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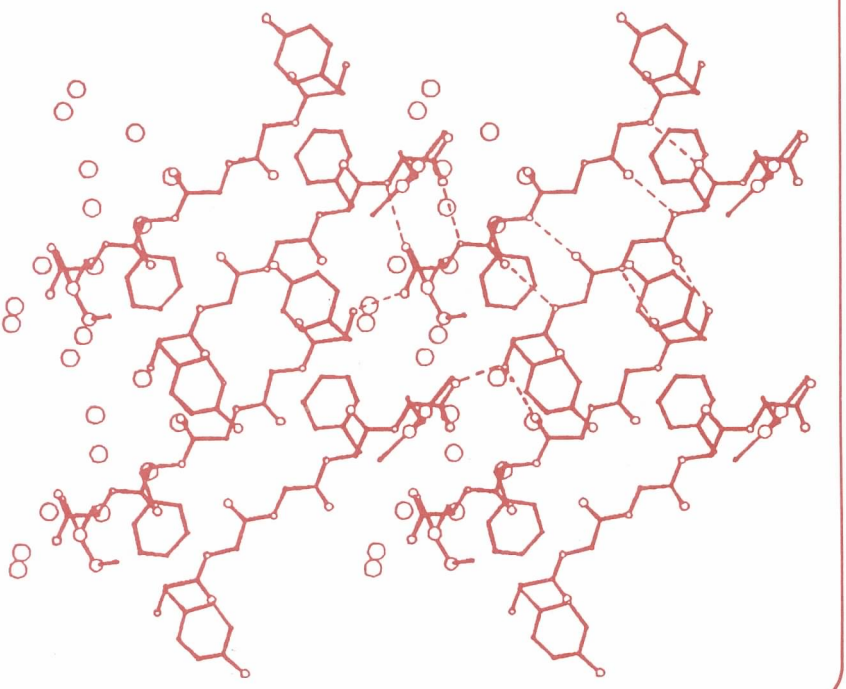

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES • Public Health Service • Alcohol, Drug Abuse, and Mental Health Administration

Opioid Peptides: An Update

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Opioid Peptides: An Update

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Preface

To bring into focus the rapidly expanding areas of research associated with the opioid peptides, an Indo-U.S. Symposium was held in February 1987 at the Central Drug Research Institute (CDRI), Lucknow, India. This symposium was organized by the National Institute on Drug Abuse (NIDA) and the CDRI and was jointly funded by NIDA; CDRI; the Department of Science and Technology, Government of India. and the National Academy of Sciences, New Delhi.

A number of individuals were responsible for the success of this conference. We especially acknowledge the help of Dr. Phil Schambra, Science attache, U.S. Embassy (India). Mrs Linda A. Vogel, Associate Director for Management and Program Coordination, Office of International Health; Dr. George V. Coelho, Chief, International Activities Program, ADAMHA; at NIDA, Dr. James C. Cooper, Associate Director for Medical and International Affairs; Dr. Marvin Snyder, Director, Division of Preclinical Research; and Dr. Richard L. Hawks, Chief, Research Technology Branch; and at CDRI, Dr. R. Raghubir and Mr. K.L. Gupta.

Selected papers from this symposium have appeared as a research monograph published by the CDRI, entitled Recent Progress in Chemistry and Biology of Centrally Acting Peptides. Other presentations from the symposium, along with contributions from invited authors, comprise the present monograph.

We are grateful to all the contributors for their cooperation in preparing this publication. We hope that this volume will serve as a useful reference source on various aspects of the medicinal chemistry, pharmacology, and biochemistry of opioid peptides and will provide new incentives for drug abuse researchers in the opioid peptide field.

Rao S. Rapaka
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Neuropeptides, A Personalized History

Sidney Udenfriend, Ph.D.

The full significance of the important roles in neurobiology played by peptides is still emerging. It seems that each day we read of the discovery and characterization of new peptides in extracts of neural tissue. In fact the chemical structures of neuropeptldes are now elucidated before their biological significance becomes apparent. What is responsible for this rapidly growing field are the recent advances in microprotein chemistry and molecular biology. Therefore a brief history of peptide chemistry is important to understand how we have advanced in so relatively short a period of time in our ability to isolate and characterize trace amounts of peptides in biological extracts. In this review, I will not differentiate between small peptides and large peptides (proteins). I will also discuss peptides in the nervous system as well as in the endocrine system because of the interrelationship of these two systems.

The earliest neuroactive substances to be characterized were the amlnes and substituted amlnes--first acetylcholine, then adrenaline, noradrenaline, and serotonln. Although dopamine was known for some time, it was considered to be an intermediate rather than a neuroregulator in Its own right until somewhat later. However, in some of the earliest studies (late forties and early fifties) it was recognized that other substances were active in nerve and muscle preparations that were not amlnes and whose activities were destroyed by proteases.

In 1950 peptide chemistry was still emerging as a discipline. While synthetic chemists could synthesize small peptides, methods for their purification, even from synthetic mixtures, were not yet very efficient. Sequencing of peptides had not yet been introduced. In fact, there was still controversy as to whether each peptide and protein was a stoichlometrlc entity (i.e., had a unique and invariable sequence) or whether a given protein or peptide was composed of molecules each varying somewhat from the other but the average being fairly constant for that peptide. The dlpeptldes carnosine and anserine had been isolated from muscle. Homocarosine, the GABA analog of carnosine. was found to be uniquely present In brain. However. the high concentration (mg of peptide per g of

tissue) and small size of these compounds required no unusual methodology. The first findings that specific proteins have an invariant molecular composition came from end-group analysis when it was shown that the same protein isolated from highly diverse species always had the same amino terminus. Not until the late 1950s. after Fred Sanger had devised a method for elucidating the amino acid sequence of a protein and applied it to insulin, was it generally conceded that all proteins and peptides had exact molecular compositions.

Several years later, Vincent du Vigneaud isolated and characterized oxytocin and vasopressin from extracts of the posterior pituitary gland. It should be noted, however, oxytocin and vasopressin are present in relatively large amounts in the pituitary gland (ca l Pmole/g). Column chromatography became generally available in the late fifties, and Edman introduced the manual procedure for sequencing peptides at about the same time. These procedures were used in the isolation and characterization of angiotensin and bradykinin. In the late sixties Roger Guillemin and Andrew Schally used similar procedures to isolate TRH from hypothalamus. However, the hormone was so potent that to isolate the amounts needed at the time for analysis and sequencing (ca 50 nmoles) they had to extract tons of tissue. This was very costly and time consuming and required all the patience and skill of these two outstanding scientists. At about the same time many other biologically active factors that were apparently peptide in nature were also reported--substance P, many growth factors, including nerve growth factor, and a host of lymphokine activities including the interferons. Isolation and characterization of these had to await newer developments.

Several major discoveries and advances in technology in the late sixties and seventies, advances that are still continuing today, completely changed our approach to dealing with biologically active peptides. High performance liquid chromatography (HLPC) with ultrasensitive detection systems made it possible to achieve many thousandfold degrees of purification in a relatively few steps and with relatively small amounts of material. The continued improvement in sensitivity and speed of commercial automated sequenators now make it possible to determine the primary structure of a modest sized peptide within a day, starting with 100 pmole or less of pure material. With monoclonal‘antibody techniques, it is now possible to prepare specific antibodies to a biologically active peptide while it is still present in a crude mixture. These monoclonal antibodies can then be used for rapid purification of the peptide. With monoclonal antibodies it is frequently unnecessary to actually isolate the biologically active peptide to determine its sequence. When expression vectors are used for cloning the cDNA derived from the tissue of interest, monoclonal antibodies can be used for clone selection. Sequencing is then carried out on specific cDNA from which the primary structure of the peptide precursor can be deduced. Having isolated and determined the sequence of a peptide or its cDNA, the peptide can be readily synthesized for further study. The synthetic peptide can then be

used to prepare site directed polyclonal antibodies, which provide additional tools for studying the localization and regulation of that peptide.

