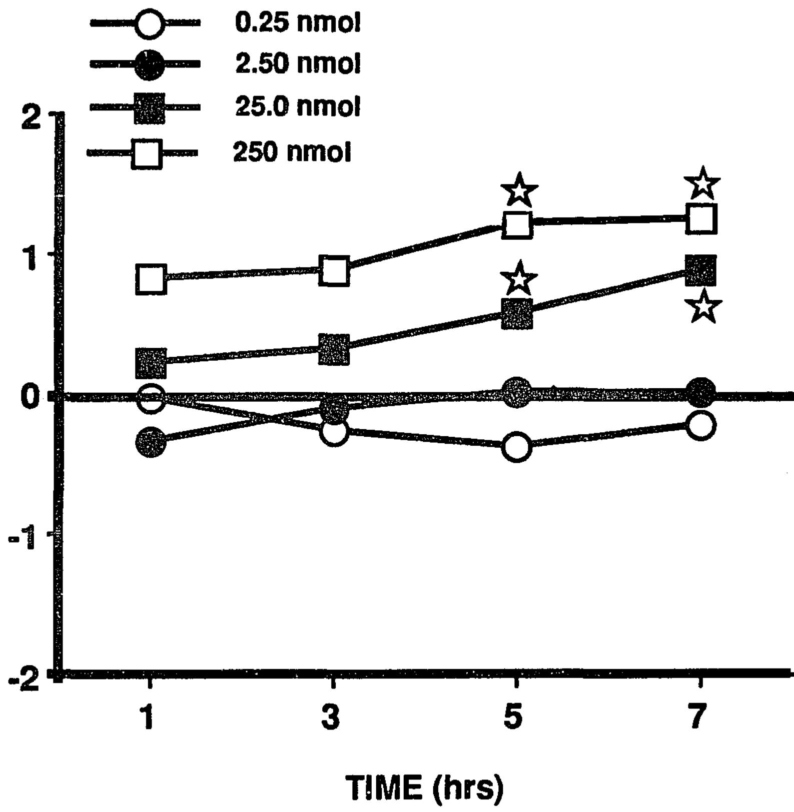

THRESHOLD DIFFERENCE SCORES (mA)
0.2
0.1
0.0
-0.1
1
3
5
7
TIME (hrs)

FIGURE 3. Alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) following icv administration of cFP-AAF-pAB. Significant differences relative to vehicle were observed after the 250 and 25 nmol doses on both tests, and are indicated by stars (Dunnett comparisons, $p < .05$).


0.25 nmol
2.50 nmol
25.0 nmol
250 nmol
LATENCY DIFFERENCE SCORES (sec)
2
1
0
-1
-2
1
3
5
7
TIME (hrs)

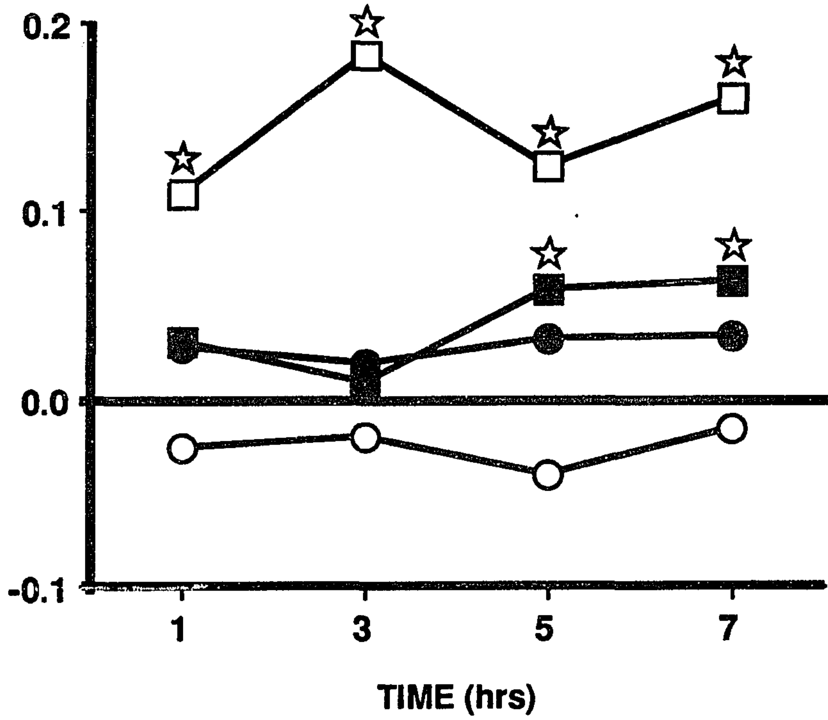
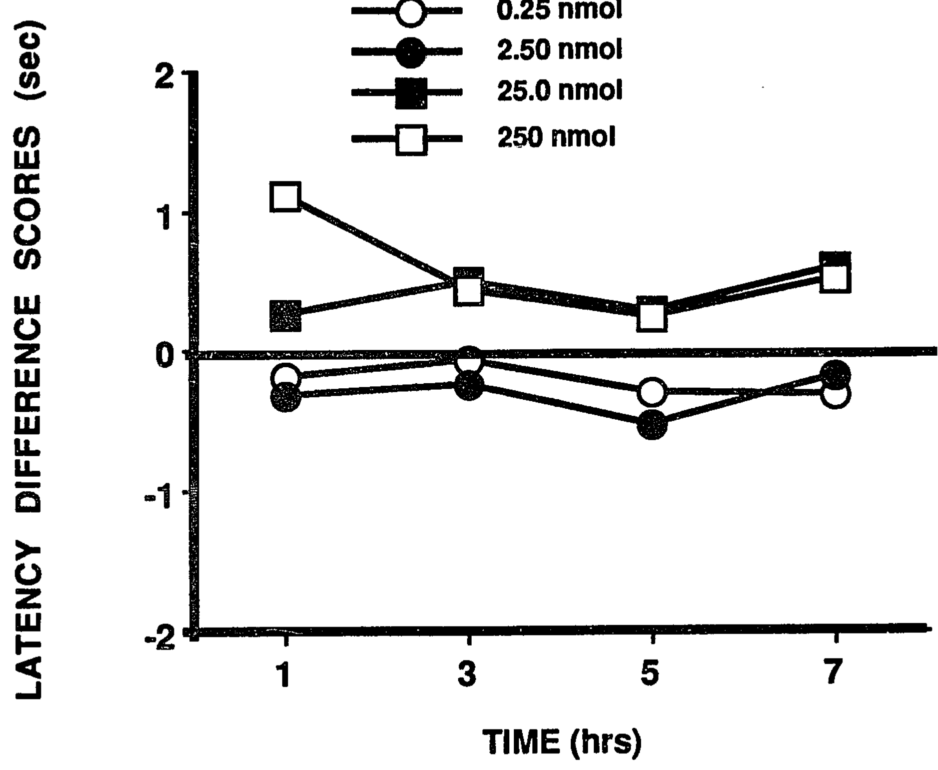
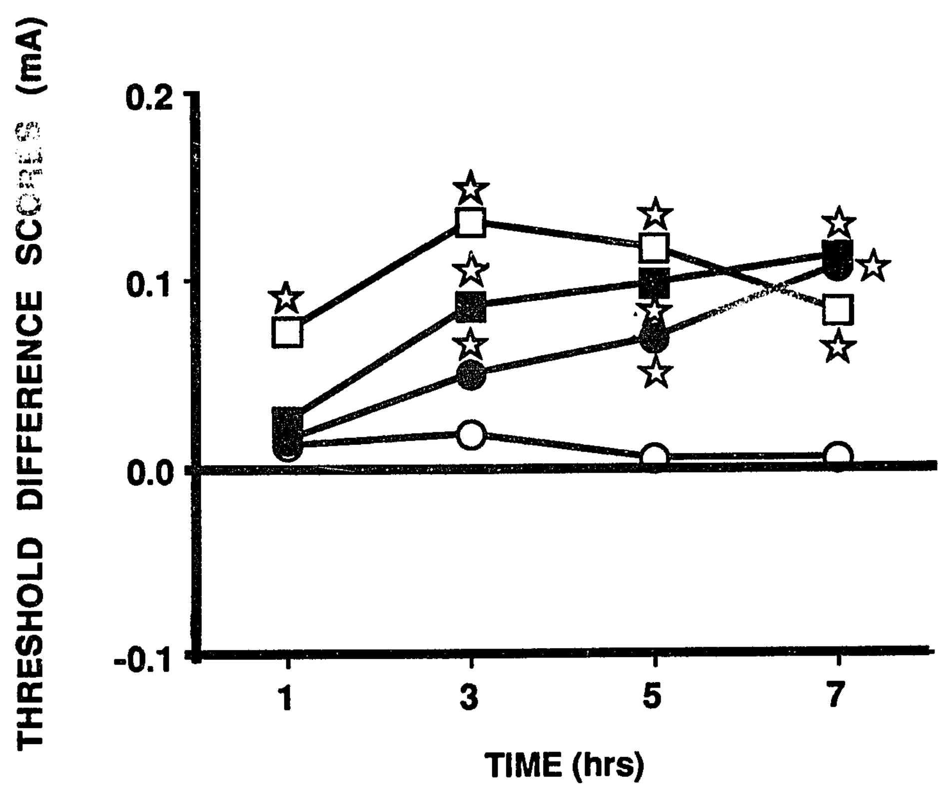

THRESHOLD DIFFERENCE SCORES (mA)
0.2
0.1
0.0
-0.1
1
3
5
7
TIME (hrs)

FIGURE 4. Alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) following icv administration of cFP-F-pAB. Significant differences relative to vehicle were observed after the 250, 25, and 2.5 nmol doses on the jump test only, and are indicated by stars (Dunnett comparisons, $p < .05$).


0.25 nmol
2.50 nmol
25.0 nmol
250 nmol
LATENCY DIFFERENCE SCORES (sec)
2
1
0
-1
-2
1
3
5
7
TIME (hrs)


THRESHOLD DIFFERENCE SCORES (mA)
0.2
0.1
0.0
-0.1
1
3
5
7
TIME (hrs)

Significant time effects were not observed on the tail-flick test, but were observed on the jump test following the 250 (F (3,9)= 14.6, $p < .001$), 25 (F (3,18)= 5.44, $p < .01$), and 2.5 (F (3,15)= 5.50, $p < .01$) nmol doses. Interactions between time and dose failed to occur on the tail-flick test, and occurred only after the 25 (F (3,18)= 7.52, $p < .001$) and 2.5 (F (3,15)= 10.3, $p < .001$) nmol doses on the jump test.

Naloxone Reversibility. Figure 5 illustrates alterations in tail-flick latencies and jump thresholds by naloxone following injection of the three endopeptidase inhibitors. Significant increases in tail-flick latencies after 5 h failed to occur following cFP-A(D)A)F-pAB (F (1,8)= 0.35), but were observed following cFP-AAF-pAB (F (1,6)= 8.54, $p < .05$) and cFP-F-pAB (F (1,8)= 13.3, $p < .01$). Only the antinociception observed following cFP-F-pAB was significantly reversed by naloxone on the tail-flick test (F (1,8)= 5.4, $p < .05$; reduction: 80%). Significant increases in jump thresholds after 5 h were observed following cFP-A(D)AF-pAB (F (1,8)= 9.10, $p < .01$), cFP-AAF-pAB (F (1,6)= 9.72, $p < .05$), and cFP-F-pAB (F (1,8)= 6.25, $p < .01$). Naloxone significantly reversed the antinociception on the jump test induced by cFP-A(D)AF-pAB (F (1,8)= 23.6, $p < .001$; reduction: 63%), cFP-AAF-pAB (F (1,6)= 6.03, $p < .05$; reduction: 72%) and cFP-F-pAB (F (3,24)= 6.25, $p < .01$; reduction: 90%). Vehicle thresholds failed to be affected by naloxone on either the tail-flick or jump tests.

Motor Performance and Activity Levels. Table 1 summarizes locomotor activity counts after central cFP-AAF-pAB injection. The inhibitor failed to significantly alter locomotor activity counts relative to vehicle after 1 (t (6) = 1.39), 3 (t (6) = -0.6), 5 (t (6) = -1.14), or 7 (t (6) = -0.06) hrs. Normal righting reflexes were observed in all animals following administration of both vehicle and cFP-AAF-pAB across the entire 7 h time course (Wilcoxon test). Similarly, performance on an inclined plane failed to differ between vehicle and cFP-AAF-pAB treatments across the entire 7 h time course (Wilcoxon test).

Discussion

Central administration of cFP-A(D)AF-pAB and cFP-AAF-pAB, two inhibitors of brain endopeptidase-24.15 activity, produced a dose- and time- dependent increase in basal nociceptive thresholds which was more consistent on the jump test than on the tail-flick test. Further, this antinociception was comparable in magnitude to that obtained after central administration of cFP-F-pAB, an inhibitor of endopeptidase-24.11, an enzyme generally believed to be involved in the in vivo degradation of enkephalins (De Labaume et al. 1983; Malfroy and Schwartz, 1982, a & b). The antinociception produced by

FIGURE 5. Comparison of naloxone (1 mg/kg, sc) effects upon alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) following icv administration of vehicle and 25 nmol of cFP-A(D)AF-pAB, cFP-AAF-pAB, and cFP-F-pAB respectively. Light and dark stars indicate significant differences (Dunnett comparisons, $p < .05$) between vehicle and corresponding drug conditions, and between pre- (5 hr) and post- (7 hr) naloxone data values respectively.

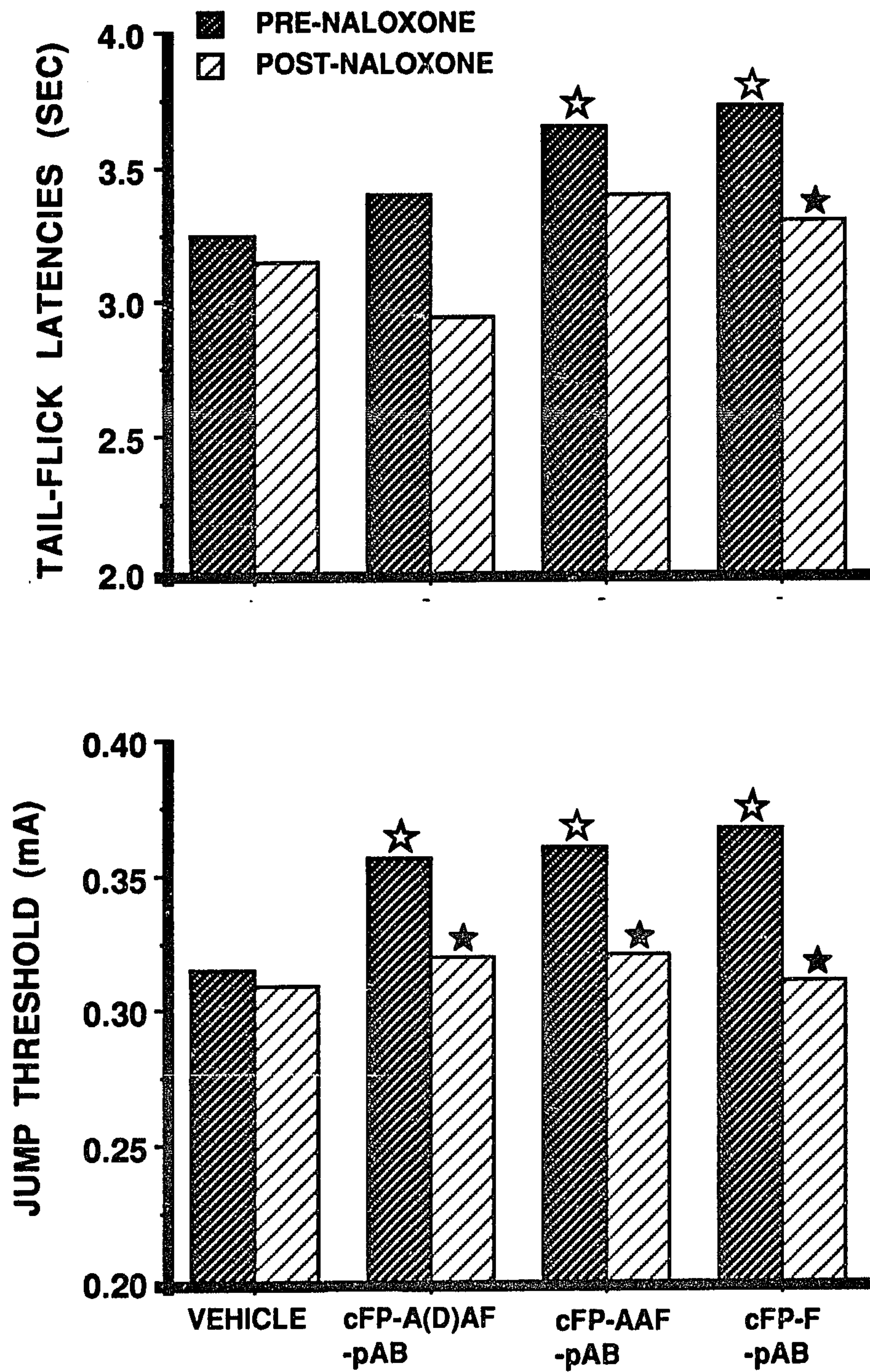

PRE-NALOXONE
POST-NALOXONE
TAIL-FLICK LATENCIES (SEC)
4.0
3.5
3.0
2.5
2.0
JUMP THRESHOLD (mA)
0.40
0.35
0.30
0.25
0.20
VEHICLE
cFP-A(D)AF
-pAB
cFP-AAF
-pAB
cFP-F
-pAB

TABLE 1. Vehicle and Endopeptidase-24.15 Inhibitor cFP-AAF-pAB (250 nmol) Effects Upon Activity Levels Over 7 Hrs in Rats.

HR (post-inj)	1		3		5		7	
TREATMENT	Veh	EPI	Veh	EPI	Veh	EPI	Veh	EPI
MEAN	413	208	166	207	164	307	261	307
SD[1]	295	263	97	196	141	206	155	199

[1] SD: Standard Deviation

cFP-A(D)AF-pAB and cFP-AAF-pAB was also comparable in magnitude to the previously-reported antinociceptive effects induced by other inhibitors of endopeptidase-24.11, acetorphan, thiorphan, and SCH34826 (Chipkin et al. 1982, 1988; Murthy et al. 1984; Roques et al. 1980, Yaksh and Harty 1982). Since some of the antinociceptive effects were significantly reduced by the general opioid antagonist naloxone, it can be assumed that the actions of the inhibitors on these effects are mediated through the opioid peptide system. The antinociceptive effects were not accompanied by deficits in either locomotor activity, righting reflexes, or performance on an inclined plane, suggesting that the alterations in nociceptive thresholds were due to sensory rather than motor system changes.

While the inhibitors of endopeptidase -24.15 and -24.11 were all effective at a dose of 25 nmol and produced somewhat comparable dose-response curves, their potency was quite modest as compared to central administration of opiates or synthetic opioid compounds. Optimal doses (250 nmol) of each inhibitor produced peak antinociception which resulted in an approximately 50% threshold increase. These antinociceptive effects were also time-dependent, with peak effects appearing 5-7 h after injection. Endogenous pain inhibitory systems have been postulated to act in a phasic, rather than tonic, manner (Fields and Basbaum 1978; Yaksh and Rudy 1978). This suggests that relatively low levels of endogenous opioids would be released over time. Endogenous peptide pools protected from degradation by endopeptidase inhibitors would be initially low, but might rise slowly in time. Hence, a threshold level of protected peptides necessary to elicit antinociception would be available only after several hours, which agrees with the present results.

Test-specific effects were observed for the inhibitors with greater antinociception observed on the supraspinally-mediated jump test than on the spinally-mediated tail-flick test. Such effects might reflect a greater sensitivity to the central ventricular route relative to the intrathecal route of drug administration, or might indicate greater modulation by the peptidases and hence the inhibitors of supraspinal pools of endogenous opioid peptides.