I would like to briefly discuss some of the developments in the chemistry and biology of neuropeptides that took place in the Roche Institute of Molecular Biology over the past 20 years. Although the structures of oxytocin and vasopressin were already known, it was Sachs and Takabatake (1964) who first demonstrated that each of these was produced as part of a larger polypeptide containing the neurophysin sequence. This information laid the groundwork for the subsequent cloning and sequencing of the cDNAs for oxytocin-neurophysin and vasopressin-neurophysin (Richter 1987).

The dipeptide carnosine, which was originally considered to be restricted to muscle, was found to be present in the olfactory bulb by Margolis (1974). Carnosine is now considered to be a transmitter with a presynaptic site of action. This peptide along with the enzyme carnosine synthetase and the degradative enzyme carnosinase are now used as markers of olfactory nerves and to monitor nerve regeneration. Margolis has also isolated, sequenced, and cloned a protein that is uniquely expressed in the olfactory mucosa (Keller and Margolis 1976).

In my own laboratory (Bohlen et al 1975) we developed HPLC and introduced the fluorescent reagent, fluorescamine, for high sensitivity. These procedures made possible many studies that were subsequently carried out in our Institute and elsewhere. Shortly after the discovery of the enkephalins, my colleagues and I used the fluorescamine HPLC procedure in the discovery of the prescursor of beta-lipotropin (Rubinstein et al. 1978a). Chromatograms of pituitary extracts revealed a ß-endorphin-containing peptide that was larger than beta-lipotropin, about 30K. This appeared simultaneously with the report of Mains et al. (1977), who found that the 30K protein also contained within it the sequence of ACTH. We coined the name proopiocortin for this common precursor of ß-endorphin and ACTH. Subsequently, to emphasize the sequences of MSH in the 30K protein, it was renamed pro-opiomelanocortin, now better known as POMC. In their studies in cell culture, Mains and Eipper found that POMC was produced as a glycosylated protein. For this reason it was generally believed that all forms of POMC are glycosylated and that the latter may have something to do with biological activity. However, we showed that in vivo glycosylation is highly variable from individual to individual within a given species. In pituitaries taken from individual camels, we showed that in some glands isolated POMC was almost entirely in the glycosylated form while in others the molecule contained little or no glycosyl residues (Kimura et al. 1979). Apparently in the case of POMC as in other proteins and peptides the significance of glycosylation remains to be determined.

It is of interest that Rubinstein, who had isolated POMC, subsequently used the same procedures in my laboratory in

collaboration with Pestka to isolate a-interferon from human leukocytes (Rubinstein et al. 1978b). This represented the first purification and characterization of any form of interferon and quite a feat because the amount of interferon in these cells was very small. Although interferon was discovered in the early fifties and many had tried to purify it over the years, it was not until 1978 with the availability of these newer peptide methods that its purification and characterization were achieved.

Getting back to the enkephalin pentapeptides, many believed that they were derived from POMC However, we reasoned that POMC could not give rise to free Leu-enkephalin since it did not contain that sequence. Furthermore, although the B-endorphin derived from POMC contained a Met-enkephalin sequence at its amino terminus, there was no known processing site adjacent to this sequence that could lead to its processing to Met-enkephalin. For those reasons we decided to look for other enkephalin-containing compounds that could serve as precursors. We devised a bioassay that involved homogenizing tissue, then treating the extract with trypsin followed by carboxypeptidase B. By analogy with other peptide precursors, we felt that there was a good possibility that the enkephalin sequence within its precursor would be bracketed by pairs of Lys and/or Arg residues, i.e., -Lys-Arg-enkephalin-Lys-Lys. Trypsin cleaves basic residues at their carboxyl termini and would be expected to cleave out a hexapeptide. enkephalin-Lys or enkephalin-Arg. The latter would be converted to free enkephalin by the carboxypeptidase and so detectable by the binding assay. The first application of the above procedure to guinea pig and rat striatum revealed many large peptides from which both Met- and Leu-enkephalin were released on treatment with the two proteases (Lewis et al. 1978). Following a report that bovine adrenal medulla is rich in enkephalins (Schultzberg et al. 1978), we turned to that tissue from which we isolated and sequenced about ten different enkephalin-containing peptides, some containing withln them as many as four enkephalin sequences (Lewis et al. 1980). Others contained both Met- and Leu-enkephalin. Even before these enkephalin-containing peptides were characterized, we had demonstrated the presence in brain and adrenal medulla of a very large peptide which, on treatment with trypsin and carboxypeptidase B, yielded approximately six Met-enkephalin residues per Leu-enkephalin residue. This led to the concept of a multivalent proenkephalin. It appeared that proenkephalin like POMC was a polyprotein, one containing the sequences of more than one active peptide, and that these sequences were designed to be released by processing.

The many enkephalin-containing peptides that we had isolated and sequenced at the time were like pieces of a jigsaw puzzle. Although we had most of the pieces, some were still missing and their exact order was not clear. However, one of the pieces, Peptide F, contained a sequence that showed no degeneracy at the nucleic acid level, enabling us to make an excellent cDNA probe. The same probe was used by us (Gubler et al. 1982) and by Numa and his colleagues

(Noda et al. 1982) to clone and sequence bovine proenkephalin. The latter was indeed shown to contain six [Metlenkephalin and one [Leu]enkephalin sequence as predicted from the peptides we had isolated. Two of the sequences were designed to be processed out as heptapeptides.

With POMC and proenkephalin sequenced, there still remained two [Leu]enkephalin-containing peptides that were present in the posterior pituitary and whose sequences did not appear in either of these precursors. These were a-neoendorphin (Kangawa et al. 1981) and dynorphin (Goldstein and Ghazarossian 1980). It was already apparent that a third enkephalin-containing precursor must exist. Before it was characterized we (Kilpatrick et al. 1982) and Fischli et al. (1982) independently isolated and characterized a third [Leu]enkephalin-containing peptide (rimorphin) and showed it to be present in posterior pituitary extracts in amounts comparable to a-neoendorphin and dynorphin. Using the sequence information provided by these peptides, Numa's laboratory cloned and sequenced their precursor. prodynorphin (Kakidani et al. 1982). The significance of the redundancy of enkephalin precursors is still not clear, nor are the exact functions of these neuropeptides known. Later, Howells in my laboratory cloned and sequenced rat proenkephalin cDNA (Howells et al. 1984) so that we would be able to study the biology of the opioid peptides in the species that has been used most in studies on opiate drugs. We used the cDNA, antibodies, and HPLC in many different ways. Proenkephalin derived peptides and the corresponding mRNA were found in several nonneural tissues including the heart ventricles (Howells et al. 1986) and the testis and ovary (Kilpatrick et al. 1985) as well. They are also present in the intestine and pancreas. It is apparent that the biological functions of proenkephalin derived peptides, like those of many other neuropeptides. are not limited to the central nervous system. Adrenal proenkephalin transcription and translation are markedly increased by denervation (Howells et al. 1984; Lewis et al. 1981) and are apparently under the control of glucocortlcoids (Yoburn et al. 1987; Mocchetti et al. 1985). Brain proenkephalin transcription and translation are increased by several centrally acting drugs (Tang et al. 1983; Romano et al. 1987).

Antibodies to opioid peptides had been used to trace the proenkephalln innervation in the CNS. However, a more precise and complete localization of proenkephalin neurons in the brain was recently carried out with the corresponding rat cDNA. The in situ hybridization studies were carried out by Howells in my laboratory in collaboration with Pfaff and his colleagues (Harlan et al. 1987). In situ hybridization methods utilizing cDNA are becoming more sensitive and may even be more specific than immunocytochemistry. The latter visualizes nerve bodies as well as fibers, whereas hybridization visualizes nerve bodies exclusively. When dealing with antibodies directed to small molecules, such as the opioid peptides or the catecholamines, diffusion may limit the precision of localization by immunocytochemistry. Localization of

macromolecules, whether precursors, specific enzymes, or specific mRNA, is generally more precise.

I will conclude with two recent examples of the advances that modern biotechnology has brought to the neurosciences. In the late sixties Guillemin had to use tons of beef hypothalami to isolate hundreds of nanomoles for characterizing the first releasing factor, TRH. More recently Guillemin's laboratory was able to isolate a few hundred picomoles of growth hormone releasing factors from several grams of human tissue (Guillemin et al. 1982). This was sufficient for sequencing. Shortly thereafter, in collaboration with Roche scientists they cloned the corresponding cDNA (Gubler et al. 1983). A fraction of the amount of tissue was used, and purification and sequencing were accomplished on far less material and in a much shorter time.

Horecker, while at our Institute, had shown that extracting tissues with 6M guanidine hydrochloride prevents proteolysis (Hannappel et al. 1982). Under these conditions most larger proteins are denatured and precipitate and supernatant solutions contain peptides that are characteristic of a tissue and are not experimental artifacts. A brain extract prepared in this manner yields many peptides on HPLC. Some of these peptides are present in other tissues and are probably known. Others are unique to brain. When Morgan applied this extraction procedure along with HPLC to different portions of the brain, the resulting chromatographic eluates contained relatively few peptides. Two of the unidentified minor components in extracts of cerebellum when isolated and sequenced were shown to be unique molecules and limited to the cerebellum (Slemmon et al. 1984). These two related peptides, which he named cerebellins, are localized in Purkinje cells (Slemmon et al. 1985). The cerebellins are now used as markers for maturation of these cerebellar cells. No doubt many other "minor peptides" present in extracts recovered from specific brain areas will prove to be of interest.

One of the most interesting developments relating to peptides in the brain concerns oncogene products. Oncogenes were originally discovered through their association with tumors, and the translations products of oncogenes are considered to be aberrant versions of normal regulatory proteins or peptides. Protooncogenes code for normal proteins the functions of which are still being determined. Morgan and Curran in our Institute showed that the c-fos protooncogene is rapidly and transiently induced by receptor-ligand interaction and by agents that affect voltage dependent calcium channels (Morgan and Curran 1986). The magnitude of these effects is modulated by pharmacologic agents. More recently they applied immunocytochemical methods to c-fos protooncogene induction as a means of determining the exact cells in brain where specific drugs act.

With all the advances that have been made in methodology and

instrumentation and with our increasing knowledge of how proteins and peptides are made, there is still a great future for peptide research in neurobiology.

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Synthesis and Biological Activity of Novel Met-enkephalin Analogs

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INTRODUCTION

The discovery of enkephalins (Hughes 1975) ushered in a new era in which peptides emerged as a novel class of potent analgesics. Soon after their isolation and characterization was reported by Hughes et al. (1975), the search for other endogenous peptides possessing opioid activity was further intensified and several of them could be isolated from different mammalian tissues and fluids within a short span of time (Rossier 1982). Almost simultaneously, structure-activity relationship studies on these pentapeptides were undertaken in a number of laboratories, primarily with the object of getting a synthetic peptide that could be utilized as a pain reliever in clinical practice (Morley 1980, 1983; Hansen and Morgan 1984). Endeavors of this sort were also started at this Institute, and the interesting results obtained by us are being briefly reviewed in this communication.

RATIONALE FOR THE DESIGN OF ANALOGS

Since the analgesia evoked by enkephalins was found to be only weak and transient after they were injected directly into the cerebral ventricles (Chang et al. 1976; Belluzzi et al. 1976), our emphasis was mainly on the design and synthesis of such analogs of enkephalins that would elicit profound and long-lasting analgesia even after their administration by the systemic routes. For designing new congeners, special care was also taken while introducing novel structural features in the molecules so that the resulting peptides could be synthesized rather conveniently even on a large scale when required. At the very outset, we were struck by the finding that β-endorphin, which is a 31 amino acid peptide having the Met-enkephalin sequence at its N-terminus (Li and Chung 1976; Bradbury et al. 1976), produces a powerful and long-lasting analgesia even after intravenous (i.v.) injection (Tseng et al. 1976). It is nearly 100 times more potent than morphine by intracerebroventricular (i.c.v.) route and 3 to 4 times as potent as morphine when administered intravenously (Li and Chung 1976; Tseng et al. 1976). Based on a comparison of the analgesic properties of β-endorphin and two synthetic congeners

of Met-enkephalin whose N- and C- termini were stabilized against enzymatic degradation (Pert et al. 1976; Bradbury et al. 1977), Bradbury et al. (1976) and Cox et al. (1976) suggested that the high order of activity of β-endorphin was not merely due to its being impervious to brain enzymes. According to them, it could be more of a reflection of its higher binding affinity with the opioid receptors that might be arising from the sequence of amino acids that extends from its N-terminal pentapeptide (Bradbury et al. 1977). If that was true, derivatization of the terminal carboxy function of Met-enkephalin to get alkylamides could be expected to lead to derivatives that would not only be resistant to the attack of carboxypeptidases, but would also possess higher binding affinity for the opioid receptors due to enhanced hydrophobicity at the C-terminus (Beddell et al. 1977). Moreover, the presence of alkyl chains in these derivatives was also expected to increase their overall lipophilic character so that their penetration through the blood-brain barrier would be possible (Belluzzi et al. 1978). To investigate this hypothesis, a series of alkylamides of Met-enkephalin were synthesized and examined for their morphinomimetic activity. As reported earlier, all the newly synthesized compounds of this series exhibited marked opioid activity (Patnaik et al. 1982). The most active compounds were, however, the n-propyl and isopropyl amide derivatives. Encouraged by the initial results, we launched the synthesis of another series of alkylamides in which the N-terminus of the pentapeptides was also protected against the attack of brain enzymes (Hambrook et al. 1976). In this series, the Gly^2 residue of enkephalins was replaced by D-Ala as Pert et al. (1976) and Beddell et al. (1977) had already demonstrated that such a substitution not only protected the Tyr^1-Gly^2 bond against cleavage by aminopeptidases but also potentiated the receptor-binding affinity of the peptides. As expected, all the pentapeptides thus obtained showed more pronounced and sustained antinociceptive activity following intracerebral (i.c.) administration (Mathur et al. 1979). The most promising compounds here again were the isopropyl- and n-propyl derivatives.

With a view to further optimize the morphine-like activity of these pentapeptide derivatives, we proceeded in two ways. On the one hand, a few substituted hydrazides of ($D\text{-}Ala^2$, Met^5)-enkephalin were synthesized as we found that the hydrazide derivative (11) was twice as potent an analgesic as the corresponding amide (compd. 10, table 2). On the other hand, we decided to substitute Phe^4 in peptides 3 and 4 (table 1) with a MePhe residue so that the Gly^2 -Phe^4 bond would be stabilized against the action of enkephalinases (Schwartz et al. 1981; Gorenstein and Snyder 1980) and the hydrophobic site provided by the phenyl ring of Phe^4 would be retained in the molecules for interaction with the opiate receptors (Corin et al. 1980).

The incorporation of this modification could be expected to yield highly potent and systemically active peptides (Römer et al 1977; Römer and Pless 1979). The sulfoxides of derivatives 5 and 6 were also synthesized (compds. 7 and 8, table 1) for studying their activity profile.

TABLE 1

Comparative Morphine-Like Activity of New Enkephalin Analogs

S.No.	Compound	Relative Molar Potency	
		Analgesia (i.c.)	GPI
1.	Tyr-Cly-Gly-Phe-Met-NH.C_3H_7(n)	0.82	0.52
2.	_____ C_3H_7(i)	2.88	0.36
3.	____D-Ala_____ C_3H_7(n)	2.53	4.55
4.	_____ C_3H_7(i)	8.84	5.60
5.	_____MePhe_____ C_3H_7(n)	24.17	30.00
6.	_____ C_3H_7(i)	716.08	12.95
7.	_____Met(0) C_3H_7(n)	20.85	24.78
8.	_____ C_3H_7(i)	353.62	19.06
9.	_____ Gly-__ C_3H_7(i)	706.09	2.98
	Tyr-Gly-Cly-Phe-Met	0.01	2.00
	Morphine hydrochloride	1.00	1.00

ED_{50} Morphine analgesia in mice(i.c.): 0.11±0.01 µg;
IC_{50} (10^{-8}M):5.7±0.07mg/ml

TABLE 2

Analgesic Activity of (D-Ala^2, Met^5)-Enkephalin Amide and Hydrazides in mice (i.c.)