The ability of naloxone to reverse antinociception after 7 hrs was equivocal. While all of the inhibitors produced antinociception on the jump test that was naloxone reversible, only cFP-F-pAB produced antinociception that was reversible on the tail-flick test. However, this may be attributable to the test-specific effects observed in other protocols of experiment 1. For example, all of the inhibitors produced significant antinociception on the jump test after 5 hrs. In contrast cFP-A(D)AF-pAB failed to produce significant antinociception on the tail-flick test. While cFP-AAF-pAB antinociception on the tail-flick

test was not significantly reversed by naloxone after 7 hrs, naloxone did attenuate the antinociception by more than 65%. This stands in contrast to the previously observed increase (25%) in tail-flick latencies produced by cFP-AAF-pAB after 7 hrs (experiment 1a). Still, while no conclusive statements can be made regarding the opioid nature of the tail-flick latency increases, the significant naloxone-reversals observed on jump test antinociception suggests that opioid synapses are involved. It is however conceivable that these inhibitors prevent degradation of nonopioid peptides which produce opioid-mediated antinociception. For example, both endopeptidase -24.15 and -24.11 degrade neurotensin and substance P. Although the centrally-mediated antinociception induced by substance P is blocked by naloxone (Frederickson et al. 1978), neurotensin antinociception is insensitive to naloxone effects (Osbahr et al. 1981).

Finally, experiment 1c demonstrated that the increases in basal nociceptive thresholds after cFP-AAF-pAB administration are due to sensory rather than motor changes since the largest dose of the endopeptidase-24.15 inhibitor cFP-AAF-pAB failed to alter motor abilities on three different measures of motor ability.

The major conclusions stemming from experiment 1 are: a), that the endopeptidase-24.15 inhibitors cFP-A(D)AF-pAB and cFP-AAF-pAB produce gradually appearing alterations upon basal nociceptive thresholds over 7 hrs; b), that these antinociceptive effects are comparable to the antinociception produced by the endopeptidase-24.11 inhibitor cFP-F-pAB; c), that these effects appear to utilize an opioid synapse; and d), that endopeptidase-24.15 inhibitor effects are due to changes in sensory rather than motor systems.

VI. EXPERIMENT 2: DYNORPHIN A $_{1-8}$ ANTINOCICEPTION: INTERACTIVE EFFECTS WITH ENDOPEPTIDASE -24.15 AND -24.11 INHIBITORS.

Dynorphin has been shown to produce poor antinociceptive actions which is accompanied by motor dysfunction, after intracerebroventricular or intrathecal administration (Freidman et al. 1981; Chavkin et al. 1982; Walker et al. 1982; Caudle and Isaac 1987; Han and Xie 1982; Herman and Goldstein 1985). However, in vivo and in vitro pharmacological studies show that dynorphin is rapidly degraded, and that inhibition of endopeptidase-24.15 can halt this process (Molineaux and Ayala 1990; Acker et al. 1987; Young et al. 1986; Chu and Orlowski 1985). Furthermore, stable dynorphin analogues have been shown to possess good antinociceptive potency (Walker et al. 1987). It is therefore suggested that the inability of dynorphin-related peptides to alter nociceptive thresholds is due to their rapid inactivation by proteolytic enzymes, especially endopeptidase-24.15. Furthermore, it is possible to distinguish the antinociceptive potency of dynorphin-related peptides from their motor dysfunctional effects (Nakazawa et al. 1989; Walker et al. 1989), which have been attributed to non-opioid dynorphin metabolites (Herman et al. 1980; Long et al. 1988; Stevens and Yaksh 1986). Experiment 2a thus examined the antinociceptive potency of dynorphin A $_{1-8}$ alone and after central co-administration with the respective inhibitors of endopeptidase -24.15 and -24.11, cFP-AAF-pAB and cFP-F-pAB. Subsequent parts of this experiment (protocols 2b and c) also characterized the opioid nature of the observed effects, as well as the involvement of specific opioid receptor subtypes. Finally, subjects were evaluated for motor impairment following co-administration protocols possessing antinociceptive potency (2d).

Methods

Protocol 2a. In order to characterize possible endopeptidase-24.15 modulation of dynorphin A $_{1-8}$ antinociception, naive rats received subsets of the following pairs of icv microinjections in addition to vehicle-vehicle (n= 60): a) vehicle-dynorphin A $_{1-8}$ (20 ug; n= 5); b) vehicle-dynorphin A $_{1-8}$ (5 ug; n= 8); c) cFP-AAF-pAB (25 nmol)-vehicle (n= 5); d) cFP-F-pAB (25 nmol)-vehicle (n= 5); e) cFP-AAF-pAB (25 nmol)-dynorphin A $_{1-8}$ (5 ug; n= 11); f) cFP-AAF-pAB (2.5 nmol)-dynorphin A $_{1-8}$ (5 ug; n= 8); g) cFP-AAF-pAB (25 nmol)-dynorphin A $_{1-8}$ (1 ug; n= 10); h) cFP-F-pAB (25 nmol)-dynorphin A_{1-8} (5 ug; n= 8). Latencies and thresholds were assessed at 15, 30, 45, 60, and 90 minutes

following the second injection. This shorter time course relative to experiment 1 was chosen for two reasons. First, previous in vivo pharmacological work indicates that cFP-AAF-pAB protection of dynorphin from endopeptidase-24.15 is at a maximum over 1 hr. Second, the results of experiment 1 suggest that antinociception produced by the inhibitors themselves is minimal over 90 min. By limiting the antinociception produced by the inhibitors themselves, the assessment of the antinociceptive actions of dynorphin are greatly facilitated. Each rat received a maximum of four injection conditions, including the vehicle-vehicle condition. In this and subsequent protocols of experiment 2, a) the order of drug injections was randomized across animals according to an incompletely counterbalanced design, and b) an interval of seven days were employed between conditions in order to minimize carry-over effects (Yaksh et al. 1976).

Protocol 2b. In order to assess whether changes in nociceptive thresholds were opioid-mediated, six naive rats were co-administered a) vehicle (sc)-vehicle (icv)-vehicle (icv), b) vehicle (sc)-cFP-AAF-pAB (25 nmol; icv)-dynorphin A $_{1-8}$ (5 ug; icv), c) naloxone (1 mg/kg; sc)-vehicle (icv)-vehicle (icv), or d) naloxone (1 mg/kg, sc)-cFP-AAF-pAB (25 nmol; icv)-dynorphin A $_{1-8}$ (5 ug; icv). Latencies and thresholds were assessed at 15, 30, 45, 60, and 90 minutes following the second microinjection.

Protocol 2c. In order to characterize receptor subtype mediation in nociceptive threshold alterations after central co-administration of cFP-AAF-pAB (25 nmol) with dynorphin A $_{1-8}$ (5 ug), naive rats were pretreated with each of the following separated by a one week interval: a) vehicle (n= 9); b) ß-FNA, a long-lasting mu-receptor-specific antagonist (20 ug; n= 9); c) nor-BNI, a kappa-receptor-specific antagonist (20 ug; n= 9); and d) naltrexone, a general opioid antagonist (20 ug; n= 8). All antagonists were administered 1 hr before co-administration of cFP-AAF-pAB with dynorphin A $_{1-8}$ except for ß-FNA, which was administered 24 hr in advance. This schedule is based on time courses previously reported to maximize antagonist effects (Zimmerman et al. 1987; Takemori et al. 1988).Nociceptive thresholds were assessed at 15, 30, 45, 60, and 90 minutes after the second injection.

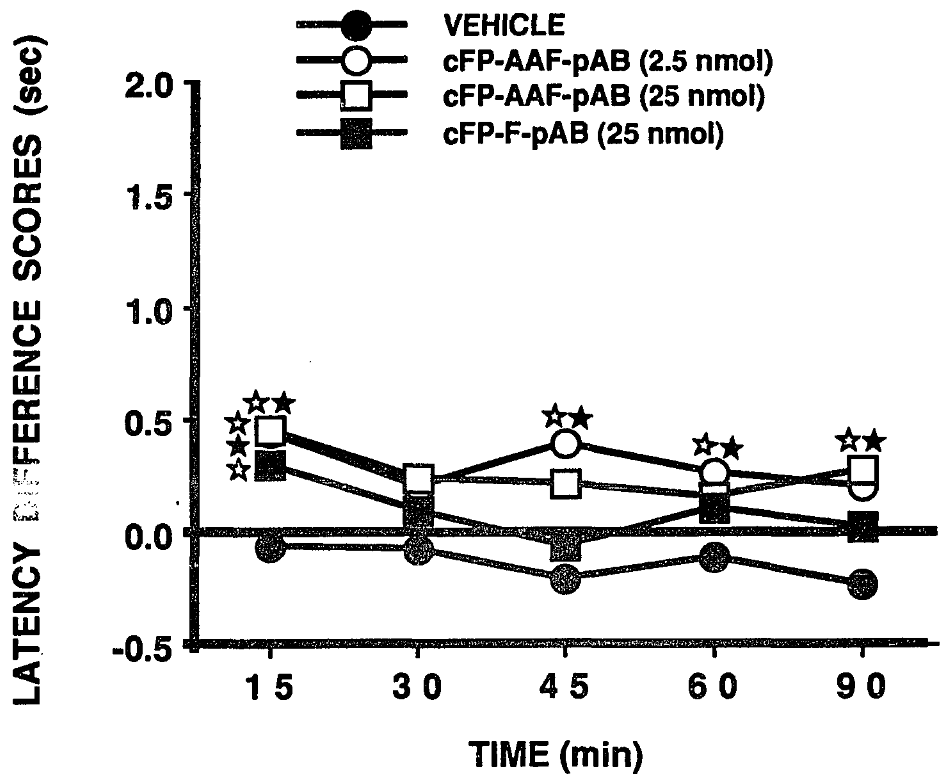
Protocol 2d. Dynorphin has been reported by some authors to induce motor dysfunction (Herman et al. 1980; Long et al. 1988). In order to differentiate between sensory- and motor- mediated alterations in nociceptive thresholds, six naive rats were assessed for locomotor activity, righting reflexes, and performance on an inclined plane after central co-administration of vehicle-vehicle and cFP-AAF-pAB (25 nmol) paired with dynorphin A $_{1-8}$ (5 ug). Immediately after the last injection, animals were assessed for righting reflexes and performance on an inclined plane. Locomotor activity counts were taken at 10 min intervals starting 10 min after the second injection and ending 60

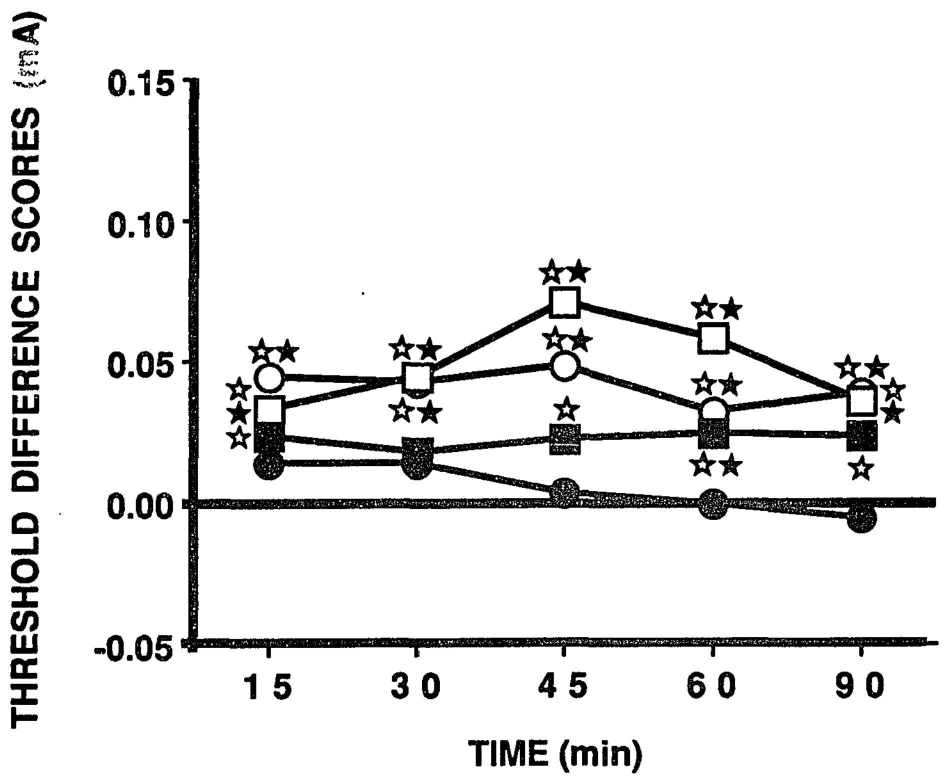
min after (total = 5 readings). Animals were then assessed again for righting reflexes and performance on an inclined plane.

Results

Dynorphin A_{1-8} / Inhibitor Co-administration. Significant treatment effects were observed on both the tail-flick (F (1,52)= 8.72, $p < .01$) and jump (F (1,52)= 17.1, $p < .01$) tests. Significant differences across test times were observed on the jump test (F (4,204)= 4.89)= $p < .001$), but not the tail-flick test (F (4,208)= 0.58). Significant interactions between time and condition failed to occur on either test (tail-flick: F (4,208)= 2.25; jump: F (4,204)= 1.49). Figure 6 illustrates the significant alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) induced by co-administration of dynorphin A_{1-8} (5 ug) with vehicle, cFP-AAF-pAB (25 and 2.5 nmol), or cFP-F-pAB (25 nmol). Vehicle-dynorphin A_{1-8} produced no significant increases in either tail-flick latencies or jump thresholds relative to vehicle-vehicle across the entire 90 min testing time course. In contrast, co-administration of dynorphin A_{1-8} with 25 nmol of cFP-AAF-pAB produced significant increases relative to vehicle-vehicle on tail-flick latencies after 15 (15%) and 90 (9%) min, while the significant increases observed on the jump test lasted throughout the entire 90 min time course (15 min: 10%; 30: 15%; 45: 22%; 60: 18%; 90: 11%). cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5 ug) treatment also increased nociceptive thresholds relative to vehicle-dynorphin A_{1-8} (5 ug) on the tail-flick test after 15 (18%) and 90 (16%) min, and across 90 min on the jump test (15 min: 9%; 30: 13%; 45: 24%; 60: 22%; 90: 17%). The co-administration of dynorphin A_{1-8} with the smaller dose of cFP-AAF-pAB (2.5 nmol) was also effective in producing antinociception. Significant increases relative to vehicle-vehicle were observed on the tail-flick test after 15 (12%), 45 (10%) and 60 (7%) min. Significant jump test increases were observed to last throughout the 90 min of testing (15 min: 14%; 30: 13%; 45: 14%; 60: 10%; 90: 12%). The increases in latencies and thresholds after cFP-AAF-pAB (2.5 nmol)-dynorphin A_{1-8} (5 ug) were also significantly greater than vehicle-dynorphin A_{1-8} (5 ug) on the tail-flick test after 15 min (38%), and between 45-90 min (45 min: 42%; 60: 40%; 60: 37%). Again, the significant increases on the jump test lasted throughout the 90 min testing time course (15 min: 19%; 30: 21%; 45: 29%; 60: 25%; 90: 25%). Co-administration of dynorphin A_{1-8} with cFP-F-pAB (25 nmol) however produced a significant though transient increase in tail-flick latencies relative to vehicle-vehicle at 15 min (8%), and on jump thresholds after 15 (7%) min, and between 45-90 min (45: 6%; 60: 7%; 90: 6%).These increases observed after co-administration with cFP-F-pAB-dynorphin A_{1-8}

FIGURE 6. Alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) following co-administration of dynorphin A $_{1-8}$ (5ug) with vehicle, cFP-AAF-pAB (25 or 2.5 nmol) and cFP-F-pAB (25 nmol). Light and dark stars denote significant differences (Dunnett comparisons, $p < .05$) relative to vehicle-vehicle and vehicle-dynorphin respectively.


VEHICLE
cFP-AAF-pAB (2.5 nmol)
cFP-AAF-pAB (25 nmol)
cFP-F-pAB (25 nmol)
LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
15
30
45
60
90
TIME (min)


THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
15
30
45
60
90
TIME (min)

failed to increase tail-flick latencies relative to vehicle-dynorphin A_{1-8} and did so transiently on jump thresholds after 60 min (24%). Thus, cFP-AAF-pAB produced antinociception that was greater in magnitude and more consistent than cFP-F-pAB. Only the increases produced by cFP-AAF-pAB were observed to be significantly greater than those produced by vehicle-dynorphin A_{1-8}.

The intrinsic antinociceptive potencies of cFP-AAF-pAB and cFP-F-pAB alone were also compared. Table 2 summarizes the alterations in nociceptive thresholds for both cFP-AAF-pAB and cFP-F-pAB over this shorter (90 min) time course. cFP-F-pAB, but not cFP-AAF-pAB, produced a transient though significant increase in tail-flick latencies after 45 min (8%), and increases on the jump thresholds after 45 (6%), 60 (6%) and 90 (6%) min. Furthermore, the increases observed for cFP-F-pAB-dynorphin A_{1-8} were significantly different than those observed for cFP-F-pAB-vehicle at 15 min only. This suggests that the small antinociceptive effects of cFP-F-pAB-dynorphin A_{1-8} may be attributable to the small antinociceptive potency of the inhibitor itself.

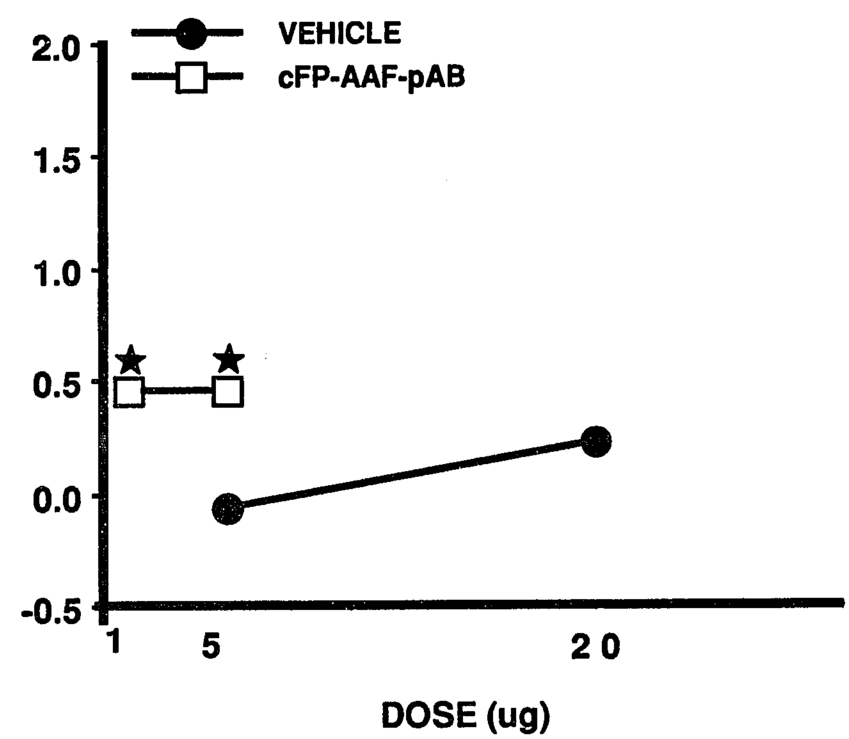
Changes were observed in the effective dose range of dynorphin A_{1-8} antinociception following pretreatment with cFP-AAF-pAB (25 nmol) relative to vehicle. Neither 5 nor 20 ug of dynorphin A_{1-8} pretreated with vehicle produced significant antinociception on the tail-flick or jump tests at any time point along the 90 min testing time course. In contrast, dynorphin doses as low as 1 ug produced antinociception following pretreatment with cFP-AAF-pAB (25 nmol) after 15 (13%) and 45 (9%) min on the tail-flick test, and after 45 (13%) min on the jump test. As described above, 5 ug of dynorphin A_{1-8} was also effective in producing antinociception virtually across 90 min after co-administration with cFP-AAF-pAB (25 and 2.5 nmol). A comparison of peak dynorphin A_{1-8} antinociception (1-20 ug) after cFP-AAF-pAB (25 nmol) and vehicle pretreatment on the tail-flick (peak: 15 min; top panel) and jump (peak: 45 min; bottom panel) tests are illustrated in Fig. 7. Low doses of dynorphin A_{1-8} (1 and 5 ug) co-administered with cFP-AAF-pAB (25 nmol) produced peak antinociception that was significant relative to vehicle-vehicle on both the tail-flick and jump tests. However, larger doses of dynorphin A_{1-8} (5 and 20 ug) co-administered with vehicle did not produce significant antinociception relative to vehicle-vehicle on either test. Thus, for a 5 ug dose of dynorphin A_{1-8}, cFP-AAF-pAB pretreatment produced 7- and 23- fold peak threshold increases relative to vehicle pretreatment on the tail-flick and jump tests respectively. Figure 8 illustrates total dynorphin A_{1-8} antinociception (1-20 ug) after cFP-AAF-pAB (25 nmol) and vehicle pretreatment on the tail-flick (top panel) and jump (bottom panel) tests. Again, low doses of dynorphin A_{1-8} (1 and 5 ug) produced greater total antinociception following cFP-AAF-

TABLE 2. Short-Term Alterations in Tail-Flick Latencies and Jump Thresholds Following Central cFP-AAF-pAB (25 nmol) and cFP-F-pAB (25 nmol) Relative to Vehicle Treatment.