S.No.	Compound	Relative Molar Potency
10.	Tyr-D-Ala-Cly-Phe-Met-NH_2	0.31
11.	_____ $NH.NH_2$	0.56
12.	_____ $NH.NH.C_6H_5$	21.52
	Tyr-Gly-Gly-Phe-Met	0.01
	Morphine hydrochloride	1.00

ED_{50} Morphine analgesia: 0.10 ± 0.01µg

It is evident from the molar. potencies of peptides 6 and 8 (table 1) that the oxidation of Met residue to Met(0) leads to a drastic reduction of the intrinsic activity of the parent peptide (Raghubir et al. 1982). An attempt was, therefore, made to substitute the Met side-chain of analog 6 by other stereochemically equivalent side-chains by replacement of the thiomethylene group of Met with an amide residue and several analogous peptides were obtained (Sharma 1983). In addition, the side-chain of Met^5 was altogether deleted in one of the analogs. As discussed in the subsequent sections, some highly potent systemically active enkephalin analogs could thus be obtained. Finally, a few analogs in which Nval, D-Nval and Gly residues were incorporated as the fifth amino acid and their carboxy functions were derivatized as isopropylamide or phenyl hydrazide were also synthesized in an effort to get an analog which may be more selective for the δ-subtype of opiate receptors. The synthetic strategies and biological activities of some of the more promising analogs of enkephalin obtained under this study are being discussed in the following sections.

SYNTHESIS OF PEPTIDES

All the enkephalin analogs were synthesized in the solution phase by well-established procedures of peptide synthesis. In general, coupling of amino acids and peptides[2] was achieved by the DCC/HOBt, mixed anhydride and 2,4,5-trichlorophenyl ester methods. Except in the case of Met-enkephalin alkylamides, where the carboxy function of Met was initially protected by p-nitrobenzyl group, methyl or ethyl esters were employed for protecting the carboxyl groups. The α-NH_2 functions of amino acids and intermediate peptides were protected either with a carbobenzoxy (Z) or t-butyloxycarbonyl (Boc) group. The cleavage of Boc group was accomplished by treatment of the protected derivatives with TFA, HCOOH or HCl/dioxane in presence of ethanedithiol and anisole. Z-group was removed either by hydrogenolysis over Pd/C or by catalytic transfer hydrogenation using HCOOH as the hydrogen donor.

The synthetic strategy for getting alkylamides of Met-enkephalin involved sequential peptidation of Met-ONBzl with appropriate Boc-amino acid-2,4,5-trichlorophenylesters to get the protected pentapeptide Boc-Tyr-Gly-Gly-Phe-Met-ONBzl. Treatment of this pentapeptide ester with the required amines followed by cleavage of Boc group from the resulting amides gave the desired analogs (Dhotre et al. 1984).

The synthesis of alkylamides of ($D\text{-}Ala^2$, Met^5)-enkephalin was also carried out in a stepwise manner starting from Met-ONBzl in the same way as described for Met-enkephalin alkylamides (Dhotre and Mathur 1984). An alternate strategy involving (3+2) fragment condensation technique was also adopted for the synthesis of peptides of this series as well as for the n-and isopropylamides of ($D\text{-}Ala^2$, $MePhe^4$, Met^5)-enkephalin (figure 1). In this procedure, the C-terminal dipeptide ester Boc-Phe-Meta-OMe or Boc-MePhe-Met-OMe was synthesized by either DCC/HOBt or mixed anhydride method and converted into the required alkylamides via the free peptide acid. The N-terminal tripeptide fragment was coupled with the appropriate C-terminal dipeptide alkylamides via the 2-4-5-trichlorophenyl ester for getting ($D\text{-}Ala^2$, Met^5)-enkephalin

alkylamides and via the mixed anhydride for getting (D-Ala^2, MePhe^4, Met^5)-enkephalin propylamides 5 and 6. Synthesis of Tyr-D-Ala-Gly-MePhe-Gly-NH.C_3H_7(i) (compd.9) was also achieved in the same fashion by coupling the N-terminal tripeptide with . MePhe-Gly-NH.C_3H_7(i). The sulfoxides 7 and 8 (table 1) could be obtained by direct oxidation of compounds 5 and 6 using approximately IN H_2O_2 in acetic acid (Raghubir et al. 1982).

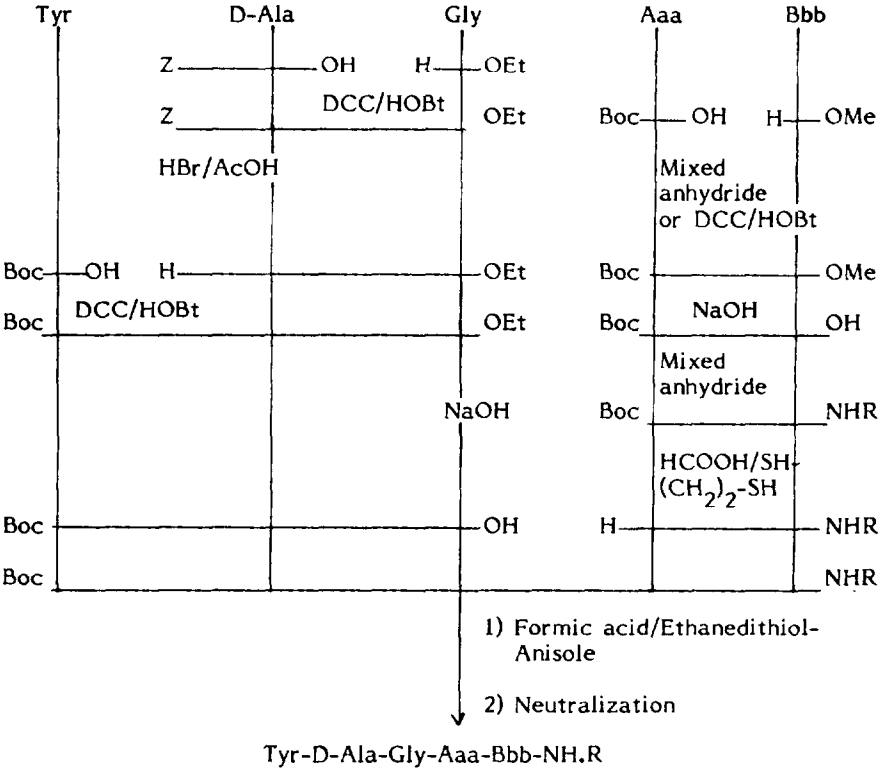

FIGURE 1

Synthetic Strategy For Various Enkephalin Analogs

Aaa:Phe or MePhe; Bbb: Met or Gly; R: n-propyl or isopropyl

Various (D-Ala^2, Met^5)-enkephalin hydrazides (table 2) were synthesized by a slightly modified procedure. The common intermediate Boc-Tyr-D-Ala-Gly-Phe-Met-OMe, was obtained first by coupling the mixed anhydride of Boc-Tyr-D-Ala-Gly with Phe-Met-OMe. Treatment of the pentapeptide ester with hydrazine hydrate directly gave the corresponding hydrazide which was deblocked with HCl/dioxane to give the compound 11. For the synthesis of the phenyl hydrazide 12, the pentapeptide ester, mentioned above, was hydrolyzed and the resulting acid

coupled with phenylhydrazine by the mixed anhydride procedure (Sharma 1983). Cleavage of the Boc group by treatment with HCOOH gave the final product. Homogeneity of all the peptides was checked by chromatography including HPLC.