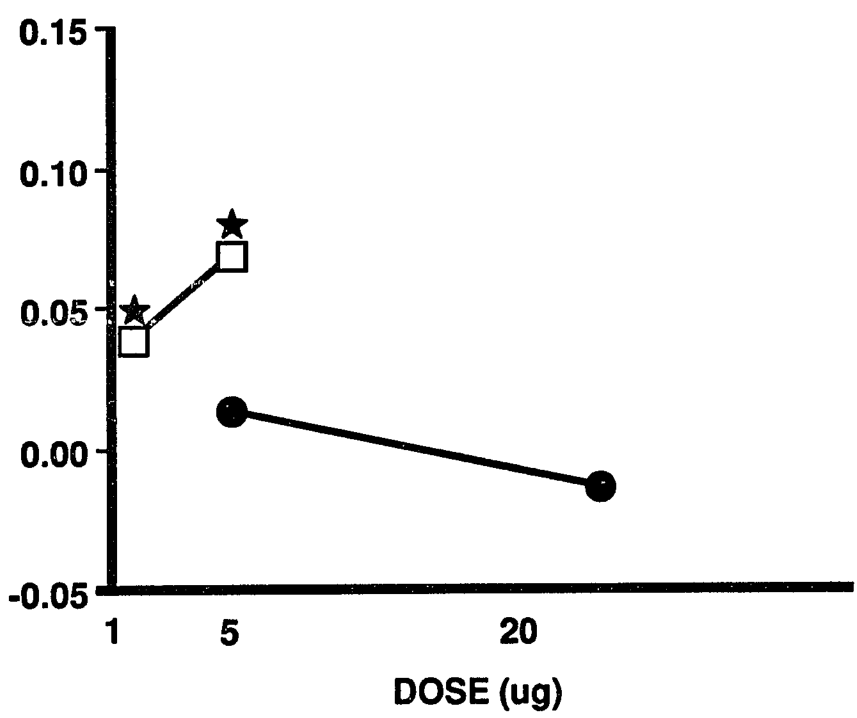
CONDITION	POST-INJECTION (min)				
	15	30	45	60	90
A. Tail-Flick Latencies (sec)					
Vehicle	3.62	3.70	3.59	3.77	3.61
cFP-AAF-pAB	3.81	3.79	3.69	3.75	3.67
Vehicle	3.60	3.40	3.33	3.43	3.44
cFP-F-pAB	3.68	3.57	3.61★	3.59	3.67
B. Jump Thresholds (mA)					
Vehicle	.363	.352	.355	.358	.347
cFP-AAF-pAB	.357	.355	.358	.350	.350
Vehicle	.358	.338	.343	.348	.353
cFP-F-pAB	.357	.353	.362★	.368★	.373★

Note: Stars denote significant differences relative to corresponding vehicle treatment.

FIGURE 7. Alterations in peak antinociception as measured by the tail-flick (15 min; top panel) and jump (45 min; bottom panel) tests after co-administration of dynorphin A $_{1-8}$ (1, 5, and 20 ug) with either vehicle or cFP-AAF-pAB (25 nmol). Stars indicate significant differences relative to vehicle-vehicle (Dunnett comparisons, $p < .05$)

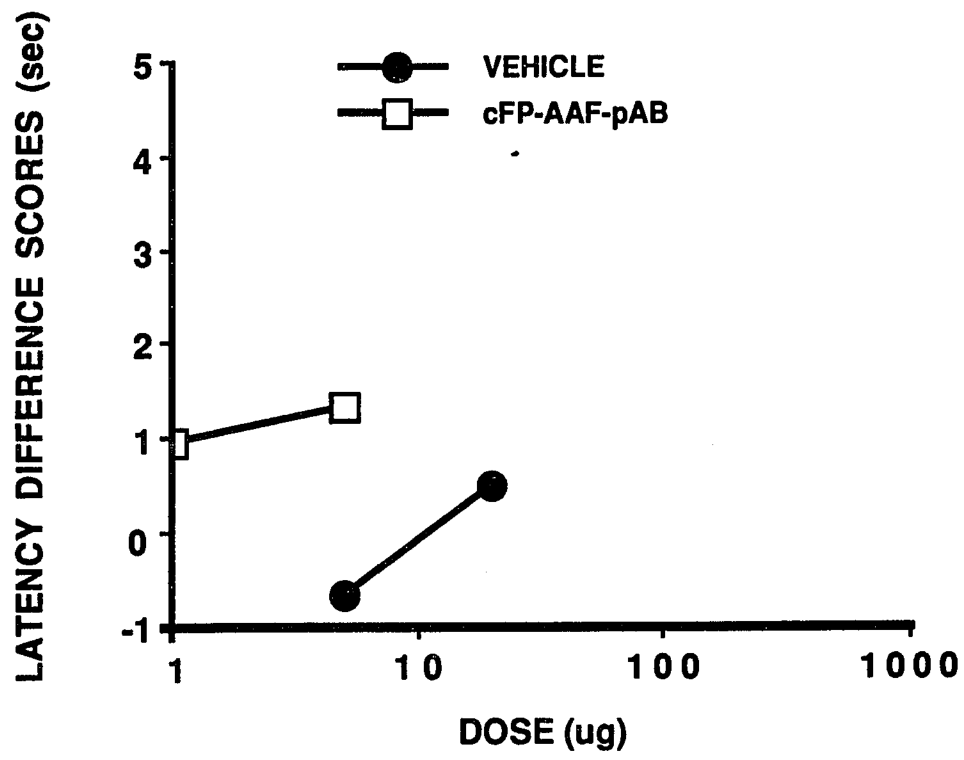


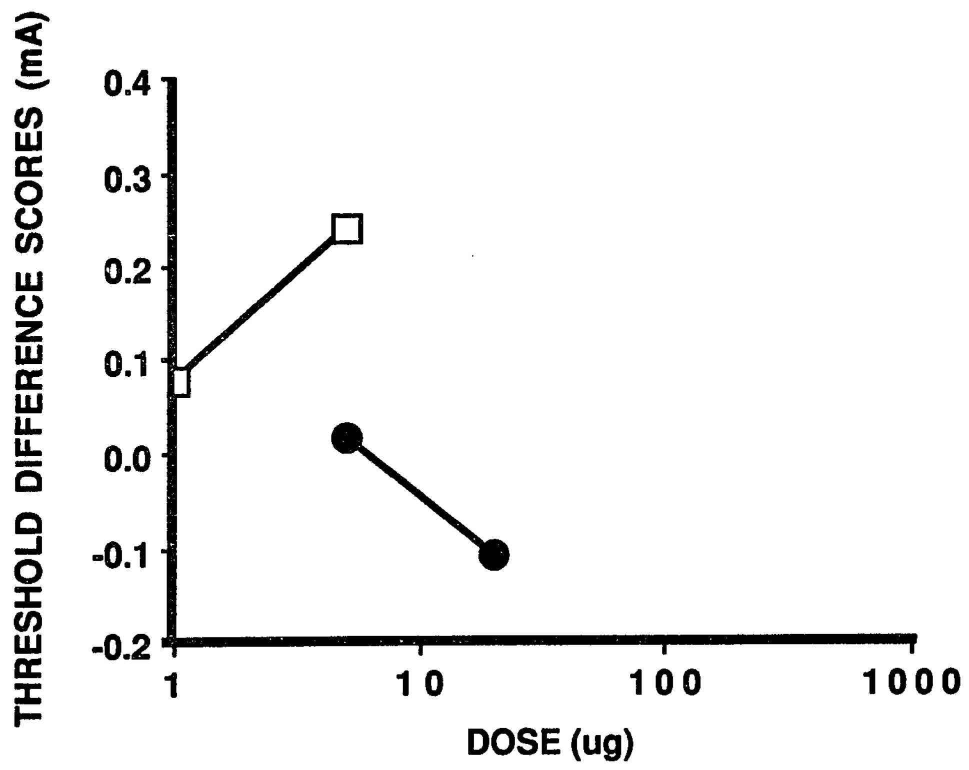
LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
VEHICLE
cFP-AAF-pAB
1
5
2 0
DOSE (ug)



THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
1
5
20
DOSE (ug)

FIGURE 8. Alterations in total antinociception as measured by the tail-flick (top panel) and jump (bottom panel) tests following co-administration of dynorphin A $_{1-8}$ (1, 5, and 20 ug) with either vehicle or cFP-AAF-pAB (25 nmol).


LATENCY DIFFERENCE SCORES (sec)
5
4
3
2
1
0
-1
VEHICLE
cFP-AAF-pAB
1
10
100
1000
DOSE (ug)


THRESHOLD DIFFERENCE SCORES (mA)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
1
10
100
1000
DOSE (ug)

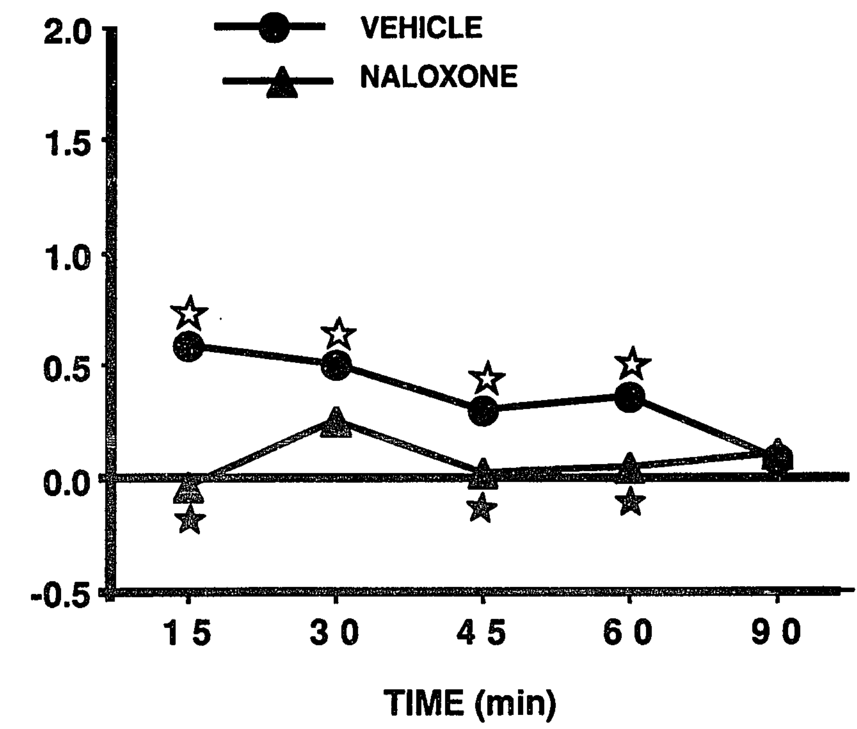
pAB pretreatment than did the larger doses of dynorphin A_{1-8} (5 and 20 ug) following vehicle pretreatment. Thus, for a 5 ug dose of dynorphin A_{1-8}, cFP-AAF-pAB pretreatment produced 2- and 14- fold increases in total antinociception relative to vehicle pretreatment on the tail-flick and jump tests respectively.

Naloxone Reversibility. Significant differences were observed among treatments (tail-flick: (F (2,10)= 7.59, $p < .01$; jump: (F (2,8)= 39.69, $p < .001$)), test times (flick: (F (4,20)= 3.10, $p < .05$; jump: (F (4,16)= 3.58, $p < .05$)) and for the interactions between time and treatment (tail-flick: (F (8,40)= 2.27, $p < .05$; jump: (F (8,32)= 5.55, $p < .001$). Figure 9 illustrates the antinociceptive effects of cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5 ug) co-administration. Significant increases were observed after 15 (18%), 30 (17%), 45 (9%), and 60 (12%) min on tail-flick latencies, and after 15 (6%), 30 (10%), 45 (19%), and 60 (15%) min on jump thresholds. Pretreatment with naloxone (1 mg/kg, sc) however significantly blocked cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5 ug) antinociception after 15 (reduction: >100%), 45, (94%), and 60 (89%) min on the tail-flick test, and between 15 and 60 (>100%) min on the jump test.

Central Antagonism. Significant differences were observed for the main antagonist effects upon dynorphin antinociception on both the tail-flick (F (4,32)= 23.90, $p < .001$) and jump (F (4,32)= 21.59, $p < .001$) tests. Significant effects were also revealed for time (tail-flick: F (4,32)= 24.35, $p < .001$; jump: F (4,32)= 10.04, $p < .001$), and for the interaction between antagonist conditions and time (tail-flick: F (16,128)= 4.84, $p < .001$; jump: F (16,128)= 5.49, $p < .001$) on both nociceptive tests. Figure 10 illustrates the antinociception produced by cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5ug) on both tests. Significant increases relative to vehicle-vehicle were observed in tail-flick latencies after 15 (24%), 30 (18%), and 45 (9%) min, and in jump thresholds at 15 (19%), 30 (17%), 45 (15%), and 60 (7%) min. Pretreatment with ß-FNA also produced significant latency increases relative to vehicle-vehicle at 15 (24%), 30 (24%), and 45 (17%) min, and threshold increases at 15 (22%), 30 (19%), 45 (15%), 60 (8%), and 90 (6%) min. In contrast, cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5ug) failed to increase either tail-flick latencies or jump thresholds at any testing time point after pretreatment with nor-BNI or naltrexone.

Motor Performance and Activity Levels. Table 3 illustrates rat locomotor activity counts 10-60 min after cFP-AAF-pAB/dynorphin A_{1-8} co-administration. Vehicle and inhibitor/peptide did not significantly differ between 10-20 (t (6) = 2.01), 20-30 (t (6) = 2.11), 30-40 (t (6) = -0.39), 40-50 (t (6) = 1.0), or 50-60 (t (6) = -1.08) min post injection. Normal righting reflexes and performance on an inclined plane were also

FIGURE 9. Reductions in cFP-AAF-pAB (25 nmol)-dynorphin A $_{1-8}$ (5 ug) antinociception following naloxone (1 mg/kg, sc). Light and dark stars indicate significant differences (Dunnett comparisons, $p < .05$) from vehicle-vehicle and cFP-AAF-pAB (25 nmol)-dynorphin A $_{1-8}$ (5 ug) respectively on the tail-flick (top panel) and jump (bottom panel) tests.


LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
VEHICLE
NALOXONE
15
30
45
60
90
TIME (min)

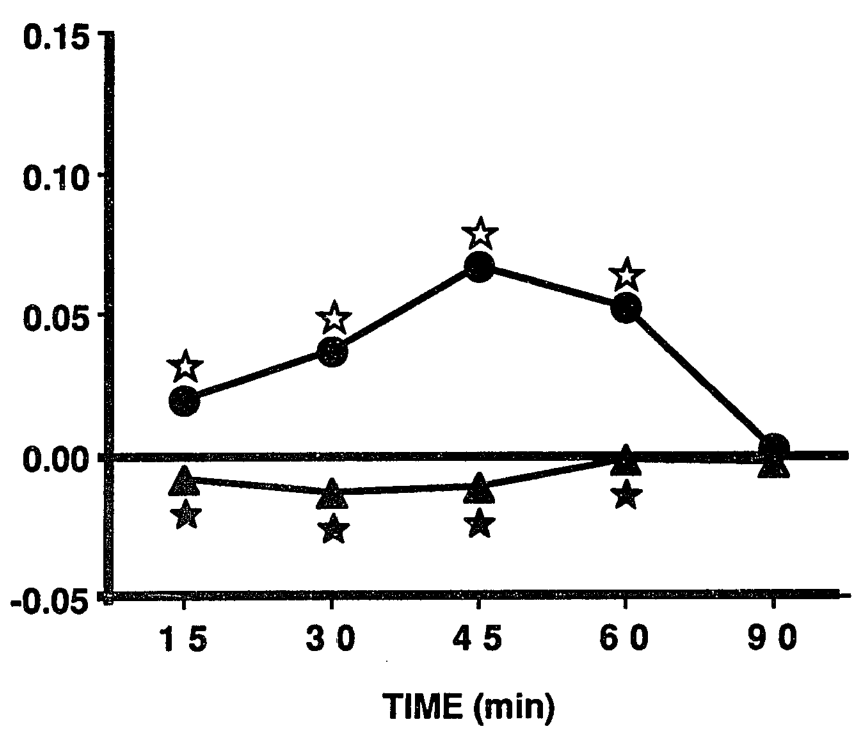
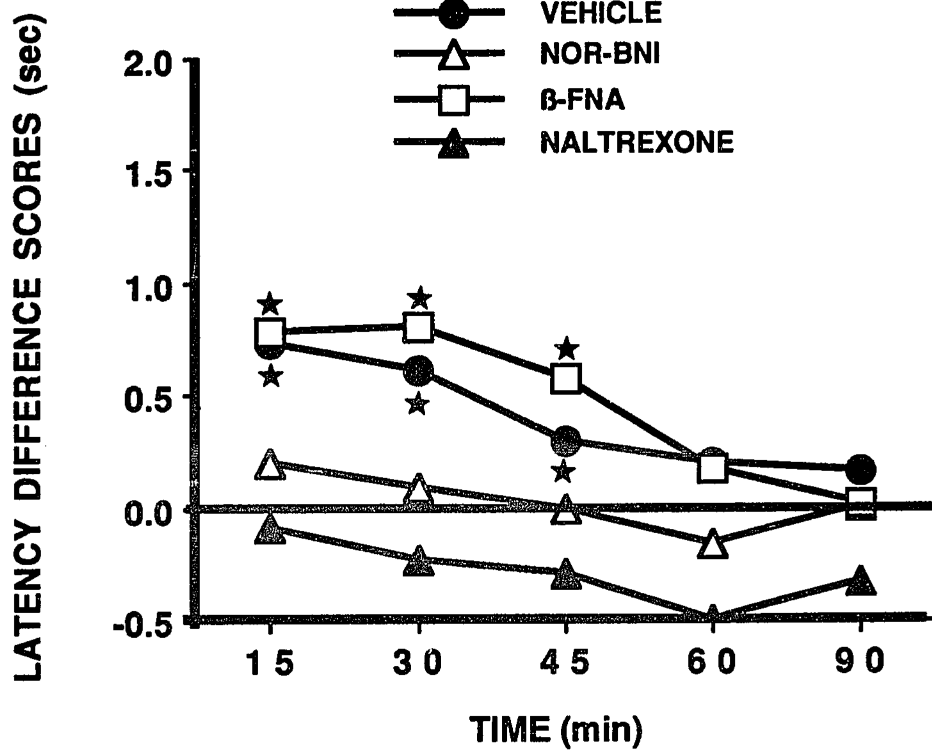

THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
15
30
45
60
90
TIME (min)

FIGURE 10. Pretreatment effects of vehicle, ß-FNA, nor-BNI, and naltrexone on cFP-AAF-pAB (25 nmol)-dynorphin A_{1-8} (5 ug) antinociception. Stars indicate significant differences on the tail-flick (top panel) and jump (bottom panel) tests relative to vehicle-vehicle (Dunnett comparisons, $P < .05$).