BIOLOGICAL ACTIVITY

Morphinomimetic activity of all the newly synthesized analogs of enkephalin was examined both in vitro and in vivo. For in vitro tests, the electrically stimulated myenteric plexus-longitudinal muscle preparation of guinea pig ileum (GPI) was prepared from adult guinea pigs of either sex as described by Kocterlitz and Watt (1968). Graded concentrations of various compounds were added to the bath and agonist activity calculated from inhibition of the contraction (IC_{50} values). Morphine hydrochloride and Met-enkephalin were used as standard for comparison. Naloxone antagonism was used to investigate the specificity of action.

For assaying analgesic activity of peptides, the method of Eddy and Leimbach (1953) was employed using mice of either sex in groups of 10 for each dose. The compounds were administered in graded doses intracerebrally (i.c.) and the control group received an equal volume of normal saline by the same route. Percentage of animals showing analgesia was determined at each dose level and ED_{50} calculated according to Finney's Probit analysis (1952). Morphine hydrochloride and Met-enkephalin were used as standard drugs and naloxone was used to antagonize the analgesic effect.

The relative molar potencies of some of the more promising analogs of enkephalin obtained by us in analgesia and GPI tests are presented in tables 1 and 2. As mentioned earlier, the most active compounds among the alkylamides of Met-enkephalin were the n-propyl and isopropylamide derivatives, being 80 and 270 times more potent than the parent pentapeptide in the analgesia test. Lengthening and shortening of the alkyl chain was found to have an adverse effect on the antinociceptive activity. However, the most potent compound of this series in the GPI test was the n-hexyl amide, its activity being 12 times higher than the isopropylamide (Patnaik et al. 1982).

In the (D-Ala^2,Met^5)-enkephalin alkylamide series too, the highest order of analgesic activity was exhibited by the isopropylamide (compd.4) followed by the n-propylamide (compd.3). It can be seen that replacement of Gly^2 with D-Ala has led to a threefold increase in the activity of these peptides (Mathur et al. 1979). Peptides 3 and 4 are approximately 240 and 800 times more potent than Met-enkephalin on a molar basis. Here again, there is no correlation in the in vivo and in vitro activities of compounds. The most active derivative of this series in the GPI test was the ethylamide. The hydrazide of (D-Ala^2, Met^5)-enkephalin was found to be twice as potent as the corresponding amide. When the hydrazide nitrogen was substituted with alkyl groups, no significant change in the activity was observed. In the case of phenyl hydrazide, however, a substantial enhancement of analgesic activity was achieved (table 2). It is nearly 2,000 times more active than Met-enkephalin.

When Phe^4 was substituted by a MePhe residue in the propylamides 3 and 4, highly potent analogs could be obtained. The antinociceptive activity of peptides 5 and 6 is nearly 12 and 80 times higher than that of compounds 3 and 4, respectively. As compared to Met-enkephalin, ($D\text{-}Ala^2$, $MePhe^4$, Met^5)-enkephalin isopropylamide is nearly 70,000 times more potent as an analgesic (Raghubir et al. 1982). The corresponding sulfoxide retains only half the activity of the parent peptide. It has, however, been found to be active even when administered by systemic routes. This is in conformity with the results obtained by Römer et al. (1977) and Römer and Pless (1979).The most significant finding of this study is that the side chain of Met^5 in ($D\text{-}Ala^2$, $MePhe^4$, Met^5)-enkephalin isopropylamide does not seem to play any role in the manifestation of the opioid activity of this analog. As such, the Met residue can be replaced by Gly without any loss of activity. ($D\text{-}Ala^2$, $MePhe^4$, Gly^5)-Enkephalin isopropylamide is, in fact, a highly potent analog and elicits profound and longer-lasting analgesia even after systemic administration (Raghubir et al. 1984). Both the systemically active compounds mentioned above have been taken up for detailed investigation, and their pharmacological profile is discussed elsewhere (Raghubir et al. 1988). We have also found that the corresponding phenyl hydrazide, ($D\text{-}Ala^2$, $MePhe^4$, Gly^5)-enkephalin phenyl hydrazide, is comparatively more selective for s-subtype of opiate receptor.

CONCLUDING REMARKS

It is evident from this review that incorporation of such structural modifications that would inhibit metabolic deactivation of enkephalins and enhance their receptor-binding affinity leads to peptides that show a high order of antinociceptive activity after systemic administration. The aromatic side chain of Phe^4 in enkephalins plays an important role in the manifestation of opioid activity. Stabilization of Gly^5-Phe^4 peptide bond against the action of enkephalinases, so that the hydrophobic site for receptor interaction provided by the Phe residue is not lost, enhances the activity of peptides remarkably. Introduction of hydrophobic chains at the C-terminus of the pentapeptides certainly has a favorable effect on their activity, but the presence of such groups that would lead to an optimum hydrophobicity of the solvent facing part of the molecule is most desirable. The deletion of Met^5 side-chain without causing any loss in the biological activity of the analog ($D\text{-}Ala^2$, $MePhe^4$, Met^5)-enkephalin isopropylamide may be viewed in this context.

It is now well established that enkephalins play several important roles in the CNS in addition to their role in the process of analgesia. They are known to be involved in learning and behavior, modulation of other neuropeptides and putative neurotransmitters, modulation of neuroendocrinal activities and central control of autonomic activities. It will be useful to study the effect of the modified enkephalins on these central parameters as well. This may provide useful leads for potential drugs/tools beyond their present significance in analgesia.

FOOTNOTES

[1]Communication no.4192 from Central Drug Research Institute, Lucknow, India.

[2]Abbreviations for amino acid and peptide derivatives are according to IUPAC-IUB Commission on biochemical nomenclature, Biochemistry 11:1726,1972; other abbreviations are: DCC, NN-dicyclohexylcarbodiimide; HOBt, I-hydroxy-benzotriazole; TFA, trifluoroacetic acid.

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Approaches to Studying Structure-Activity Relationships in Peptide Hormones Through the Expression of Synthetic Genes

John W. Taylor, Ph.D.

INTRODUCTION

The primary structures of a very large number of peptide hormones having diverse pharmacological properties have now been elucidated. In order to understand the functions of these peptides and to exploit their potential as pharmaceutical agents, it is essential to study structure-activity relationships in each case. To this end, the techniques of peptide synthesis have commonly been applied, allowing the direct preparation of deletion analogues and analogues incorporating natural and nonnatural amino acid substitutions. However, many peptide hormones are too large to consider extensive investigations of the role of individual amino-acid residues, as the preparation of multiple analogues in this way would be too time-consuming and expensive, and even the synthesis of the natural structure alone may represent a significant synthetic achievement or produce material that is too poorly characterized to be useful (Clark-Lewis et al. 1986). In such cases, the alternative approach of employing synthetic genes and recombinant DNA technology is becoming increasingly more attractive as new methods in this area are developed. The latter approach consists of three main stages: (a) the assembly from synthetic oligonucleotides of a gene coding for the peptide hormone being studied; (b) the preparation of mutant genes coding for the desired hormone analogues; (c) the efficient expression of the original and mutant genes in overproducing cells from which the hormone analogues are recovered and purified. Compared to peptide synthesis, this approach has the major advantage that all possible deletion analogues or single residue substitution analogues may be prepared simply and rapidly, at little extra cost, by mutation of the original gene once it has been assembled. Furthermore, the purity of the final peptide products prepared in this way, and the likelihood that they will be contaminated with pharmacologically active impurities, is not a function of the size of the hormone or its amino acid composition, as it is when direct chemical synthesis is employed. The purpose of this article is to review the methods that are currently available for preparing peptide hormones by gene expression in *Escherichia coli* and to discuss how they might be employed to perform structure-activity analyses.