VEHICLE
NOR-BNI
ß-FNA
NALTREXONE
LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
1 5
3 0
4 5
6 0
9 0
TIME (min)

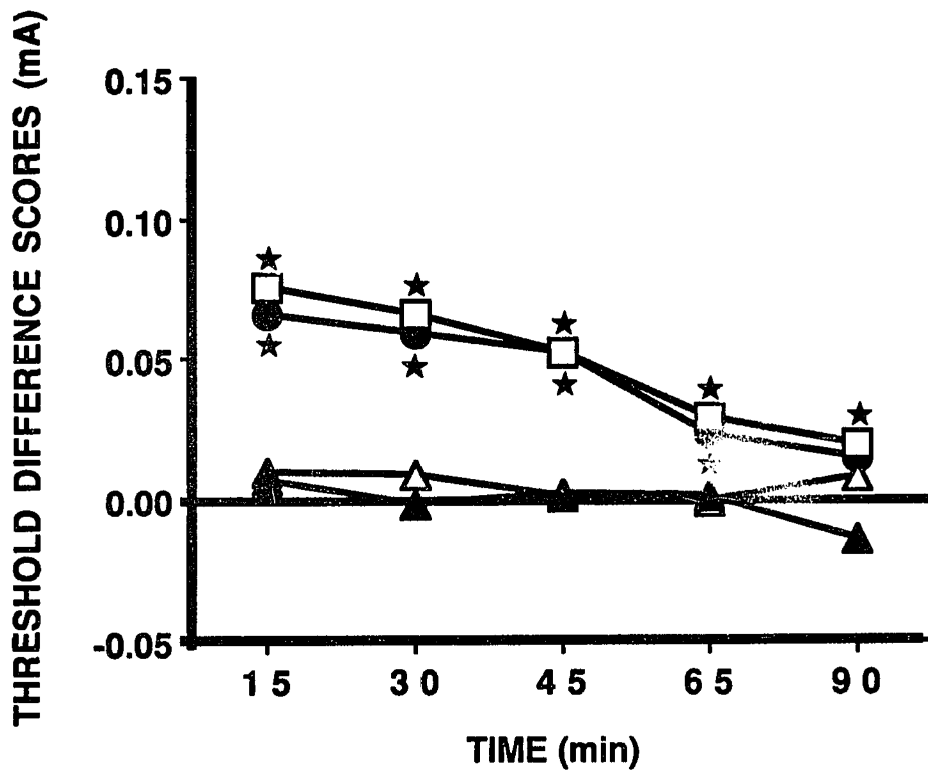

THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
1 5
3 0
4 5
6 5
9 0
TIME (min)

TABLE 3. Effects Upon Locomotor Activity Levels after Vehicle and cFP-AAF-pAB (25 nmol)-Dynorphin A $_{1-8}$ (5 ug) Co-administration.

	POST-INJECTION (min)				
TREATMENT	20	30	40	50	60
Vehicle	546	478	392	582	445
(SD[1])	257	366	295	273	211
EPI/DYN[2]	432	518	498	381	656
(SD)	219	234	333	235	232

[1] SD: Standard Deviation

[2] EPI/DYN: Co-administration of endopeptidase inhibitor cFP-AAF- pAB with dynorphin.

observed in all animals following co-administration relative to vehicle across the entire 90 min time course (Wilcoxon tests).

Discussion

The present study demonstrates that dynorphin A_{1-8} (5 ug) produces significant increases in tail-flick latencies (peak: 15%) and jump thresholds (peak: 22%) following co-administration with the endopeptidase-24.15 inhibitor cFP-AAF-pAB (25 nmol). Furthermore, increases were observed for dynorphin A_{1-8} doses as low as 1 ug (peak flick, jump: 13%) and for cFP-AAF-pAB doses as low as 2.5 nmol (peak flick: 12%; jump: 14%). cFP-AAF-pAB was itself ineffective in increasing nociceptive thresholds at this shorter 15-90 min testing interval. Although the endopeptidase-24.11 inhibitor cFP-F-pAB also elicited antinociception when co-administered with dynorphin A_{1-8}, the magnitude and duration of antinociception were significantly more modest than that observed for cFP-AAF-pAB, and occurred in the presence of intrinsic antinociception induced by the inhibitor itself. Furthermore, caution is advised before drawing inferences from this experiment about effective dynorphin dose levels. Dynorphin displays unusually high binding to plastics and glass, and thus some loss of the peptide may have occurred during preparation or delivery.

Importantly, the increases in nociceptive thresholds were observed in the absence of any alterations upon rat motor performance, activity level, or reflexes (experiment 2d). Thus, although previous studies report that the prototypical prodynorphin-derived peptide dynorphin A fails to produce central supraspinal antinociception, and produces only weak spinal antinociception which is accompanied by motor dysfunction (Caudle and Isaac 1987; Herman and Goldstein 1985; Long et al. 1988; Stevens and Yaksh 1986), our results indicate that such an inability may be due to its rapid enzymatic degradation (Young et al. 1987). Four lines of evidence support these results. First, a stable analogue of dynorphin, [(D-ala^2, F^5) Phe^4]-dynorphin A_{1-13} -NH2, increases nociceptive thresholds (Walker et al. 1987). Second, the motor dysfunctions associated with intrathecal dynorphin antinociception have been attributed to the nonopioid actions of breakdown products (Long et al. 1988). Third, the aminopeptidase inhibitor bestatin potentiates the antinociception, but not the motor dysfunction, induced by intracerebral administration of dynorphin B in mice (Nakazawa et al. 1989). Finally, the functional interaction of cFP-AAF-pAB with dynorphin A_{1-8} reported here to produce antinociception parallels the inhibitor's effects upon dynorphin degradation in vitro (Acker et al. 1987) and in vivo (Molineaux and Ayala 1990). In the latter study, a loss of more than 95% of central exogenous dynorphin A_{1-8} was observed immediately after

administration. Co-administration with cFP-AAF-pAB however increased the concentration of dynorphin A $_{1-8}$ in the perfusate by as much as 40-fold five minutes after treatment. Co-administration also increased the time (up to 50 min) during which significant amounts of dynorphin A $_{1-8}$ could be recovered. Importantly, cFP-AAF-pAB failed to significantly increase basal dynorphin A $_{1-8}$ over this interval, paralleling the failure of this inhibitor to increase basal nociceptive thresholds during the short time course used in experiment 1. Although the magnitude of antinociception was small, amounting to 14% and 22% increases in tail-flick latencies and jump thresholds respectively, it was still significantly greater than that observed following the pairing of the endopeptidase-24.11 inhibitor cFP-F-pAB with dynorphin A $_{1-8}$, and comparable to the small antinociceptive properties of the stable dynorphin analogue [(D-ala^2, F^5) Phe^4]-dynorphin A $_{1-13}$ -NH2 (Walker et al. 1987), and the intrathecal combination of dynorphin and bestatin (Nakazawa et al. 1989).

In the present experiment, pretreatment with the general opiate receptor antagonists naloxone and naltrexone, and the kappa receptor-specific antagonist nor-BNI significantly blocked cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception. The ability of these drugs to antagonize opioid antinociception are commonly understood to be effected through opioid receptor blockade. Alternatively, if these antagonists possess intrinsic hyperalgesic potency, the reversal of cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception reported here maybe more attributable to cancellation than to receptor blockade. Although naloxone has indeed been observed to produce hyperalgesia (Jacob et al. 1974; Woolf 1980), this effect is dependent on the measure of nociception (Jacob et al. 1974; Frederickson et al. 1977) and is not observed in studies of experimental pain threshold (El-Sobky et al. 1976; Grevert and Goldstein 1978). The extent to and the conditions under which naltrexone or nor-BNI may or may not produce hyperalgesia are not currently known, and future research should address this question. One characteristic of naltrexone is the general nature of its antagonistic effects. Thus, its antagonism of the present antinociceptive effects may be mediated through any one of the opioid receptors. Dynorphin-related peptides however have been most often characterized as exhibiting a pronounced selectivity for kappa receptors (Kosterlitz and Paterson 1985; Holt et al. 1983; Goldstein and James 1984; Holt et al. 1985, and as possessing kappa receptor-like activity (Akil et al. 1984). It is likely that the cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception observed here is kappa mediated. First, mu blockade via pretreatment with the specific mu receptor antagonist ß-FNA failed to block the observed increases in nociceptive latencies and thresholds after co-administration. Although no attempt was made to verify mu blockade in the present experiment, data from other work with ß-FNA show large significant

reductions in mu-receptor binding when the same combination of dose, route of administration, and pretreatment interval used here are employed (Arjune et al. 1990). Second, pretreatment with the irreversible kappa receptor anatagonist nor-BNI also produced significant and potent blockade of cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception, thus implicating kappa receptors. Additional support for kappa receptor mediation of cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception stems from the magnitude of the present effects. If indeed mu receptors mediated the antinociceptive effects of cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception, these effects should be equipotent or similar to other mu receptor agonists. Mu receptor agonists produce the most consistent and efficacious antinociception (Yaksh and Rudy 1987; Wood et al. 1981a; Millan 1986). In the present experiment, cFP-AAF-pAB-dynorphin A $_{1-8}$ antinociception produced significant but only very modest effects when compared to mu acting agonists like morphine. The small increases produced by cFP-AAF-pAB-dynorphin A $_{1-8}$ are at least consistent with the minor role ascribed to kappa receptors in mediating supraspinal antinociception (Bodnar et al. 1988; Jensen and Yaksh 1986; Yaksh 1984 a, b).

Contrary to results obtained in previous studies (Herman et al. 1980; Long et al. 1988; Stevens and Yaksh 1986; Faden and Jacobs 1984), dynorphin antinociception, as induced by co-administration with cFP-AAF-pAB, was not accompanied by motor dysfunction as assessed through locomotor activity levels, righting reflexes, and performance on an inclined plane. Since the behavioral and physiological effects of dynorphin have been attributed to nonopioid metabolites (Faden and Jacobs 1984; Przewlocki et al. 1983; Walker et al. 1982), it is reasonable not to expect motor impairment in light of the protection afforded dynorphin after central co-administration with the endopeptidase-24.15 inhibitor cFP-AAF-pAB. Indeed, only naloxone-reversible increases in nociceptive thresholds, but not motor impairment, were previously observed after co-administration of dynorphin with bestatin (Nakazawa et al. 1989).

The major conclusions stemming from experiment 2 are: a), that dynorphin A $_{1-8}$, if protected from endopeptidase-24.15 enzymatic metabolism, can induce modest supraspinal antinociception; b), that these antinociceptive effects are due to sensory rather than motor system alterations; and c), that this modest dynorphin antinociception, at least in this instance, is mediated by kappa but not mu receptor activation.

VII. EXPERIMENT 3: MERGL AND MET-ENKEPHALIN ANTINOCICEPTION: POTENTIATION BY ENDOPEPTIDASE -24.15 AND -24.11 INHIBITORS.

Met-enkephalin-Arg^{6}-Gly^{7}-Leu^{8} (MERGL) and met-enkephalin have been shown to produce short-lived weak antinociceptive effects in rats (Inturrisi et al. 1980; Iadorola et al. 1985; Meek et al. 1977; Belluzzi et al. 1976). It is widely believed that the major factor limiting the antinociceptive actions of these opioid peptides is their deactivation by enzymatic catabolism. While endopeptidase-24.15 rapidly hydrolyses MERGL (Acker et al. 1987; Chu and Orlowski 1985), endopeptidase-24.11 is the primary enzyme in met-enkepahlin metabolism (Almenoff et al. 1981; Altstein et al. 1981; Fulcher et al. 1982; Malfroy et al. 1978; Orlowski and Wilk 1981). Although MERGL contains the met-enkephalin sequence, and thus susceptible to hydrolysis by endopeptidase-24.11, endopeptidase-24.11 is less effective than endopeptidase-24.15 upon peptides with C-terminal extensions. It was therefore expected that co-administration of the endopeptidase-24.15 inhibitor cFP-AAF-pAB will potentiate the antinociception induced by MERGL to a greater extent than would the endopeptidase-24.11 inhibitor cFP-F-pAB. Conversely, it was predicted that cFP-F-pAB, but not cFP-AAF-pAB, will potentiate met-enkephalin antinociception.

Methods

Protocol 3a. In order to characterize the modulatory role endopeptidase-24.15 has upon MERGL-mediated antinociception, naive rats received subsets of the following pairs of icv microinjections in addition to vehicle-vehicle (n= 24): a) vehicle-MERGL (200 ug; n= 9); b) vehicle-MERGL (100 ug; n= 9); c) vehicle-MERGL (25 ug; n= 10); d) vehicle-MERGL (5 ug; n= 5); e) cFP-AAF-pAB (25 nmol)-vehicle (n= 6); f) cFP-AAF-pAB (25 nmol)-MERGL (100 ug; n= 7); g) cFP-AAF-pAB (2.5 nmol)-MERGL (100 ug; n= 9); h) cFP-AAF-pAB (25 nmol)-MERGL (25 ug; n= 7); i) cFP-F-pAB (25 nmol)-MERGL (100 ug; n= 9). A methodological description of the MERGL dose-response curve in the presence and absence of cFP-AAF-pAB is presented in Table 4. Latencies and thresholds were assessed at 5, 10, 15, 30, 45, and 60 minutes following the second injection. This time course allowed for the observation of the very brief MERGL antinociception as established in previous degradation, antinociception and inhibitor studies

TABLE 4. Methodological Description of MERGL Antinociceptive Dose-Response Curve in the Presence and Absence of cFP-AAF-pAB.

MERGL (dose)	PRETREATMENT Vehicle	cFP-AAF-pAB (25 nmol)
5		X
25	X	X
100	X	X
200	X	

(Mellstrom et al. 1987; Patey et al. 1983; Iadorola et al. 1986). Each rat received a maximum of four injection conditions, including the vehicle-vehicle condition. In this and experiment 3b, the order of drug injections was randomized across animals according to an incompletely counterbalanced design, and an interval between conditions of seven days was employed in order to minimize carry-over effects of opioids (Yaksh et al. 1976).

Protocol 3b. This experiment examined the effects of endopeptidase -24.11 and -24.15 inhibitors upon antinociception induced by met-enkephalin after co-administration. Naive rats received subsets of the following pairs of icv microinjections in addition to vehicle-vehicle (n= 24): a) vehicle-met-enkephalin (200 ug; n= 11); b) vehicle-met-enkephalin (100 ug; n= 8); c) vehicle-met-enkephalin (50 ug; n= 8); d) vehicle-met-enkephalin (25 ug; n= 7); e) cFP-AAF-pAB (25 nmol)-met-enkephalin (100 ug; n= 8); f) cFP-F-pAB (25 nmol)-met-enkephalin (100 ug; n= 9); g) cFP-F-pAB (25 nmol)-met-enkephalin (50 ug; n= 8); h) cFP-F-pAB (25 nmol)-met-enkephalin (25 ug; n= 7); i) cFP-F-pAB (25 nmol)-vehicle (n= 7). A methodological description of the met-enkephalin dose-response curve in the presence and absence of cFP-F-pAB is presented in Table 5. Latencies and thresholds were assessed at 5, 10, 15, 30, 45, and 60 minutes following the second injection. This time course allowed for the observation of the very brief met-enkephalin antinociception as established in previous degradation, antinociception and inhibitor studies (Belluzi et al. 1976; Meek et al. 1977; Chaillet et al. 1983).

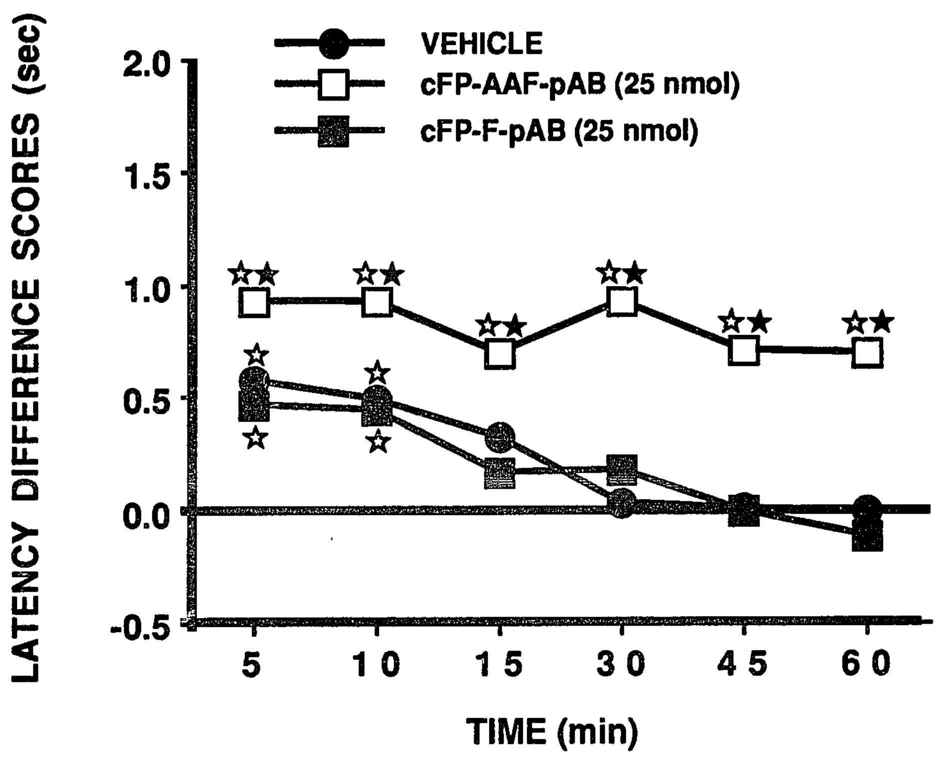
Results

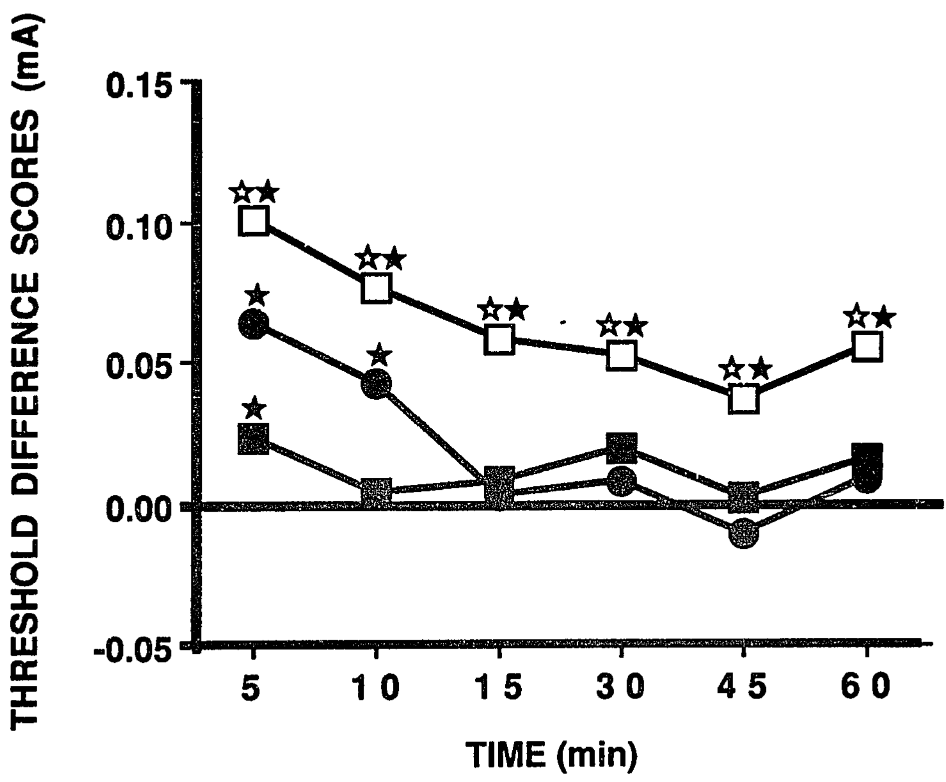
MERGL Antinociception. Significant effects were observed for treatment (tail-flick: F (8,60)= 12.21, $p < .001$; jump: F (8,60)= 6.07, $p < .001$), time (tail-flick: F (5,300)= 18.66, $p < .001$; jump: (F (5,300)= 14.68, $p < .001$), and for the interaction between treatment and time (tail-flick: F (40,300)= 2.98, $p < .001$; jump: F (40,300)= 3.05, $p < .001$) on both measures of antinociception. Figure 11 illustrates the alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) induced by the central co-administration of MERGL (100 ug) with vehicle, cFP-AAF-pAB (25 nmol), or cFP-F-pAB (25 nmol). Vehicle-MERGL produced significant increases in nociceptive thresholds relative to vehicle-vehicle on both the tail-flick and jump tests at 5 (tail-flick: 17%; jump: 19%) and 10 (tail-flick: 14%; jump: 13%) minutes. Significant increases were not observed between 15-60 min on either test. cFP-AAF-pAB-MERGL however produced antinociception relative to vehicle-vehicle that lasted across 60 min on the tail-flick (5 min: 27%; 10: 27%; 15: 20%; 30: 27%; 45: 21%; 60; 21%) and jump (5 min: 30%; 10: 22%; 15: 16%; 30: 15%; 45: 11%; 60; 16%) tests. These increases in nociceptive thresholds

TABLE 5. Methodological Description of Met-Enkephalin Antinociceptive Dose-Response Curve in the Presence and Absence of cFP-F-pAB.