OLIGONUCLEOTIDE SYNTHESIS AND GENE CONSTRUCTION

Oligomeric DNA must be synthesized in order to construct a gene that codes for the desired peptide hormone and can be incorporated into a suitable plasmid vector for expression. In addition, synthetic oligonucleotides are employed in the subsequent site-directed mutagenesis of that gene and the sequence characterization of all of the gene constructs produced, as described below. There are two synthetic strategies in common use, each involving the stepwise addition of protected nucleotides to the 5' end of the

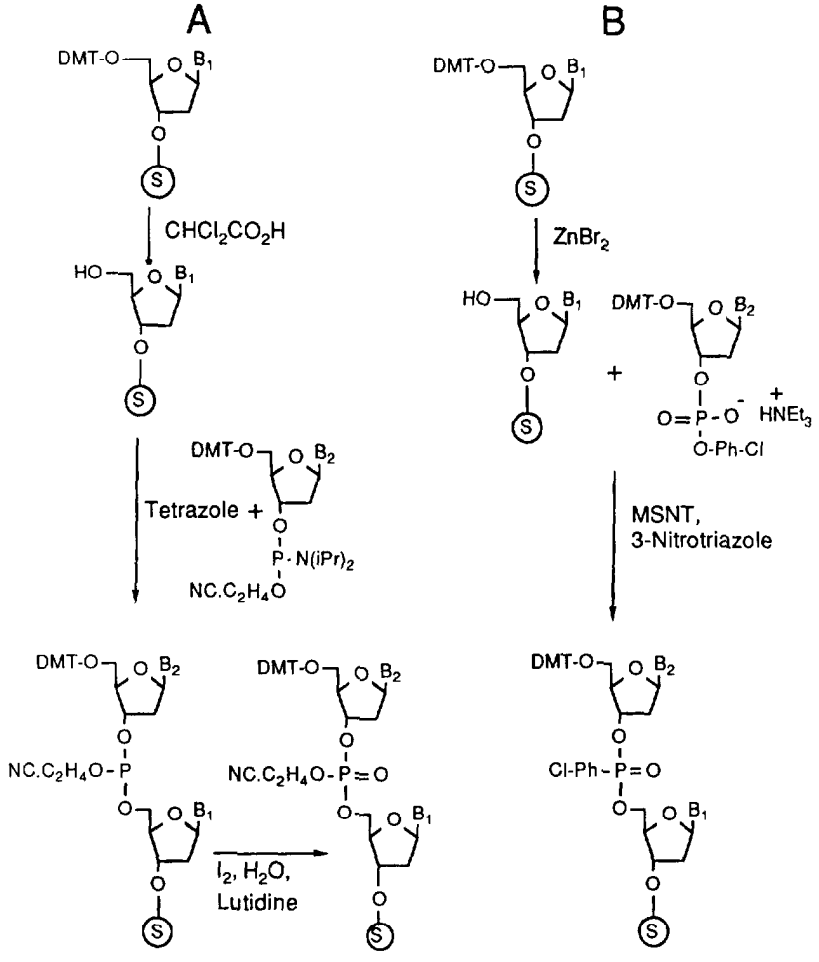

FIGURE 1

Oligonucleotide synthesis by the phosphite method (A) and the phosphate method (B) Oligonucleotides are built up on a solid phase support through the repetitive application of these reactions for each base addition. DMT, dimethoxytrityl; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro 1,2,4-triazole.

growing oligonucleotide chain which is anchored to a solid support at its 3' end (reviewed by Itakura et al. 1984 and Sonveaux 1986). The two methods are distinguished by the use of either a phosphite analogue of the nucleotide (a phosphoramidite) in the addition step which is subsequently oxidized to the phosphate form, or the introduction of the protected nucleotide in the phosphate form directly (figure 1). After completion of the final synthetic cycle, the base- and phosphate backbone-protecting groups are removed, and the linkage to the solid support is cleaved to yield the crude oligonucleotide. If an automated synthesizer is employed under optimal conditions, the synthesis of oligonu-

cleotides as long as 100 bases may be achieved. However, manual syntheses that employ apparatus as simple as a small syringe are quite adequate for the preparation of oligonucleotides up to 20 bases long.

Purification of the correct oligonucleotides from crude synthetic mixtures may be achieved by HPLC on ion-exchange or reversed-phase columns, or by electrophoresis through polyacrylamide gels under denaturing conditions (Sonveaux 1986). The HPLC methods are suitable for oligonucleotides shorter than about 20 bases long and can be used for larger quantities, but often require the assumption that the major product from the synthesis is the correct one. In contrast, polyacrylamide gel electrophoresis can readily provide adequate purification of oligonucleotides 10-100 bases long in sufficient quantities for the uses described here, and can be performed on multiple samples simultaneously in the presence of reliable standards to identify the desired products.

The solid supports that are most commonly used are derivatized silica, which is useful for preparing relatively large quantities of shorter oligonucleotides, and derivatized controlled-pore glass, which gives higher yields per synthetic cycle and is more useful for the present purposes (Sonveaux 1986). In addition, however, Helmut Blocker's group has demonstrated that paper disks may also be used as a solid support for oligonucleotide synthesis (Frank et al. 1983). If these disks are used in conjunction with a synthetic apparatus that incorporates one reaction vessel dedicated to the addition of each of the four base types, then the simultaneous synthesis of multiple oligonucleotides (as many as 40 is reasonable) having unique base sequences can be achieved by numbering the paper disks and resorting them between the four reaction vessels after each successive base addition. Both the phosphite and the phosphate chemistries have been applied to this process, which can result in considerable savings in time and expensive synthetic reagents (Frank et al. 1983; Matthes et al. 1984; Ott and Eckstein 1984). In our laboratory, for example, we have successfully employed the phosphoramidite chemistry to the simultaneous manual synthesis on paper disks of 12 unique oligonucleotides that ranged from 13 to 19 bases in length. These were then purified in a few hours from the crude synthetic products by simultaneous electrophoresis on a single polyacrylamide gel in sufficient quantities and purities for use in oligonucleotide-directed mutagenesis or DNA sequencing experiments. An analysis of the crude and purified products of these syntheses by gel electrophoresis and autoradiography, after 5'-end labelling with ATP-y-^{32}P and polynucleotide kinase, is presented in figure 2. Since the preparation of each mutant gene coding for a new peptide hormone analogue requires a unique oligonucleotide of this size range, the application of Blocker's approach should be particularly useful in this regard.

Several semisynthetic approaches to the assembly of the double-stranded DNA comprising the synthetic gene and its incorporation into a double-stranded plasmid or phage DNA vector may be considered. Most commonly, both strands of the gene are synthesized in their entirety as oligonucleotide fragments that have overlapping base complementarities. These oligonucleotides are enzymatically phosphorylated at their 5' ends, and then annealed together and ligated using T4 DNA ligase and ATP. The resulting segment of double-stranded DNA is designed to have unpaired "sticky ends" that allow another enzymatic ligation into vector DNA linearized with a restriction enzyme that produces complementary "sticky ends." This approach, illustrated in figure 3, can be refined by (a) the use of two restriction enzymes producing different "sticky ends" so that the orientation of the inserted DNA is unambiguous, and (b) using the synthetic DNA in excess over the vector (about five- to tenfold has given us good results) and leaving the oligonucleotides that provide the 5' ends of the synthetic gene unphosphorylated so that ligation of either the insert or the vector to itself is minimized. Using this approach, synthetic genes can be efficiently assembled into vectors in segments of 100-300 base pairs, punctuated by unique restriction enzyme sites (for examples, see Ferretti et al.

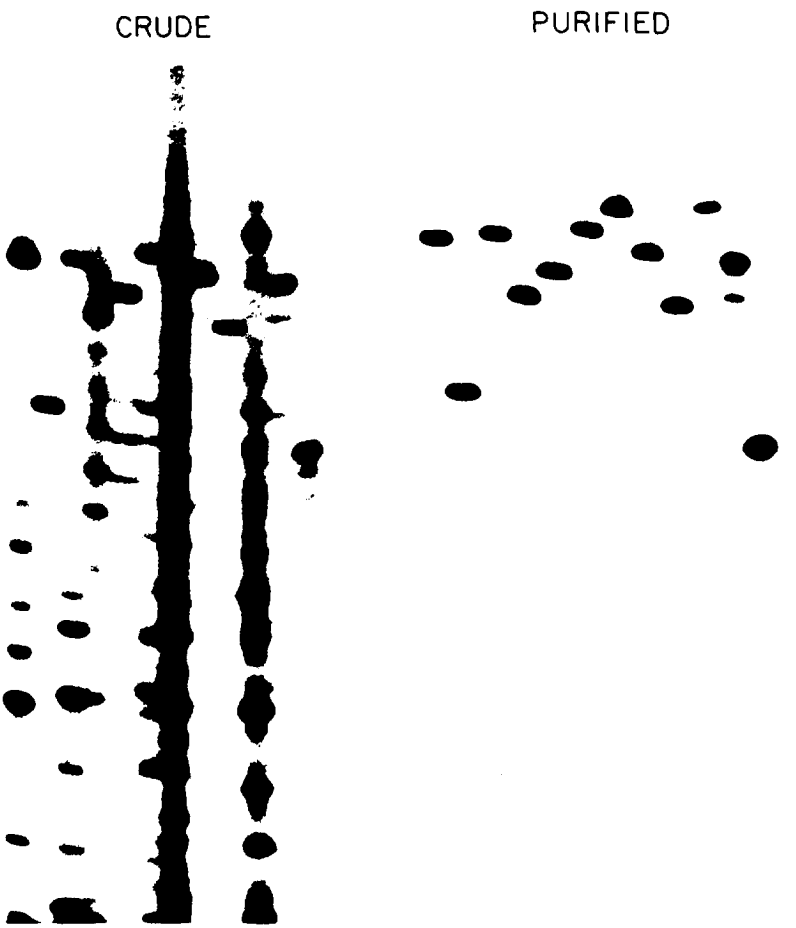

FIGURE 2

Analysis of the products from a manual synthesis of twelve unique oligonucleotides. The oligonucleotides were synthesized simultaneously on individual paper disks using the phosphite method. They were then desalted on Sep-Pak C_{18} cartridges (Waters Co. Ltd.), and then purified by electrophoresis on a 20% polyacrylamide gel and desalted again, as before. The crude (left pane/) and gel-purified (right pane/) synthetic products were analyzed by autoradiography, after 5'end labeling with ^{32}P and electrophoresis on a 15% gel. The oligomers ranged in length from thirteen to nineteen base residues, as follows (left to right): 18mer; 14mer; 18mer; 17mer; 18mer; 18mer; 19mer; 18mer; 16mer; 19mer; 18mer; 13mer.

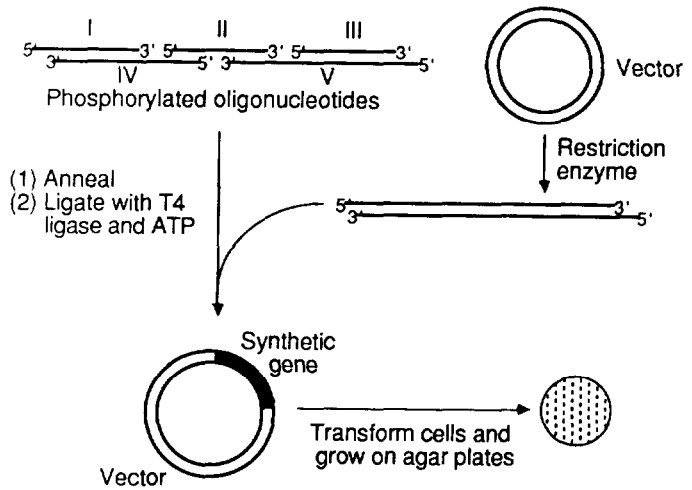

FIGURE 3

Assembly of a synthetic gene and its incorporation into a vector. For best results, the oligonucleotides at the 5'-end of each strand (in this case, I and V) are left unphosphorylated, and the "sticky ends" of the synthetic gene are different, so that the vector must be cut with two restriction enzymes that produce complementary "sticky ends."

1986 and von Bodman et al. 1986). Thus, a synthetic gene coding for a given peptide hormone can usually be assembled in a single step. Oligonucleotides about 50 bases long and overlaps of about 8-12 bases appear to provide the most reliable combination for obtaining the correct annealing and ligation and the fewest sequence errors after cell transformation and characterization of the amplified products. We have also found that it is quite reliable to perform the two ligation steps simultaneously, by adding both the vector and ligase directly to the annealed oligonucleotides. The use of longer oligonucleotides can result in more sequence errors, since there will inevitably be more impurities present from their synthesis even after purification, and the use of shorter oligonucleotides and/or overlaps makes the design of unique overlapping segments difficult and increases the difficulties of obtaining correct annealing. Nevertheless, synthetic genes have been successfully assembled from shorter oligonucleotides prepared by the paper disk method of Blocker described above (Grundstrom et al. 1985; Brodin et al. 1986).

Two other notable strategies have been employed. First, the approach of ligating the synthetic oligonucleotides one strand at a time was successful in a synthesis where the presence of a repeated codon made direct assembly of the duplex difficult (Smith et al. 1984). In this case, the oligonucleotides comprising one strand only were phosphorylated with kinase. These were then ligated together after annealing to the unphosphorylated overlaping oligonucleotides comprising the opposite strand. After denaturing this mixture, the ligated strand was then purified by polyacrylamide gel electrophoresis. This process was performed simultaneously for the second strand, and the two purified strands were then annealed and ligated into the vector in a separate step. The second alternative strategy is one in which oligonucleotide synthesis is minimized by limiting the synthesis to longer overlapping single-stranded segments of the gene, so that the gaps between the overlaps can be filled enzymatically using a suitable polymerase and the

deoxynucleotide triphosphates (dNTPs), before the segments are ligated. This ap roach has also been quite successfully employed (see, for example, Bergmann et al. 1986 but is generally found to be less reliable due to the difficulties of obtaining correct annealing and strand synthesis.

MUTAGENESIS

The preparation of peptide hormone analogues for structure-function analysis requires that the synthetic gene coding for the natural structure be mutagenized. This may be achieved in a random or partially random fashion by a number of chemical or physical methods, including treatments with sodium bisulfite, nitrous acid, hydrazine or UV light, that involve damaging the DNA so that its sequence is incorrectly repaired or replicated upon cell transformation giving rise to mutant progeny (Myers et al. 1985). Alternatively, restriction enzyme recognition sites purposefully introduced into the synthetic gene at strategically useful points may be exploited to introduce more specifically chosen mutations. In this case, either the DNA is cleaved in one strand only by restriction enzyme digestion under suboptimal conditions and then "misrepaired" enzymatically (Shortle et al. 1982), or else two such restriction sites are employed in order to cut out a "cassette" of double-stranded DNA that can then be replaced by any new "cassette" consisting of synthetic oligonucleotides (Lo et al. 1984). The most universally applicable methods, however, involve oligonucleotidedirected mutagenesis (Zoller and Smith 1983). In these methods, a synthetic oligonucleotide carrying a mismatch near the center of its sequence to direct the desired mutation is annealed to its partially complementary sequence on a single-stranded vector carrying the gene or gene fragment to be mutagenized. This oligonucleotide is then used to prime the synthesis of the entire complementary strand *in vitro,* using a DNA polymerase and each of the deoxynucleotide triphosphates (dNTPs). Competent cells are transformed with the resultant heteroduplex DNA and spread out on agar plates, giving rise to both forms of the DNA, from which the desired mutant form must be selected (figure 4a). In principle, all types of mutations of the target DNA sequence are achievable in this way, including deletions, additions, and substitutions of one or more bases, and there are no limitations on the sites that are accessible to change. It is necessary only that the vector carrying the gene can be prepared in the single-stranded form, and that a suitable mismatched primer oligonucleotide can be designed and synthesized so that it will anneal specifically to the correct site on the gene. Since most expression vectors are double-stranded plasmids, the requirement for the single-stranded form usually necessitates a subcloning step, where the gene or gene fragment to be mutagenized is cut out by restriction enzyme digestion and transferred to a single-stranded bacteriophage such as one of the convenient M13 vectors adapted by Messing and coworkers to contain multiple cloning sites (Messing 1983). Once the mutant has been prepared, the gene can then be transferred back to the original plasmid for the purposes of expression. In some cases, however, this process has been simplified either through the use of phage vectors for expression directly (Wilkinson et al. 1983), or through the use of plasmid expression vectors of the pEMBL type, which are normally double-stranded but contain the origin of replication of M13 phage DNA and can readily be prepared in the single-stranded form upon infection of the plasmid-carrying cells with phage (Dente et al. 1983). The requirement for specific annealing of the mismatched oligonucleotide to the desired site usually presents no problems in cases involving the addition, deletion, or substitution of a single codon. It is usually sufficient to place the mismatched bases at least eight bases from either end of the oligonucleotide, and the precise arrangement can be further optimized for specific annealing to the correct site through the use of commercially available computer programs designed for this purpose.