Met-Enkephalin (dose)	PRETREATMENT Vehicle	cFP-F-pAB (25 nmol)
25	X	X
50	X	X
100	X	X
200	X	

FIGURE 11. Alterations in tail-flick latencies (top panel) and jump thresholds (bottom panel) following co-administration of MERGL (100 ug) with vehicle, cFP-AAF-pAB (25 nmol) and cFP-F-pAB (25 nmol). Light and dark stars denote significant differences (Dunnett comparisons, $p < .05$) relative to vehicle-vehicle and vehicle-MERGL respectively.


LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
VEHICLE
cFP-AAF-pAB (25 nmol)
cFP-F-pAB (25 nmol)
5
10
15
30
45
60
TIME (min)

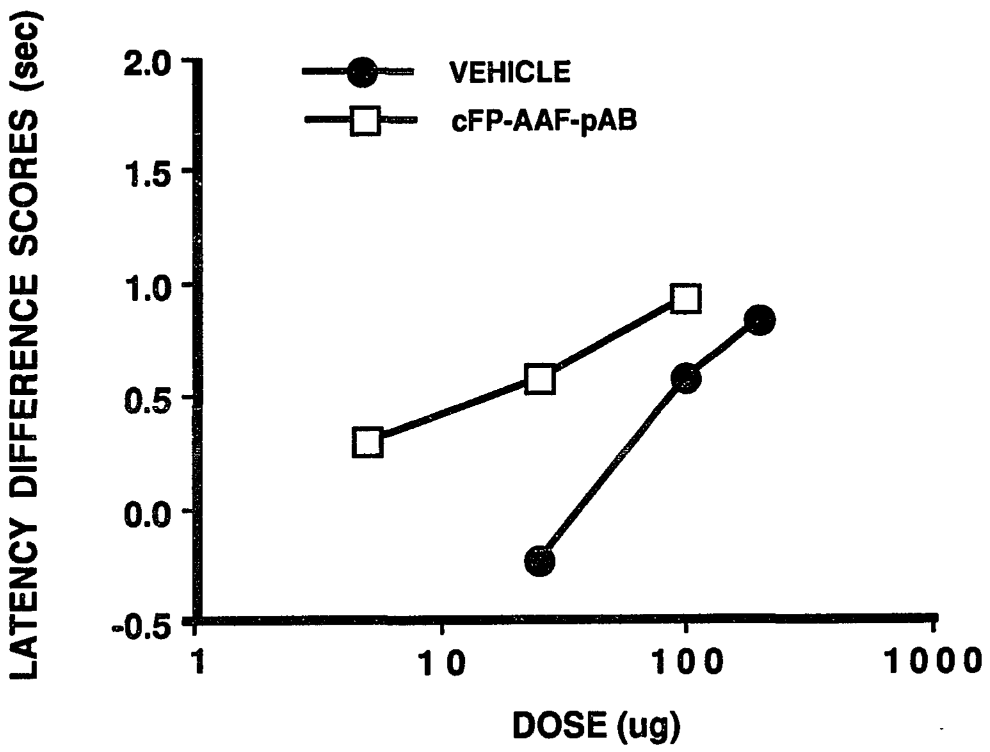

THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
5
10
15
30
45
60
TIME (min)

following cFP-AAF-pAB-MERGL were also significant relative to vehicle-MERGL on the tail-flick test after 10 (11%), 15 (10%), 30 (26%), 45 (20%), and 60 (21%) min, and on the jump test after 15 (15%), 30 (13%), 45 (14%), and 60 (13%) min. cFP-F-pAB-MERGL also produced significant antinociception relative to vehicle-vehicle on the tail-flick test at 5 (14%) and 10 (11%) min, and on the jump test at 5 (8%) min. These increases however were of smaller magnitude and shorter duration than those observed after vehicle-MERGL. Significant nociceptive threshold increases relative to vehicle-vehicle were also observed following the smaller dose of cFP-AAF-pAB (2.5 nmol) paired with MERGL (100 ug) on the tail-flick test after 5 (15%) and 10 (15%) min, and on the jump test after 5 (11%) min (data not shown). These increases however were not significantly greater than those observed after vehicle-MERGL (100 ug).

Changes in the antinociceptive efficacy of MERGL were observed across a range of doses (5-200 ug) following either vehicle or cFP-AAF-pAB pretreatment. Co-administration of vehicle-MERGL (200 ug) produced significant antinociception relative to vehicle-vehicle on the tail-flick test at 5 (24%), 10 (30%), 15 (30%) and 45 (16%) min, and on the jump test after 5 (21%), and 10 (18%) min. Whereas vehicle-MERGL (100 ug) also had antinociceptive potency relative to vehicle-vehicle (see above), vehicle-MERGL (25 ug) was without effect on either test across the entire 60 min. In contrast, the co-administration of MERGL (25 ug) with cFP-AAF-pAB (25 nmol) increased latencies relative to vehicle-vehicle after 5 (17%), 10 (12%), 15 (14%), 30 (17%), and 45 (17%) min, and jump thresholds after 5 (13%) and 30 (10%) min. Given the above stated failure of cFP-F-pAB (25 nmol) to potentiate MERGL (100 ug) antinociception, no other dose levels of MERGL were further examined.

Figure 12 illustrates the significant leftward shifts in the dose-response curve for peak (5 min) MERGL antinociception on both the tail-flick (top panel) and jump (bottom panel) tests. Thus, a 100 ug dose of MERGL paired with cFP-AAF-pAB produced peak antinociception that was similar in magnitude to that produced by a 200 ug dose of MERGL paired with vehicle. Regression analyses revealed that cFP-AAF-pAB effects upon peak MERGL antinociception were significant on the tail-flick (F (2,43)= 9.07, $p <$ .001) and jump (F (2,43)= 15.26, $p < .001$) tests with ED_{50} shifts of 4.6 and 2.4 respectively (Table 6). Shifts in the dose-response curve for total MERGL antinociception following cFP-AAF-pAB or vehicle are illustrated in Figure 13. Again, the co-administration of cFP-AAF-pAB with 25 ug of MERGL produced total antinociception that was equal or more potent than 200 ug of MERGL paired with vehicle on both the tail-flick and jump tests. Furthermore, pairing MERGL (100 ug) with cFP-AAF-pAB

FIGURE 12. Alterations in peak antinociception as measured by the tail-flick (5 min; top panel) and jump (5 min; bottom panel) tests after co-administration of MERGL (200, 100, 25, and 5 ug) with either vehicle or cFP-AAF-pAB (25 nmol).


LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
VEHICLE
cFP-AAF-pAB
1
10
100
1000
DOSE (ug)

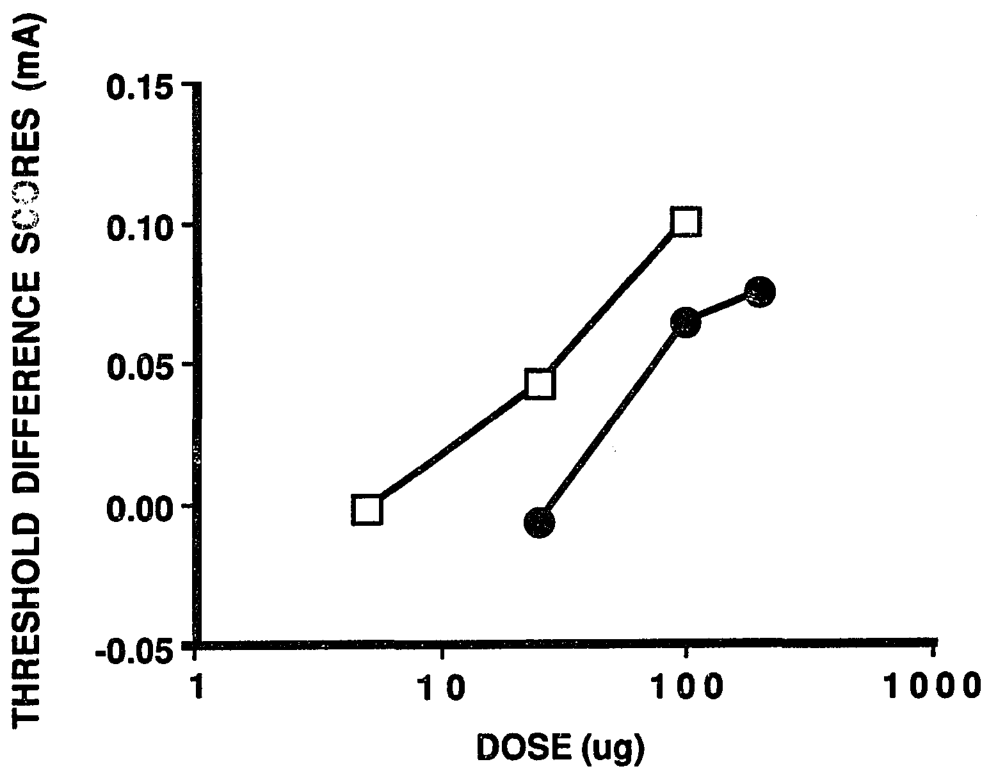

THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
1
10
100
1000
DOSE (ug)

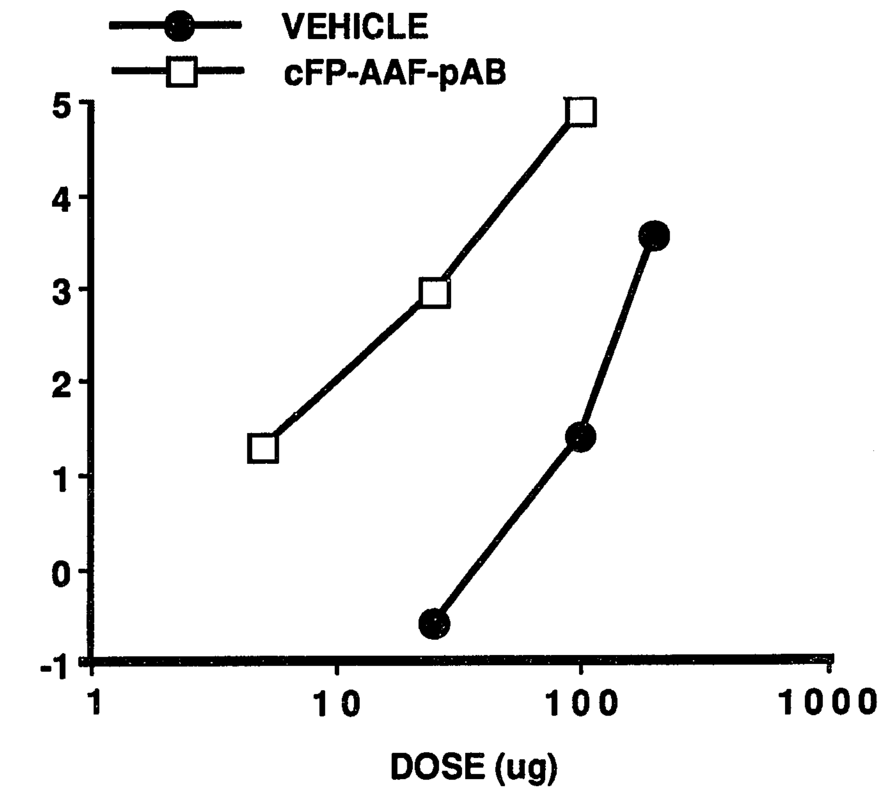
TABLE 6. Regression analyses of the dose response function of peak MERGL antinociception in rats pretreated with vehicle and cFP-AAF-pAB (25 nmol).

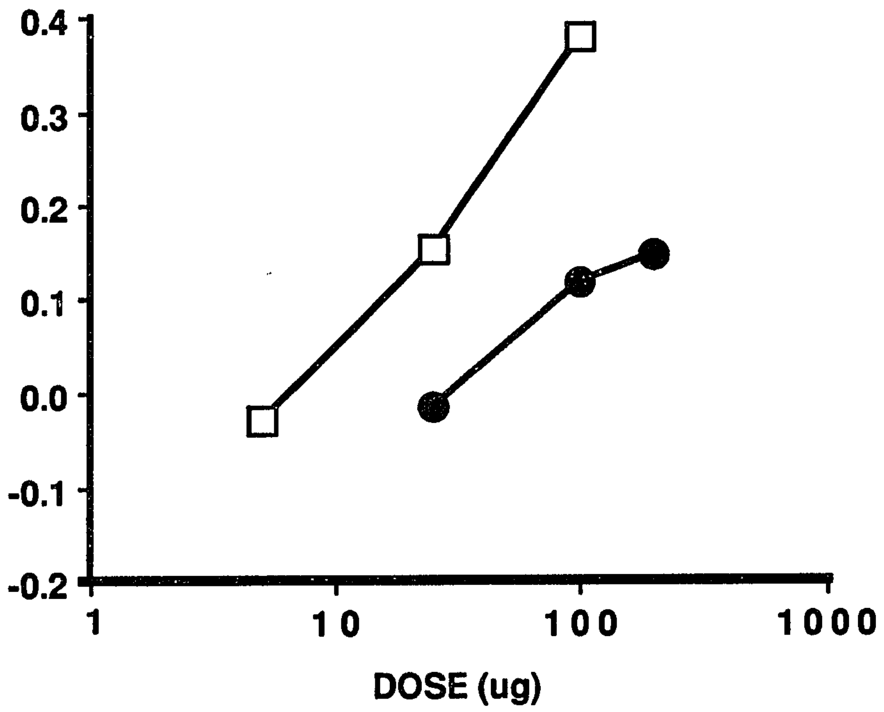
GROUP	ED_{50}[1] (ug)	SLOPE	INTERCEPT	SE[2]
A. Tail-Flick Test				
Vehicle	120	0.95	-1.48	0.17
cFP-AAF-pAB	26	0.64	-0.40	0.21
B. Jump Test				
Vehicle	68	0.12	-0.17	0.02
cFP-AAF-pAB	28	0.09	-0.08	0.01

[1] The ED_{50} for MERGL antinociception represents the value necessary to produce an increase of 0.5 sec and 0.05 mA in peak antinociception on the tail-flick and jump tests respectively.

[2] SE: Standard Error of Estimate.

FIGURE 13. Alterations in total antinociception as measured by the tail-flick (top panel) and jump (bottom panel) tests following co-administration of MERGL (200, 100, 25, and 5 ug) with either vehicle or cFP-AAF-pAB (25 nmol).


VEHICLE
cFP-AAF-pAB
LATENCY DIFFERENCE SCORES (sec)
5
4
3
2
1
0
-1
1
10
100
1000
DOSE (ug)


THRESHOLD DIFFERENCE SCORES (mA)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
1
10
100
1000
DOSE (ug)

produced increases in total antinociception that was greater than vehicle paired with MERGL at doses of 200 (flick: > 125%; jump: > 250%) or 100 (flick and jump: > 300%) ug. Regression analyses revealed that cFP-AAF-pAB effects upon total MERGL antinociception were significant on both the tail-flick (F (2,43)= 14.6, $p < .001$) and jump (F (2,43)= 15.3, $p < .001$) tests, resulting in ED_{50} shifts of 10.3 and 13.5 respectively (Table 7).

Met-Enkephalin Antinociception. Significant effects were observed for treatment (tail-flick: F (9,86)= 24.96, $p < .001$; jump: F (9,86)= 7.93, $p < .001$), time (tail-flick: F (5,430)= 77.9, $p < .001$; jump: F (5,435)= 52.6, $p < .001$), and for the interaction between treatment and time (tail-flick: F (45,430)= 5.79, $p < .001$; jump: F (45,435)= 6.69, $p < .001$) on both tests. Figure 14 illustrates the alterations induced by central administration of met-enkephalin (100 ug) on the tail-flick and jump tests after co-administration with either vehicle, cFP-AAF-pAB (25 nmol), or cFP-F-pAB (25 nmol). Vehicle paired with met-enkephalin produced nociceptive threshold increases relative to vehicle-vehicle on the tail-flick test after 5 (28%) and 10 (13%) min, and on the jump test after 5 min (11%) only. cFP-F-pAB and met-enkephalin co-administration however produced significant antinociception relative to vehicle-vehicle on tail-flick latencies across 60 min (5 min: 32%; 10: 31%; 15: 34%; 30: 21%; 45: 13%; 60: 14%) and on jump thresholds up to 30 min (5 min: 33%; 10: 19%; 15: 18%; 30: 12%) after co-administration. The pairing of cFP-F-pAB with met-enkephalin produced increases which were also significantly greater in magnitude than those observed following the vehicle-met-enkephalin pairing on the tail-flick test after 10 (16%), 15 (27%), 30 (15%), and 45 min (12%) and on the jump test after 5 (20%), 10 (20%), 15 (24%), and 30 (23%) min. In contrast, co-administration of met-enkephalin with cFP-AAF-pAB produced significant antinociception relative to vehicle-vehicle on the tail-flick test after 5 (16%), 10 (20%), and 15 (21%) min, and on the jump test after 10 (12%) min only. Furthermore, the increases following cFP-AAF-pAB and met-enkephalin were almost never greater than those observed following the vehicle-met-enkephalin condition, with significant increases occurring only after 15 (14%) min on the tail-flick test and 10 (13%) min on the jump test.

Shifts in the dose-response curve for peak and total met-enkephalin antinociception were observed in rats pretreated with vehicle or cFP-F-pAB (25 nmol). As illustrated in Fig. 15, leftward shifts were observed for peak (5 min) met-enkephalin antinociception on the tail-flick (top panel) and jump (bottom panel) tests. Thus, whereas low doses of met-enkephalin (50 and 25 ug) paired with vehicle failed to produce antinociception relative to vehicle-vehicle on the tail-flick test at this time point, met-enkephalin (50 ug)

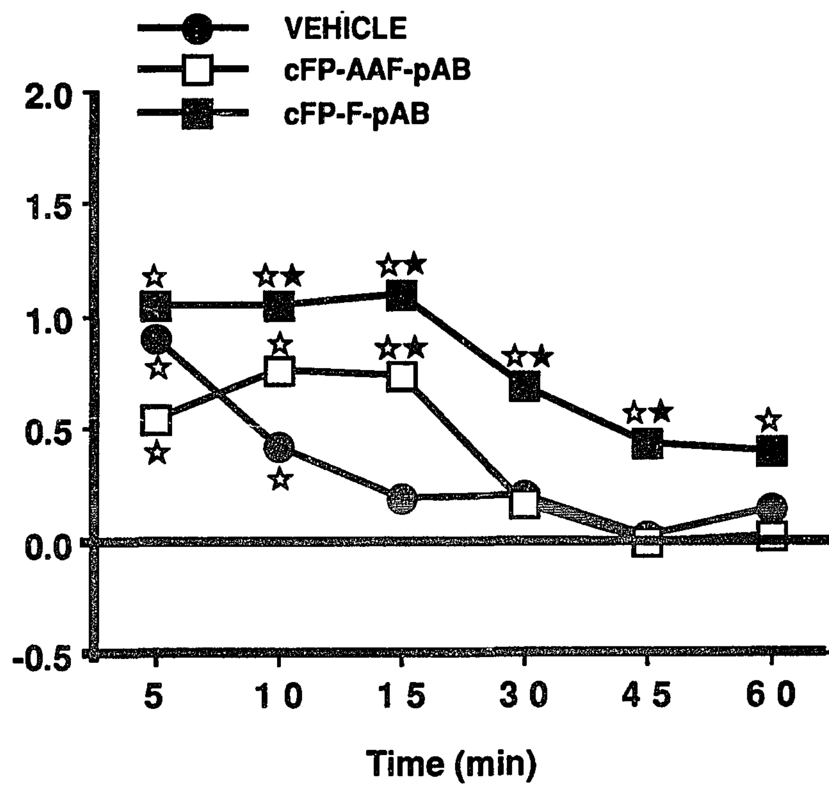
TABLE 7. Regression analyses of the dose response function of total MERGL antinociception in rats pretreated with vehicle and cFP-AAF-pAB (25 nmol).

GROUP	ED_{50}[1] (ug)	SLOPE	INTERCEPT	SE[2]
A. Tail-Flick Test				
Vehicle	370	3.26	-5.38	0.70
cFP-AAF-pAB	36	3.37	-2.21	1.08
B. Jump Test				
Vehicle	690	0.25	-0.41	0.05
cFP-AAF-pAB	51	0.34	-0.28	0.06

[1] The ED_{50} for MERGL antinociception represents the value necessary to produce an increase of 3 sec and 0.3 mA in total antinociception on the tail-flick and jump tests respectively.

[2] SE: Standard Error of Estimate.

FIGURE 14. Alterations in tail-flick latencies (left panel) and jump thresholds (right panel) following co-administration of met-enkephalin (100 ug) with vehicle, cFP-AAF-pAB (25 nmol) and cFP-F-pAB (25 nmol). Light and dark stars denote significant differences (Dunnett comparisons, $p < .05$) relative to vehicle-vehicle and vehicle-met-enkephalin respectively.


VEHICLE
cFP-AAF-pAB
cFP-F-pAB
LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
5
10
15
30
45
60
Time (min)

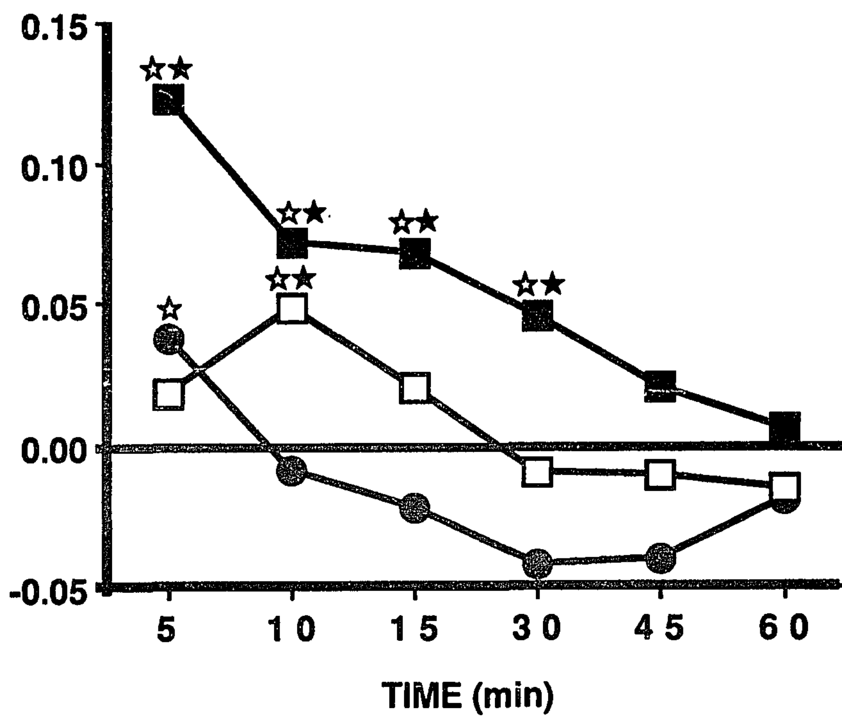
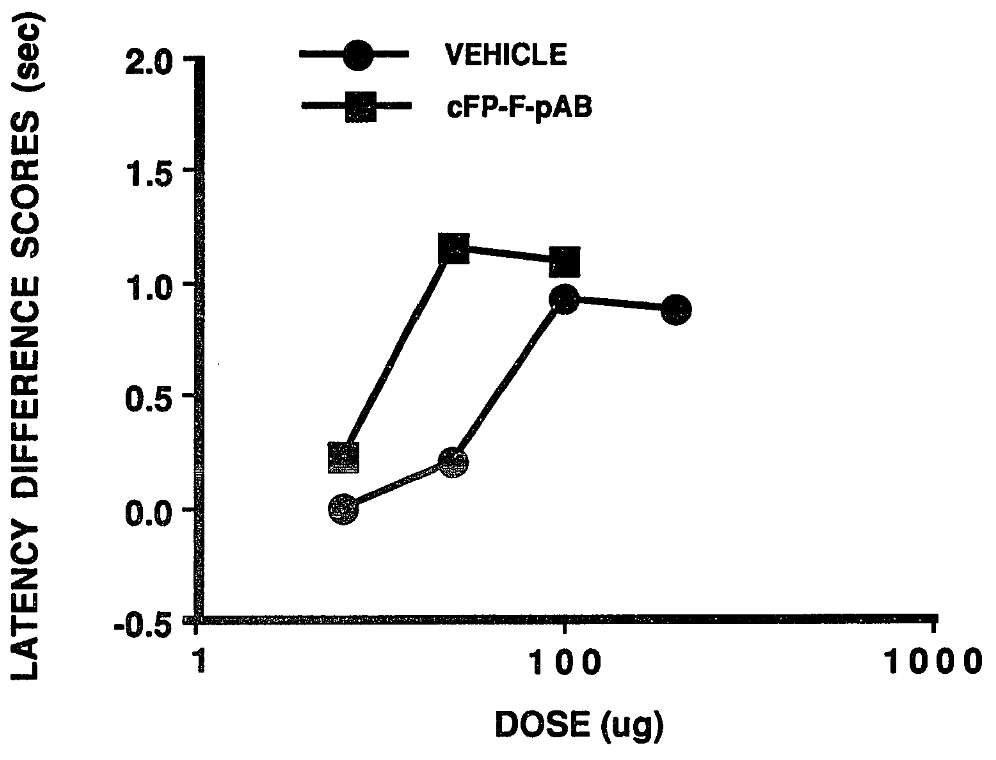
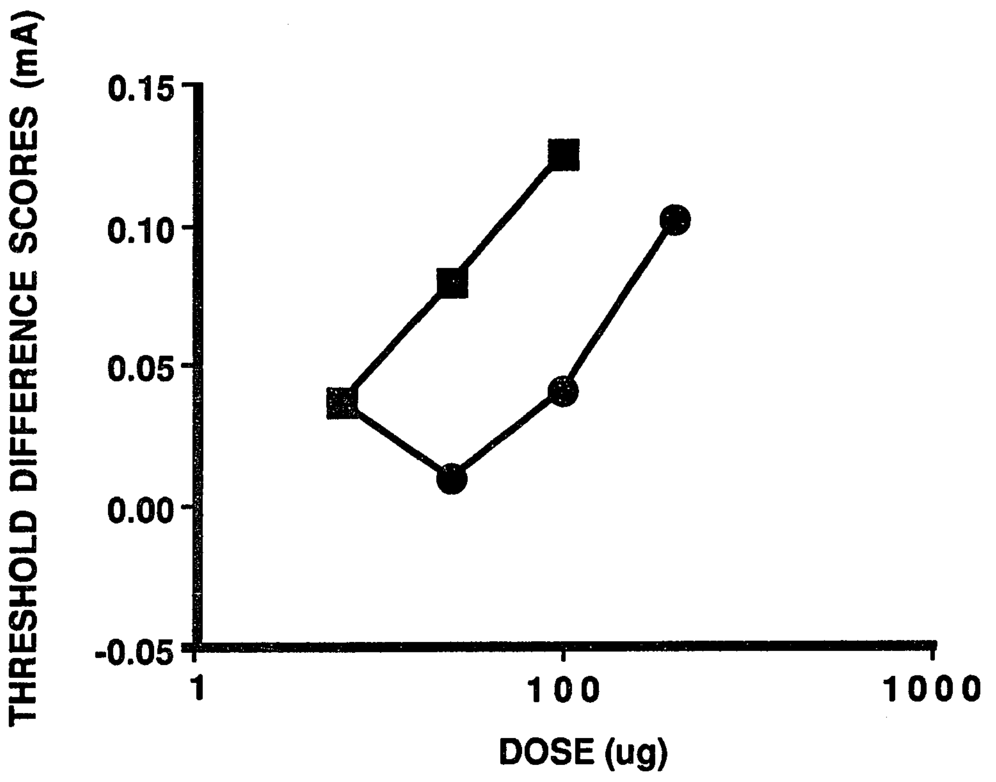

THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
5
10
15
30
45
60
TIME (min)

FIGURE 15. Alterations in peak antinociception as measured by the tail-flick (5 min; left panel) and jump (5 min; right panel) tests after co-administration of met-enkephalin (200, 100, 50, and 25 ug) with either vehicle or cFP-F-pAB (25 nmol).


LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
VEHICLE
cFP-F-pAB
1
100
1000
DOSE (ug)


THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
1
100
1000
DOSE (ug)

cFP-F-pAB produced significant antinociception (34%). Furthermore, cFP-F-pAB paired with met-enkephalin (50 ug) produced peak increases on the tail-flick test which were significantly greater (7%) than those observed following vehicle-met-enkephalin (200 ug). Whereas significant vehicle-met-enkephalin (50 ug) antinociception was not observed on the jump test, co-administration of met-enkephalin (50 ug) with cFP-F-pAB produced significant antinociception relative to vehicle-vehicle (21%), with increases that were significantly greater (10%) than those observed following met-enkephalin (10 ug) paired with vehicle. Vehicle-met-enkephalin (25 ug; 10%) and cFP-F-pAB-met-enkephalin (25 ug; 10%) produced significant increases in peak antinociception relative to vehicle-vehicle that was similar in magnitude. Regression analyses revealed that the differences in pretreatment effects upon met-enkephalin antinociception were significant in both tests (tail-flick: (F (2,54)= 7.57, p = .001; jump: (F (2,54)= 20.46, p < .001). Table 8 indicates shifts in the ED_{50} values for vehicle and cFP-F-pAB pretreatment of 2.5 on the tail-flick test, and of 2.4 on the jump test. Shifts in the dose-response curve for total met-enkephalin antinociception in rats pretreated with vehicle or cFP-F-pAB are illustrated in Fig. 16. Regression analyses also revealed significant differences in vehicle and cFP-F-pAB pretreatment effects upon total antinociception on the tail-flick (F (2,54)= 28.36, p < .001) and jump (F (2,54)= 29.8, p < .001) tests, which resulted in ED_{50} shifts of 4.3 and 3.7 respectively (Table 9).

Discussion

As expected, cFP-AAF-pAB and cFP-F-pAB differed significantly in their ability to effect MERGL and met-enkephalin antinociception. While cFP-AAF-pAB significantly potentiated and increased the duration of MERGL antinociception, cFP-F-pAB was more effective upon met-enkephalin antinociception. These results are in accord with substrate specificity studies that demonstrate that whereas met-enkephalin is resistant to degradation by endopeptidase-24.15 (Orlowski et al. 1983), MERGL is rapidly hydrolysed into met-enkephalin (Acker et al. 1987; Chu and Orlowski 1985). Thus, although MERGL contains the met-enkephalin sequence, and thus susceptible to hydrolysis by endopeptidase-24.11 as well, endopeptidase-24.11 is less effective than endopeptidase-24.15 upon peptides with C-terminal extensions. Since cFP-AAF-pAB has been shown to be an effective inhibitor of endopeptidase-24.15 (Acker et al. 1987; Chu and Orlowski 1985), it was therefore expected that co-administration of the endopeptidase-24.15 inhibitor cFP-AAF-pAB would potentiate the antinociception induced by MERGL to a greater extent than would the endopeptidase-24.11 inhibitor cFP-F-pAB. In contrast, met-

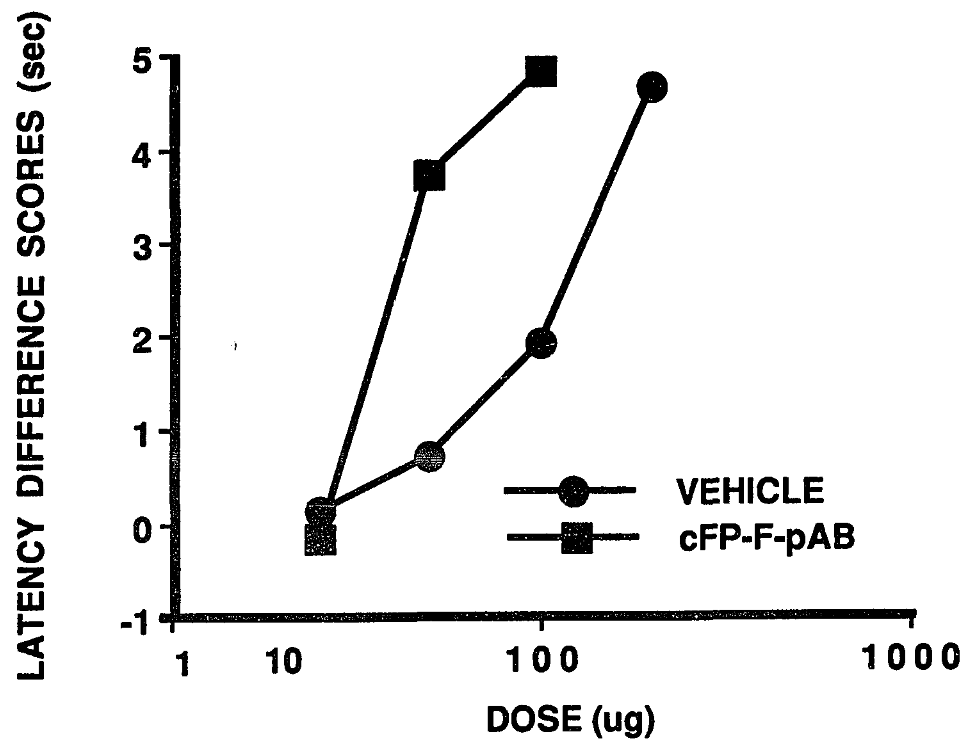
TABLE 8. Regression analyses of the log-dose response function of peak met-enkephalin antinociception in rats pretreated with vehicle and cFP-F-pAB (25 nmol).

GROUP	ED_{50}[1] (ug)	SLOPE	INTERCEPT	SE[2]
A. Tail-Flick Test:				
Vehicle	77	1.10	-1.58	0.26
cFP-F-pAB	31	1.48	-1.72	0.41
B. Jump Test				
Vehicle	100	0.09	-0.13	0.02
cFP-F-pAB	41	0.23	-0.32	0.03

[1] The ED_{50} for met-enkephalin antinociception represents the value necessary to produce an increase of 0.5 sec and 0.05 mA in peak antinociception on the tail-flick and jump tests respectively.

[2] SE: Standard Error of Estimate.

FIGURE 16. Alterations in total antinociception as measured by the tail-flick (left panel) and jump (right panel) tests following co-administration of met-enkephalin (200, 100, 50, and 25 ug) with either vehicle or cFP-F-pAB (25 nmol).


LATENCY DIFFERENCE SCORES (sec)
5
4
3
2
1
0
-1
1
10
100
1000
DOSE (ug)
VEHICLE
cFP-F-pAB

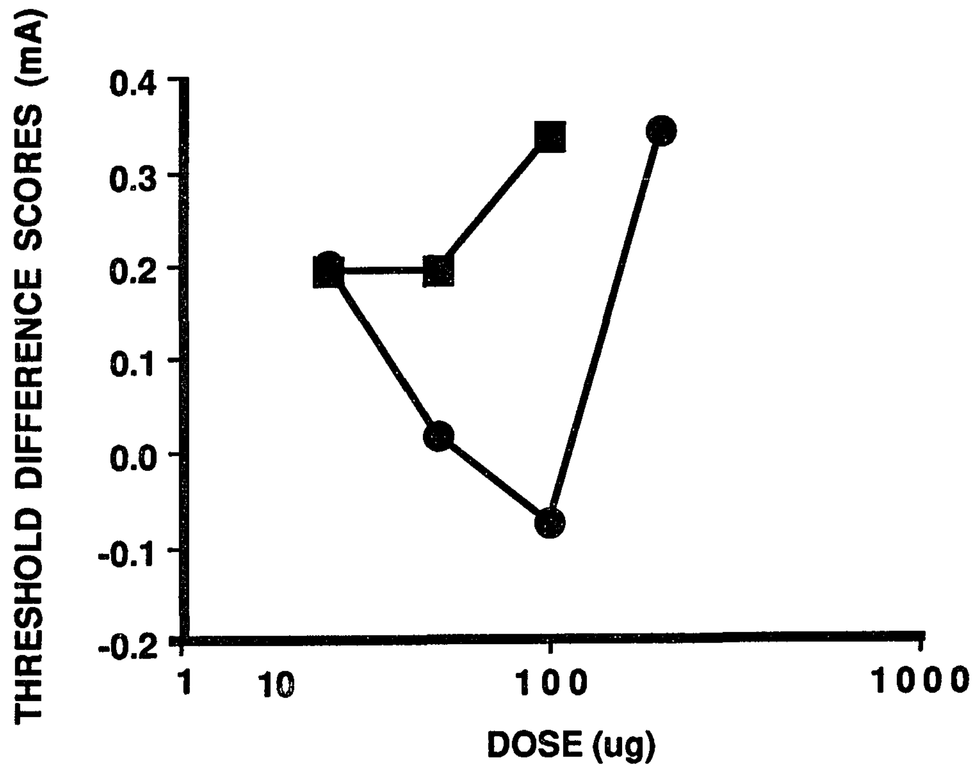

THRESHOLD DIFFERENCE SCORES (mA)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
1
10
100
1000
DOSE (ug)

TABLE 9. Regression analyses of the log-dose response function of total met-enkephalin antinociception in rats pretreated with vehicle and cFP-F-pAB (25 nmol).

GROUP	ED_{50}[1] (ug)	SLOPE	INTERCEPT	SE[2]
A. Tail-Flick Test:				
Vehicle	149	1.08	-0.84	0.66
cFP-F-pAB	35	7.93	-10.70	1.35
B. Jump Test				
Vehicle	151	0.21	-0.31	0.06
cFP-F-pAB	41	0.77	-1.09	0.12

[1] The ED_{50} for met-enkephalin antinociception represents the value necessary to produce an increase of 1.5 sec and 0.15 mA in total antinociception on the tail-flick and jump tests respectively.

[2] SE: Standard Error of Estimate.

enkephalin is highly susceptible to endopeptidase-24.11 metabolism (Almenoff et al. 1981; Altstein et al. 1981; Fulcher et al. 1982; Malfroy et al. 1978; Orlowski and Wilk 1981). cFP-F-pAB, an inhibitor of endopeptidase-24.11, has been shown to be effective in slowing the in vivo hydrolysis of exogenously applied enkephalins (Pozsgay et al. 1986). Therefore, it was expected that cFP-F-pAB, but not cFP-AAF-pAB, would potentiate met-enkephalin antinociception.

In agreement with other studies, the present series of experiments demonstrated that exogenously applied MERGL and met-enkephalin produce short-lived increases in nociceptive thresholds as assessed by the tail-flick and jump tests, with peak effects occurring at 5-10 minutes (Inturrisi et al. 1980; Iadarola et al. 1985; Belluzzi et al. 1976). The magnitude of antinociception produced by MERGL in the present study is comparable on the tail-flick test to that reported elsewhere for intracerebroventricular (Mellstrom et al. 1987), but not intrathecal (Iadorola et al. 1986) administration. This difference, may be in part due to route of administration (Iadorola et al. 1986; Hollt et al. 1982), particularly considering the spinal organization of the tail-flick test (D'Amour and Smith 1941). Significant increases in magnitude and duration of MERGL and met-enkephalin antinociception were observed after co-administration with the endopeptidase -24.15 and -24.11 inhibitors cFP-AAF-pAB and cFP-F-pAB respectively. Similar increases were generally observed after inhibitor co-administration with low doses of MERGL and met-enkephalin which were otherwise ineffective. This resulted in significant shifts in the peak and total dose-response curves of the peptides relative to vehicle pretreatment. As central administration of the inhibitors alone produced no observable alterations upon nociceptive thresholds, these effects can be assumed not to be based on any intrinsic properties of the inhibitors themselves, but attributable to peptide protection from the endogenous proteolytic endopeptidases -24.15 and -24.11.

The inhibitor of endopeptidase-24.15 used in the present study potentiated MERGL antinociception over the the entire 60 minute testing time course. In contrast, the endopeptidase-24.11 inhibitor used in the present experiment failed to potentiate met-enkephalin antinociception at 5 and 60 minutes on the tail-flick test and at 45-60 minutes on the jump test. This may reflect an instance where a peptide is afforded only partial protection to the degradatory actions of several peptidases. Peptidase inhibitor studies clearly demonstrate that met-enkephalin is indeed susceptible to the metabolic actions of not only endopeptidase-24.11 ("enkephalinase"), but also aminopeptidase and ACE (Noueihed and Yaksh 1985; Patey et al. 1983; DeLabaume et al. 1982). Their collective inhibition by kelatorphan yields the most efficacious enkephalinergic recovery and antinociception (Roques and Fournie-Zaluski 1987; Fournie-Zaluski et al. 1985; 1984).

Thus, inhibition of one of several participating peptidases, in this instance endopeptidase-24.11, may serve to initially potentiate the antinociceptive actions of a given peptide, with increases in nociceptive thresholds dependent upon such factors as peptidase-peptide and peptidase-inhibitor affinity. But the susceptibility of the peptide to degradation by other enzymes would serve to limit the potency and/or length of any antinociceptive effect. In contrast to met-enkephalin, there is evidence to suggest that other peptidases participating in MERGL degradation are limited in number. The inhibition of endopeptidase-24.11, ACE, and aminopeptidase does not increase the recovery of MERGL in vitro (Patey et al. 1983, 1985; Mellstrom et al. in press), and only bestatin (aminopeptidase inhibitor), but not Hoe 498 diacid (ACE inhibitor), produces a very brief increase in MERGL antinociception(Mellstrom et al. 1987). These data suggest that the primary inactivation of MERGL is through a peptidase acting on internal peptide bonds, characteristic of an endopeptidase. Thus, the efficacy of cFP-AAF-pAB to potentiate MERGL antinociception in the present study not only substantiates previous in vitro data implicating endopeptidase-24.15 in MERGL degradation (Acker et al. 1987), but also suggests that this enzyme has a regulatory role in MERGL antinociception.

VIII. EXPERIMENT 4: ENDOPEPTIDASE -24.11 AND -24.15 INHIBITOR EFFECTS UPON OPIOID-MEDIATED COLD-WATER SWIMS

Exposure to stressful stimuli has been shown to elicit a transient antinociceptive response (Chance et al. 1977; Hayes et al. 1978; Madden et al. 1977). The opioid and nonopioid character of the antinociceptive response has been shown to be dependent upon the type of stress as well as upon parametric variations within a particular stressor (see review: Bodnar 1990). For cold water swims, variations in temperature, duration, and patterning of swims interact so that intermittent swims at cold-temperatures produce antinociception that is opioid in nature (Girardot and Holloway 1984a, b; 1985; Romero et al. 1988). Since endogenous opioids are susceptible to rapid inactivation by proteolytic enzymes, it would be expected that inhibition of these enzymes would potentiate opioid-mediated antinociceptive effects of stress. Indeed potentiation of stress-induced antinociception is observed in mice and rats after inhibition of endopeptidase-24.11 by thiorphan (Chipkin et al. 1983; Greenberg and O'keefe 1982). However, little is currently known about the role endopeptidase-24.15 plays in stress-induced antinociception. The present experiment thus evaluated and compared the effects of the respective endopeptidase -24.15 and -24.11 inhibitors cFP-AAF-pAB and cFP-F-pAB upon intermittent cold-water swim (ICWS) antinociception. The opioid nature of the cold-water swim-induced antinociception was confirmed by ICWS pretreatment with naloxone in protocol 4b.

Methods

Protocol 4a. This experiment was designed to evaluate whether endopeptidase -24.15 and -24.11 have a modulatory role in stress-induced antinociception. Twenty-seven naive rats were exposed to a single vehicle injection and three days later, vehicle followed by an intermittent (10 x 5 sec in-5 sec out, total= 100 sec) 5 ^{0}C swim. Animals were then pretreated 10 min prior to ICWS with no more than two of the following: a) cFP-AAF-pAB (0.25 nmol; n= 8); b) cFP-AAF-pAB (25 nmol; n= 9); c) cFP-F-pAB (25 nmol; n= 7); d) cFP-F-pAB (250 nmol; n= 7). Latencies and thresholds were assessed at 30, 60, and 90 minutes post-swim. An interval between ICWS conditions of seven days was employed in order to minimize carry-over effects of opioids (Yaksh et al. 1976).

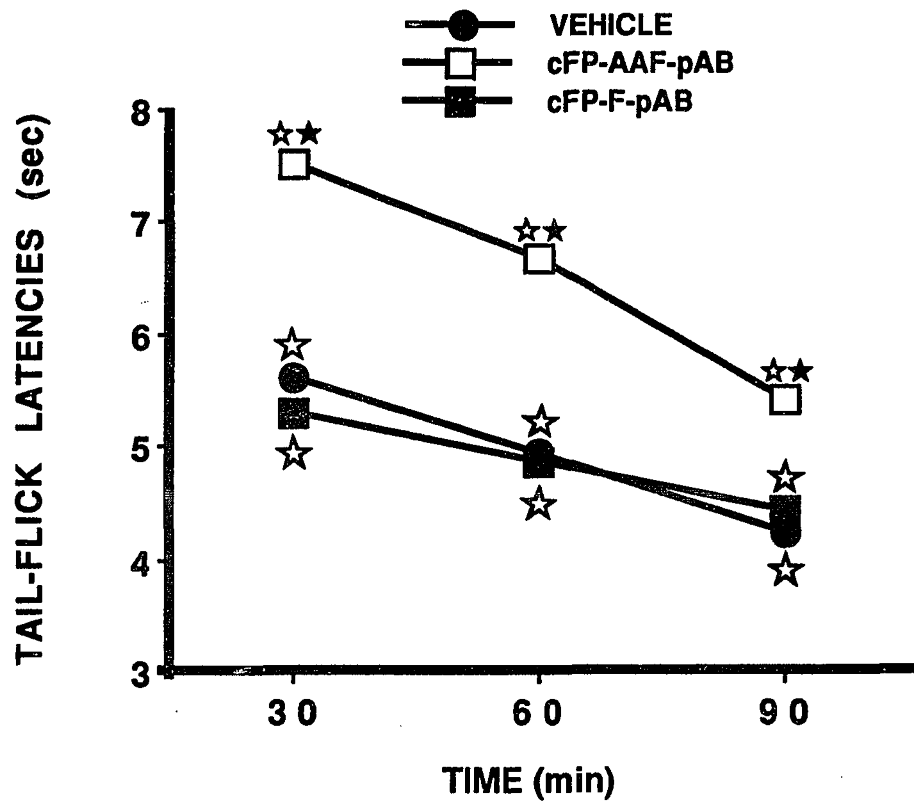
Protocol 4b. Because parametric variations such as pattern and temperature determine the physiological substrates (opioid vs. nonopioid) mediating the antinociceptive response of cold-water swims, nine rats were pretreated with naloxone (5 mg/kg) 20 min before exposure to the ICWS protocol outlined in experiment 4a. Latencies and thresholds were assessed at 30, 60, and 90 minutes post-swim. Reductions on these antinociceptive measures indicate opioid mediation in the antinociceptive effects of ICWS.

Results

ICWS Antinociception and Inhibitor Effects. Significant effects were observed for treatment (tail-flick: F (2,54)= 118.3, $p < .001$; jump: F (2,54)= 115.9, $p < .001$), time (tail-flick: F (2,54)= 57.26, p <001; jump: F (2,54)= 30.86, $p < .001$), and for the interaction between treatment and time (tail-flick: F (4,108)= 2.61, $p < .001$; jump: F (4,108)= 2.41, $p < .01$) on both tests of antinociception. Alterations in ICWS antinociception following either vehicle, cFP-AAF-pAB (25 nmol) or cFP-F-pAB (25 nmol) are illustrated in Figure 17. The vehicle-ICWS condition produced significant antinociception relative to vehicle-vehicle across 90 min on both the tail-flick (left panel; 30 min: 56%; 60: 43%; 90: 24%) and jump (right panel; 30 min: 34%; 60: 24%; 90: 14%) tests. cFP-AAF-pAB-ICWS also produced significant antinociception relative to vehicle-vehicle across the testing time course on both the tail-flick (30: 110%; 60: 93%; 90: 54%) and jump (30: 76%; 60: 54%; 90: 37%) tests. cFP-AAF-pAB-ICWS also significantly potentiated vehicle-ICWS antinociception, increasing nociceptive thresholds across 90 min (tail-flick: 30 min: 18%; 60: 30%; 90: 18%; jump: 30: 29%; 60: 21%; 90: 12%). The cFP-F-pAB-ICWS condition also produced significant antinociception relative to vehicle-vehicle across 90 min on both tests (tail-flick: 30 min: 63%; 60: 45%; 90: 25%; jump: 30: 37%; 60: 30%; 90: 21%). In contrast to cFP-AAF-pAB however, cFP-F-pAB pretreatment was limited in its ability to increase nociceptive thresholds relative to vehicle-ICWS. Significant increases were observed on the tail-flick test only after 30 min (12%).

Naloxone Reversibility. Significant effects were found between groups (tail-flick: F (1,8)= 45.8, $p < .001$; jump: F (1,8)= 21.27, $p < .001$), among treatments (tail-flick: F (2,18)= 23.27, $p < .001$; jump: F (2,16)= 17.29, $p < .001$), and for the interaction between group and treatment (tail-flick: F (2,16)= 6.45, $p < .01$; jump: F (2,16)= 4.69, $p < .05$). Figure 18 illustrates the alterations in ICWS antinociception following pretreatment with vehicle and naloxone (5 mg/kg, sc). Vehicle-ICWS produced antinociception relative to vehicle-vehicle on the tail-flick test across 90 min (30: 58%; 60: 42%; 90: 24%). Antinociception was also observed on the jump test after 30 (23%) and

FIGURE 17. Alterations in tail-flick latencies (left panel) and jump thresholds (right panel) following intermittent cold-water swims (ICWS) pretreated with vehicle, cFP-AAF-pAB (25 nmol) or cFP-F-pAB (25 nmol). Light and dark stars denote significant differences (Dunnett comparisons, $p < .05$) relative to vehicle-vehicle and vehicle-ICWS respectively.


VEHICLE
cFP-AAF-pAB
cFP-F-pAB
TAIL-FLICK LATENCIES (sec)
8
7
6
5
4
3
3 0
6 0
9 0
TIME (min)

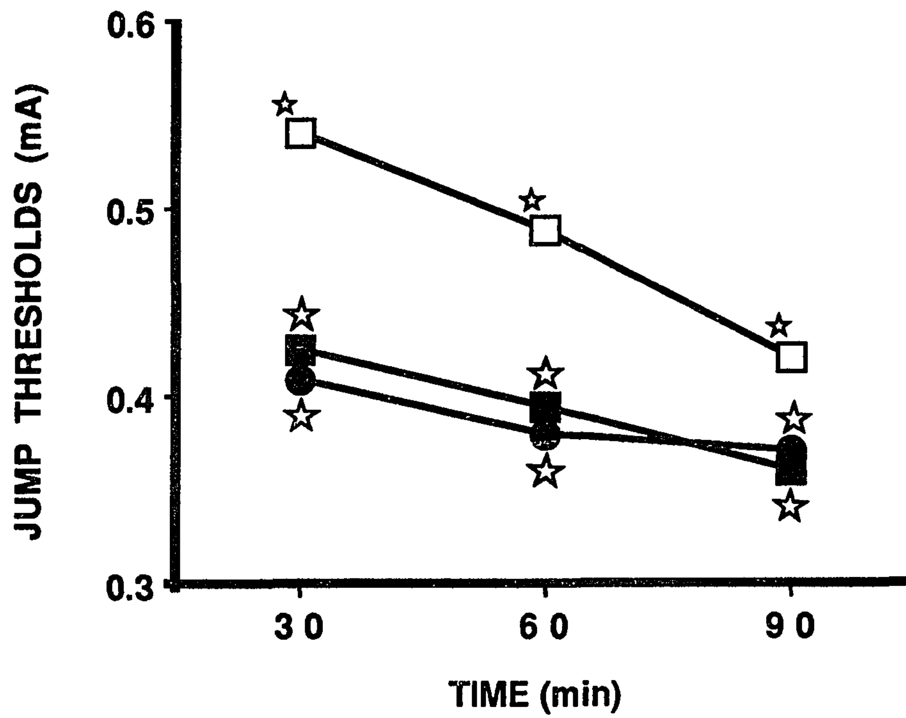
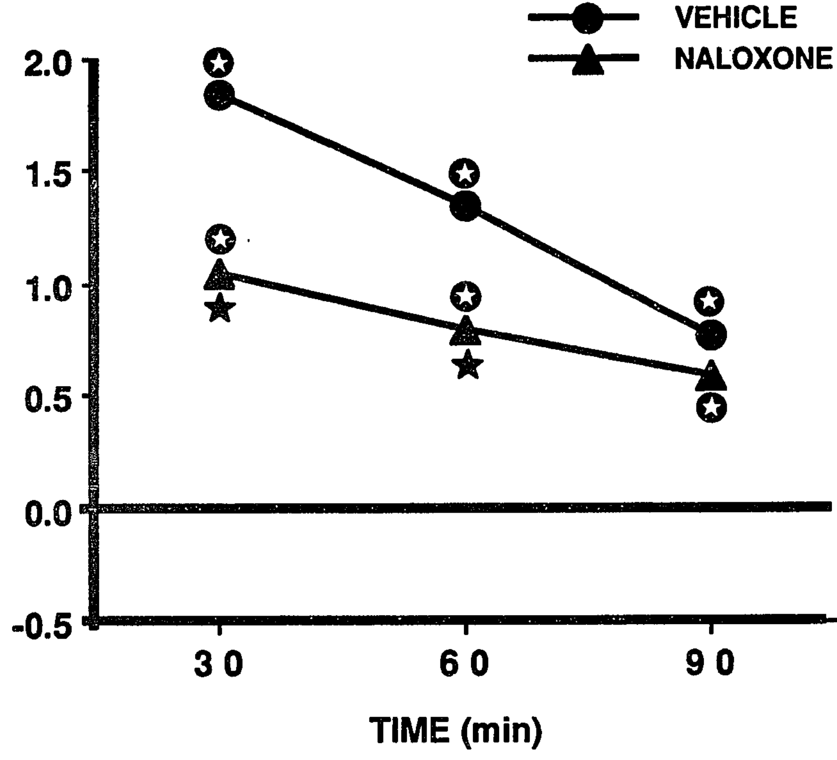
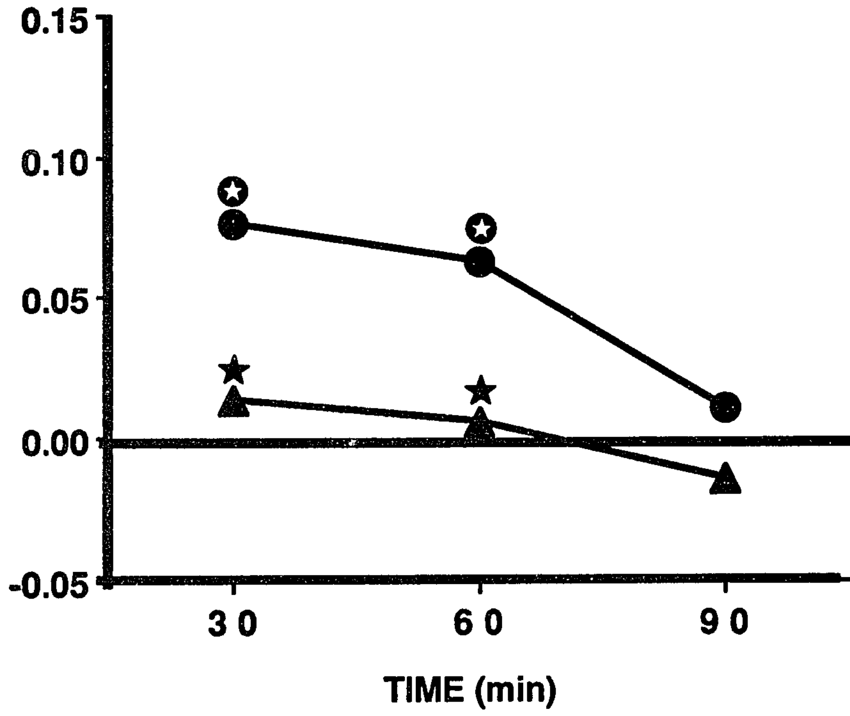

JUMP THRESHOLDS (mA)
0.6
0.5
0.4
0.3
3 0
6 0
9 0
TIME (min)

FIGURE 18. Reductions in vehicle-ICWS antinociception following naloxone (5 mg/kg, sc). Light and dark stars indicate significant differences (Dunnett comparisons, $p < .05$) relative to vehicle-vehicle and vehicle-ICWS respectively on the tail-flick (left panel) and jump (right panel) tests.


VEHICLE
NALOXONE
LATENCY DIFFERENCE SCORES (sec)
2.0
1.5
1.0
0.5
0.0
-0.5
30
60
90
TIME (min)


THRESHOLD DIFFERENCE SCORES (mA)
0.15
0.10
0.05
0.00
-0.05
30
60
90
TIME (min)

60 (20%), but not 90, min. Pretreatment with naloxone before exposure to ICWS produced significant antinociception relative to vehicle-vehicle on the tail-flick test after after 30 (33%), 60 (25%), and 90 (18%) min. This represents significant reductions in latency increases relative to vehicle-ICWS of 43% (30 min), and 41% (60 min). The increases in tail-flick latencies observed 90 min following vehicle-ICWS were not significantly greater than those observed for animals pretreated with naloxone before the swims. Naloxone-pretreated animals exposed to ICWS also did not produce significant antinociception relative to vehicle-vehicle on the jump test, representing significant reductions in jump thresholds relative to vehicle-ICWS of 82% (30 min), 99% (60 min).

Discussion

The present study demonstrated that cFP-AAF-pAB was effective in potentiating the antinociception induced by intermittent cold-water swims. cFP-AAF-pAB potentiated cold-water swim antinociception on the tail-flick and jump tests by 12-30%. In contrast, cFP-F-pAB produced a mild and transient increase (12%) in ICWS antinociception on the tail-flick test only. The opioid nature of the observed swim antinociception was confirmed by the pretreatment with the general opioid antagonist naloxone. Whereas significant ICWS antinociception was observed on the tail-flick test following naloxone, the magnitude of this antinociception was significantly reduced by 41%-43%. Naloxone pretreatment dramatically blocked swim antinociception on the jump test, reducing antinociceptive magnitudes by 82%-99%. Given the apparent opioid involvement in the above effects, it is suggested that cFP-AAF-pAB may act to protect those opioid peptides that are activated in response to intermittent cold-water swims and susceptible to endopeptidase-24.15 from inactivation, thus potentiating their antinociceptive action.

That selective inhibitors of proteolytic enzymes can potentiate the antinociceptive actions of stressors has been previously reported (Chipkin et al. 1983; Greenberg and O' Keefe 1982). Those studies report a potentiation of stress-induced antinociception by the endopeptidase-24.11 inhibitor thiorphan. This is in contrast to the failure of the endopeptidase-24.11 inhibitor used here, cFP-F-pAB, to potentiate cold-water swim induced antinociception. In addition, the endopeptidase-24.15 inhibitor cFP-AAF-pAB was much more effective in potentiating cold-water swim antinociception. The apparent success of cFP-AAF-pAB relative to cFP-F-pAB may reflect differences in the physiological systems underlying the antinociceptive response to stress. Indeed it is well established that antinociceptive responses to stress can be characterized along several physiological dimensions (see review: Bodnar 1990). Stressors therefore may also vary with respect to the opioid peptides mediating their response. Indeed, there is evidence to

suggest that there is a differential participation of opioid peptides in the response to foot-shock, and that the overall contribution of a ligand type to the response may change with the parameters of the same stimulus (Lewis et al. 1980; Terman et al. 1983; Watkins and Mayer 1982; Nabeshima et al. 1983). Furthermore, there is evidence that opioid peptide synthesis and precursor prohormone processing are dynamic, dependent upon certain parameters of the stress (Hollt et al. 1986; Kelsey et al. 1984; Shiomi and Akil 1982). Thus, it is plausible to suggest that under certain stressful conditions, but not others, there is a processing preference (or inhibition) towards particular opioid ligand subtypes and not others. Thus, it may be suggested that the effectiveness of cFP-AAF-pAB to potentiate cold-water swim antinociception, and its greater potency relative to cFP-F-pAB to do so, may reflect the greater participation of opioid peptides susceptible to endopeptidase-24.15 inactivation in response to cold-water stress. Other stressors, like foot-shock, may however elicit the greater participation of other opioid peptide family types, particularly those more susceptible to the proteolytic actions of endopeptidase-24.11. At this point however, more work is necessary to definitively characterize the physiological substrates mediating the antinociceptive response to various stressors.

IX. GENERAL DISCUSSION

Aims

There were four major goals addressed in this dissertation. The first goal was to assess what effects endopeptidase-24.15 inhibitors cFP-A(D)AF-pAB and cFP-AAF-pAB would have upon basal nociceptive thresholds after central ventricular administration, and to compare their effects to cFP-F-pAB, an inhibitor of endopeptidase-24.11. The data indicate that inhibition of endopeptidase-24.15 can produce time- and dose- dependent effects upon basal nociceptive thresholds that were test specific. Furthermore, the ability of endopeptidase-24.15 inhibitors to produce antinociception was comparable to that of cFP-F-pAB, an inhibitor of endopeptidase-24.11. The antinociceptive effects of all three inhibitors were significantly reduced by the general opioid antagonist naloxone, suggesting that the inhibitor's actions were mainly mediated through the opioid system. The antinociceptive actions of the more potent endopeptidase-24.15 inhibitor, cFP-AAF-pAB, were not accompanied by changes in either locomotor activity, righting reflexes, or performance on an inclined plane.

The second aim of the dissertation was to evaluate what, if any, role endopeptidase-24.15 may play in limiting dynorphin A $_{1-8}$ antinociception. This was addressed by examining alterations in nociceptive thresholds after central co-administration of dynorphin A $_{1-8}$ with the endopeptidase-24.15 inhibitor cFP-AAF-pAB. The data reported here indicate that cFP-AAF-pAB and even low doses of dynorphin A $_{1-8}$ produce antinociception under conditions of co-administration. Either drug administered alone was ineffective in significantly increasing nociceptive threshold values from baseline. Co-administration antinociception was significantly blocked by pretreatment with naloxone, thus revealing their opioid nature. The data obtained after pretreating subjects with long-term, opioid receptor-specific antagonists such as ß-FNA, nor-BNI, and naltrexone implicate kappa opioid receptors in dynorphin A $_{1-8}$ /cFP-AAF-pAB antinociception. The antinociception produced by co-administration was not accompanied by changes in either locomotor activity levels, righting reflexes, or performance on an inclined plane.

Another aim of the dissertation research was to evaluate the limits endopeptidase-24.15 may impose upon MERGL antinociception by examining changes in MERGL antinociception after co-administration with cFP-AAF-pAB. This was compared to the ability of cFP-F-pAB to potentiate met-enkephalin antinociception. The data show

selective potentiations in MERGL and met-enkephalin antinociception by cFP-AAF-pAB and cFP-F-pAB respectively. This argues that the inhibitors of endopeptidase -24.15 and -24.11 used in this dissertation possess some degree of specificity in their inhibitory actions upon endopeptidases, and that endopeptidase-24.15 has some modulatory role upon MERGL antinociception.

The final aim of this dissertation was to evaluate the role, if any, that endopeptidase-24.15 may play in limiting the potency or duration of antinociception induced by an environmental stressor, cold-water swims. cFP-AAF-pAB, but not cFP-F-pAB, was effective in increasing the potency and duration of cold-water swim antinociception. That the swim procedure employed here was largely mediated by endogenous opioids released in response to stress was confirmed by the significant reduction in cold-water swim antinociception by naloxone.

Implications

Endopeptidase-24.15 Modulates Opioid Antinociception. The data reported here have several implications, the most significant being the apparent role suggested for endopeptidase-24.15 in opioid-mediated antinociceptive processes. Unlike the classical cholinergic and monoaminergic systems, which utilize enzymatic and re-uptake mechanisms to regulate and terminate transmitter actions, it is commonly accepted that extracellular proteolysis is the primary method of opioid peptide inactivation in the central nervous system (Terenius 1988). Much in vivo and in vitro evidence implicates aminopeptidase, ACE, and endopeptidase-24.11 as primary participants in opioid peptide degradation (see review: Turner et al. 1985). However, in vitro studies with endopeptidase-24.15 have demonstrated that it too possesses a high affinity for several opioid peptides, and that its proteolytic actions upon them could be halted by specific and potent active-site directed inhibitors (Chu and Orlowski 1984; Orlowski et al. 1988; Acker et al. 1987). However, conclusive demonstration of the involvement of endopeptidase-24.15 in the metabolism of the opioids requires the demonstration that its inhibition in vivo leads to changes in those functions which are dependent upon opioid peptide function. In this dissertation, endopeptidase-24.15 inhibitors were used in three different but integrated lines of inquiry designed to address this question. The first line examined the effects of endopeptidase-24.15 upon background levels of opioid pools in a non-activated, quiescent state. The second line evaluated the role of endopeptidase-24.15. upon a mobilized and activated opioid system, reacting to a stressful stimulus. The final line of investigation examined the role of endopeptidase-24.15 upon exogenously applied

opioid substrates. The data indicate that inhibition of endopeptidase-24.15 in vivo leads to significant alterations in rat nociceptive processes across all line of investigation. These changes were apparently sensory in nature as in no experiment was there ever any indication of rat motor impairment, or that these changes were due to some intrinsic antinociceptive potency of the inhibitors themselves. Thus, in vivo behavioral data presented in this dissertation are indeed supportive of previous in vitro and in vivo biochemical and pharmacological findings (Acker et al. 1987; Molineaux and Ayala 1990) that suggest some modulatory role for endopeptidase-24.15, among with aminopeptidase, ACE and endopeptidase-24.11, in opioid inactivation.

Multiple Opioid Peptidases. The present demonstration of yet another peptidase involved in opioid inactivation gives rise to the second implication, that of the existence of multiple of opioid peptidases. Although studies with opioid peptidase inhibitors often suggest that peptidases may frequently overlap in modulating opioid functions, a number of observations suggest that these similarities are superficial. First, peptidases involved in opioid inactivation differ in their affinities for opioids of varying lengths (Turner et al. 1985; Orlowski et al. 1983; Chu and Orlowski 1985). For example, met-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL), met-enkephalin-Arg^6-Phe^7, and met^5-enkephalin all vary in their susceptibility to various peptidases (Iadorola et al. 1986; Mellstrom et al. 1987; Orlowski et al. 1983; Acker et al. 1987). Furthermore, all of the peptidases with an affinity for the opioids have similar, competitive affinities for other bioactive neural substrates. This has been demonstrated for endopeptidase-24.11 (Matsas et al. 1983; 1984; Deschodt-Lanckmann and Strosberg 1983), ACE (Erdos et al. 1978; Cascieri et al. 1984), the aminopeptidases (Deschodt-Lanckmann and Strosberg 1983; Matsas et al. 1984), as well as endopeptidase-24.15 (Orlowski et al. 1983; Chu and Orlowski 1985; Molineaux et al 1988). Thus, the lack of specificity of the peptidases for opioids and the variety of opioid peptide subtypes may make the participation of various peptidases in opioid inactivation necessary. A further possibility is that the differential distribution of opioid peptides throughout the neuraxis requires a multiple enzyme system also possessing a differential distribution and having variable affinities. For example, although endopeptidase-24.11 was hailed as the 'enkephalinase' of opioid synapses, membrane aminopeptidase activity exceeds that of endopeptidase-24.11 in all brain regions examined (Hudgin et al. 1981). Since the ratio of aminopeptidase: endopeptidase-24.11 varies greatly between tissues and even brain regions, the relative importance of the two activities shows marked regional and tissue differences (Geary et al. 1982; Matsas et al. 1984). Multiple, somewhat overlapping opioid peptidases also allow for flexibility in inactivating an opioid system that can be characterized as dynamic, showing shifts in

peptide processing in response to stress (Kelsey et al. 1984; Shiomi and Akil 1982). But not all opioid pools in the brain have been shown to be activated in response to stress, nor is it believed that all brain opioid peptide subtypes participate in stress antinociceptive responses (Millan et al. 1984; Millan et al. 1981; Alessi et al. 1982; Lewis et al. 1982). Thus, the specific enzyme or enzymes that are primarily responsible for the inactivation of an opioid peptide subtype at any particular time may be quite variable, depending on such factors as the physiological state of the organism and the consequential shifts in peptide and prohormone processing in response to challenge, the concurrent activation and activity levels of other peptide systems utilizing the same peptidases, and the subtypes of opioids that are involved in the opioid mediated response. Multiple, overlapping enzymatic activity may therefore provide the necessary flexibility required to regulate a complex and dynamic opioid system.

Opioid Peptidase Inhibitors. The final set of implications relate to the use of potent and selective inhibitors of endopeptidases. The results from previous in vitro and in vivo pharmacological experiments indicate that cFP-A(D)AF-pAB and cFP-AAF-pAB are potent and selective inhibitors of endopeptidase-24.15. As with potent active-site directed inhibitors of endopeptidase-24.11, ACE, and aminopeptidases (Matsas et al. 1984; Roques et al. 1980; Mellstrom et al. 1987; Chaillet et al. 1983; Noueihed and Yaksh 1985), in vivo studies with cFP-AAF-pAB have already provided investigators with significant details in understanding opioid processing (Acker et al. 19877; Molineaux et al. 1988; Orlowski et al. 1983; Chu and Orlowski 1985). Their use in the present dissertation also provide evidence that, as with inhibitors of endopeptidase-24.11, ACE, and aminopeptidase, they can be applied to the behavioral analysis of opioid antinociception. For example, experiment 2 demonstrated that dynorphin, long thought to be an opioid possessing only questionable spinal antinociceptive potency (Han and Xie 1984; Han et al. 1984; Porecca et al. 1984; Chavkin et al. 1982), was shown to elevate antinociceptive thresholds after central co-administration with the endopeptidase-24.15 inhibitor cFP-AAF-pAB. Thus, inhibitors may serve as probes useful in uncovering the antinociceptive potency of endogenous opioids whose physiological relevance in antinociceptive processes may be difficult to assess due to their rapid enzymatic inactivation. Furthermore, since inhibitors have been used here and elsewhere to induce antinociception and potentiate the antinociception of selective opioids (Matsas et al. 1984; Roques et al. 1980; Mellstrom et al. 1987; Chaillet et al. 1983; Noueihed and Yaksh 1985), their micro-injection into circumscribed areas along the neuraxis (i.e. spinal vs. supraspinal), may help to characterize which opioid subtypes participate in antinociceptive responses organized at each level. The pretreatment of subjects with cFP-AAF-pAB

before exposure to an environmental stressor, cold-water swims, yielded data which suggest a modulatory role for endopeptidase-24.15 in stress-induced antinociception (experiment 4). Other inhibitors, such as thiorphan, have also been used as a pretreatment in other stress protocols such as foot-shock. Inhibitors have similarly been used in studies of electrical brain stimulation antinociception (Lake et al. 1989). The experiments reported here also demonstrated that the use of endopeptidase inhibitors may allow for the dissociation between nonopioid- and opioid- mediated effects. For example, because relatively large doses of dynorphin are required for antinociception, it has been suggested by some investigators that its effects are non-specific, or due to a motor impairment caused by its nonopioid metabolites (Herman et al. 1980; Long et al. 1988; Stevens and Yaksh 1986). The use of inhibitors in experiment 2 however allowed for relatively small dose levels of dynorphin to be administered while still producing antinociception. In this way, the non-specific effects attributable to large dose levels was circumvented. Importantly, dynorphin antinociception was not accompanied by motor impairment, a finding supported by previous investigations utilizing the aminopeptidase inhibitor bestatin (Nakazawa et al. 1989). Thus, the studies reported here with the endopeptidase-24.15 inhibitors cFP-A(D)AF)-pAB and cFP-AAF-pAB and elsewhere with inhibitors of endopeptidase-24.11, ACE, and aminopeptidase, demonstrate that peptidase inhibitors can potentially be utilized as discrete and selective probes into the organization and physiology of opioid antinociception.

Clinical Potential. Given the clinical success of the ACE inhibitors captopril and enalapril in controlling hypertension, it is hoped that opioid peptidase inhibitors will eventually be useful in the clinical control of pain. Indeed, acetorphan, a derivative of thiorphan, has been shown to inhibit cerebral endopeptidase-24.11 and to produce antinociception and some other opioid-like effects after intravenous administration (Lecomte et al. 1986). Other investigators report that SCH 34826 is an effective, orally-active endopeptidase-24.11 inhibitor analgesic (Chipkin et al. 1988). The initial biochemical, pharmacological, and now behavioral data, from studies utilizing endopeptidase-24.15 are promising. cFP-A(D)AF-pAB and cFP-AAF-pAB have been shown to be effective in inhibiting the endopeptidase in vitro and in vivo (Chu and Orlowski 1985; Molineaux and Ayala 1990), and in producing various physiological effects (Lasdun et al. 1989; Kest et al. 1991). Furthermore, although cFP-AAF-pAB is a substrate for endopeptidase-24.11, the enzyme is not thought to significantly contribute to its degradation in brain given its low brain activity (Lasdun et al. 1989). This is important in light of the fact that many of the preferred substrates for endopeptidase-24.15 are prodynorphin-derived opioids, which show an affinity for kappa opioid receptors (Hollt

1986; Chavkin et al. 1982). Indeed, experiment 2 of this dissertation demonstrated that dynorphin A $_{1-8}$ antinociception is significantly kappa-mediated. It has been suggested that kappa-mediated antinociception can circumvent many problems associated with mu-mediated antinociception. In contrast to mu receptor pharmacology for example, the pharmacology of kappa receptors indicate less potential for abuse, milder dependence and withdrawal effects, and a decreased number of clinical side effects including respiratory depression (Cowan et al. 1988; Gmerek 1987; Von Voigtlander et al. 1983). Although there may be any number of potential problems with kappa-mediated antinociception in going from preclinical stages to therapeutic usefulness (Shippenberg et al. 1988; Traynor 1988), consideration should be given to the possible application of opioid peptidase inhibitors as future analgesics, potentially capable of selectively acting through the kappa receptor system. The clinical applications of endopeptidase-24.15 inhibitors however need not be limited to the control of pain. As there are a number of peptides that serve as good substrates for endopeptidase, in brain and peripheral organs, inhibition of their enzymatic degradation by endopeptidase may have therapeutic value. For example, endopeptidase-24.15 has been shown to be the primary enzyme responsible for the degradation of leutinizing hormone-releasing hormone (LHRH) in the brain and in peripheral tissue (Lasdun et al. 1989). Inhibitors of endopeptidase-24.15 may therefore ultimately prove to be a useful alternative or addition to the superactive LHRH analogues currently used in the treatment of reproductive and nonreproductive disorders.

Future Directions

Given the goals, results, and implications of the experiments conducted in this dissertation, a number of future research directions can be anticipated. First, the effects of co-administrating inhibitors of endopeptidase-24.15 with other opioid peptidases upon opioid antinociception should be examined. There are indications that potent synergistic effects would be observed. One such indication stems from the fact that several peptidases are involved in the degradation of opioid peptides (Hersh et al. 1981; Erdos et al. 1978; Yang et al. 1981; Benuck et al. 1981; Chu and Orlowski 1985; Acker et al. 1987; Almenoff et al. 1981; Malfroy et al. 1978), and that the concurrent inhibition of two or more peptidases produces antinociceptive effects that are more potent than any inhibitor administered alone (Patey et al. 1983; Chou et al. 1984; DeLabaume et al. 1983; 1982). This is indeed the rationale for the highly effective, multiple endopeptidase inhibitor kelatorphan (Fournie-Zaluski et al. 1984; 1985; Roques and Fournie-Zaluski 1987).Given the increasing body of literature supporting a role for endopeptidase-24.15

in opioid metabolism (Kest et al. 1991; Molineaux and Ayala 1990; Chu and Orlowski 1985), it is anticipated that the concurrent inhibition of endopeptidase-24.11, ACE, or aminopeptidase with endopeptidase-24.15 inhibition may also produce potent antinociceptive effects through additive or synergistic actions. This has already been demonstrated with nonopioid substrates of endopeptidase-24.15. For example, while the half-life of intravenously injected LHRH was increased from 10 minutes to 20 and 15 minutes after receiving inhibitors of endopeptidase -24.15 and -24.11 respectively, the concurrent administration of both endopeptidase -24.15 and -24.11 inhibitors increased the half-life of the peptide up to 80 minutes (Lasdun et al. 1989).

Another potential research direction would be to systematically micro-inject inhibitors of endopeptidase-24.15, and other inhibitors alone or in combination, to circumscribed areas along the neuraxis. Particularly relevant to this undertaking would be an examination of the PAG-rostral medulla-spinal cord axis, as this system and its related structures are known to mediate supraspinal opioid antinociception (see review: Basbaum and Fields 1978; Fields and Basbaum 1978). Although spinal and supraspinal antinociception has indeed been characterized as delta- and kappa-, and mu_1- receptor mediated respectively, there is no clear evidence that opioid receptor types have a perfect correspondence with opioid peptide subtypes (Herkenham 1987), and some peptide subtypes may participate but degrade too rapidly for analysis. Thus, which particular endogenous opioid peptides mediate the antinociceptive effects of this system is not known. Such an examination might be conducted under basal and stressful conditions. It is hoped that under these controlled conditions there would be an alteration in nociceptive thresholds to selective inhibitors only. This approach may thus reveal the relative importance of particular opioid peptide subtypes involved in organizing antinociceptive responses at the different levels of the neuraxis, and how they contribute to an integrated antinociceptive response.

In addition to antinociceptive processes, opioids have been implicated in a wide variety of biological functions. These include appetitive behavior, mood, gastrointestinal motility, reproductive endocrinology, and cardiovascular and respiratory control (Morley et al. 1983; Pfeiffer et al. 1986; Holaday 1983; Heyman et al. 1988; Millan and Herz 1985; Martin 1967). In many cases, the particular opioid receptor(s) mediating these effects are known. For example, the respiratory depression caused by opiates is mediated through the mu_2 receptor site (Ling et al. 1985). Other studies indicate that whereas mu-agonists exert a positive effect upon mood (they produce euphoria), kappa agonists appear to be actually aversive (Mucha and Herz 1985; Bals-Kubik et al. 1989; Woods et al. 1980; Tang and Collins 1985). Physiological systems mediating food intake are more

complex and as such utilize many different opioid receptors (mu, delta, and kappa), active at multiple brain sites, to stimulate feeding (Stanley et al. 1989; Levine et al. 1988; Simone et al. 1985; Jackson and Sewell 1985). However, like antinociceptive processes, the participation of a receptor type in a given response does not necessarily mean that all of the opioids possessing high affinity for that receptor can activate the response. Thus, opioid peptides with the same active "opioid core", but having different extended amino acid sequences, will have significantly different effects. For example, dynorphin $_{1\text{-}17}$ and dynorphin $_{1\text{-}13}$ increase food intake (Mann et al. 1988; Morley and Levine 1981), but dynorphin $_{1\text{-}8}$ does not (unpublished observation). The determination of which opioids participate in a given physiological action must therefore be determined by examining changes in the response after exogenous opioids are applied. However, opioid peptides are highly susceptible to rapid enzymatic degradation, thereby limiting the scope of such research. This limitation may be overcome in some instances by the use of opioid endopeptidase inhibitors. In a related example, peptide studies demonstrate that angiotensin II and III are equipotent in activating central angiotensin-sensitive neurons mediating pressor responses (Fink and Bruner 1985; Wright et al. 1984). One interpretation of these results is that both angiotensin II and III activate angiotensin receptors. An alternate interpretation is that angiotensin III is the active ligand for the receptor, and the biological response seen after angiotensin II is a result of its prior conversion to angiotensin III (Harding and Felix 1987). The aminopeptidase A inhibitor amastatin was successful in inhibiting the conversion of angiotensin II to III, and also suppressed icv angiotensin II-induced pressor responses (Sullivan et al. 1988). A similar approach was used to assess the relative importance of these two neuropeptides on drinking in rats (Wright et al. 1988). It is anticipated that such an approach to peptide pharmacology with opioid peptidase inhibitors will be similarly successful.

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