The major difficulty in oligonucleotide-directed mutagenesis is often the identification, after cel l transformation with the heteroduplex DNA, of the plaques of phage-infected cells that contain the mutant phage. *E. coli* cells have a DNA mismatch repair system

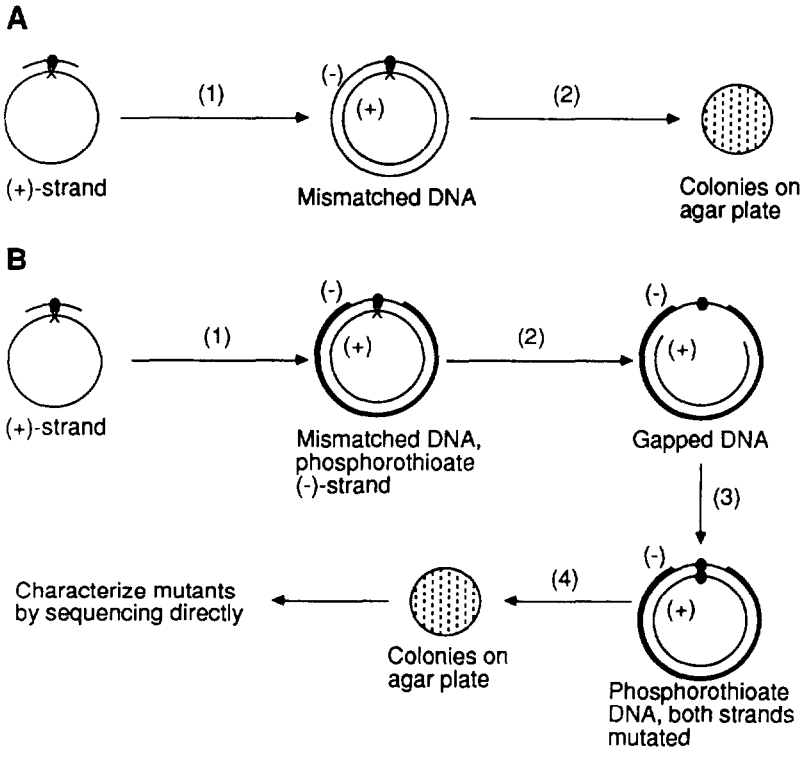

FIGURE 4

Oligonucleotide-directed mutagenesis using M13 vectors. (A) The unmodified procedure of Zoller and Smith (1983): (1) Mismatched primer extension with the Klenow fragment of DNA polymerase I and the dNTPs; ligation with T4 DNA ligase and ATP. (2) Transformation of competent cells and colony growth on agar plates. (B) The phosphorothioate method (Sayers and Eckstein 1987): (1) Mismatched primer extension with Klenow, dATP, dGTP, dTTP and dCTP-α-S; ligation with T4 ligase and ATP. (2) Nicking with Nci I; gapping with exonudease III in the 3' to 5' direction, or with A exonuclease or the T7 gene 6 exonuclease in the 5' to 3' direction, as appropriate. (3) Repair with DNA polymerase I, the dNTPs, T4 ligase and ATP. (4) Transformation and colony growth.

that depends upon the action of the dam methylase, which labels DNA in vivo by methylation of GATC sequences, in order to recognize the original (methylated) viral (+)-strand and correct mismatches that were introduced into the (-)-strand during synthesis *in vitro* (Kramer et al. 1984a). The efficiency of this system is dependent on the type of mismatch introduced, but it typically reduces the frequency of occurrence of the mutant phage to about 10% of the plaques, and much lower frequencies are quite common (Taylor et al. 1985a). Furthermore, most of the plaques containing mutant phage that were obtained by plating out transformed cells directly are contaminated by the presence of the nonmutant form and must be replated at least once to ensure that the pure mutant form is obtained. Since the mutagenesis of a hormone gene in a phage vector

carried by *E. coli* cells will not normally result in a recognizable change in plaque phenotype, the mutant phage obtained by this method must be identified by screening a large number of plaques with a hybridization assay using the ^{32}P-labelled mismatched oligonucleotide (which is perfectly complementary to the mutant page DNA). This assay involves transferring the plaques obtained in a mutagenesis experiment onto nitrocellulose filter paper, denaturing the phage and then hybridizing the radiolabelled oligonucleotide to the exposed immobilized phage DNA. Mutant and nonmutant phage are then distinguished by autoradiography after washing the nitrocellulose paper in a high salt buffer at successively higher temperatures. The radiolabel is washed off the samples of nonmutant phage at lower temperatures (Zoller and Smith 1983).

In order to avoid the time-consuming hybridization screening step described above and eliminate the use of high specific activity ^{32}P radiation, a number of modified mutagenesis procedures have been developed that increase the frequency of occurrence of the mutants to a point where they may be conveniently identified and characterized directly by DNA sequencing. For this purpose, it is necessary to increase the efficiency of the mutagenesis to a point where the random selection for sequencing of only two or three plaques from each experiment will result in a high probability of identifying the desired mutant in a pure form. The relationship between the mutagenesis efficiency and the number of plaques that must be screened to achieve a satisfactory 90% probability of success is graphically illustrated in figure 5 (Kramer et al. 1982). Clearly, any method that will be useful in this regard needs to achieve reliable efficiencies of mutagenesis that are higher than about 50%. Only a few methods are adequate in this regard. These methods depend upon either selecting against the original DNA sequence in the (+)-strand of the heteroduplex *in vivo,* after transformation, or eliminating the original DNA sequence in that strand *in vitro,* before transformation.

Selection against the (+)-strand in vivo has been successfully achieved in different ways. Kunkel (1985) has shown that single-stranded Ml 3 vectors carrying cloned genes may be prepared in host cells deficient in dUTPase (*dut* $^-$) and uracil glycosylase (*ung* $^-$) to contain several deoxyuridine residues per molecule in place of the normal thymidine residues. When this DNA is used as the template to produce heteroduplex DNA in the normal way, transformation into *ung* + cells results in glycosylation and excision of the uracil moieties, creating multiple abasic sites and a consequent strong selection against the original (+)-strand molecules during the subsequent replication. The result is that more than 50% of the plaques obtained from these transformed cells contain progeny phage that are the desired mutants derived from the (-)-strand sequence. In a similar manner, the amber nonsense mutation or the Eco K restriction site have been exploited as genetic markers that may be incorporated into the (+)-strand of M13 DNA when it is prepared in a suitable suppressor-carrying or *Eco K*$^-$ host, respectively (Carter et al. 1985). In these approaches, selection against the (+)-strand of the heteroduplex DNA that is subsequently prepared *in vitro* requires an additional mismatch in the (-)-strand opposite the marker, so that transformation into a suppressor-deficient or Eco K-restricting host, as appropriate, will select against progeny derived from the (+)-strand only. The simplest way to achieve this double mismatch is to use two mismatched oligonucleotides simultaneously to prime the (-)-strand synthesis *in vitro.* In this case, the oligonucleotide annealed to the site of the genetic marker would be identical for all the mutations directed by the second mismatched primer in the normal way, so that little extra synthetic effort is required. Other, more difficult approaches involving the preparation of heteroduplex, gapped DNA have also been applied to these selection methods (Kramer et al. 1984b). Again, the typical mutation efficiencies appear to be about 50%, although higher efficiencies have been reported, so that all of the preceding methods for in vivo selection against the nonmutant strand appear to allow the use of DNA sequencing methods to identify and characterize the mutants generated directly.

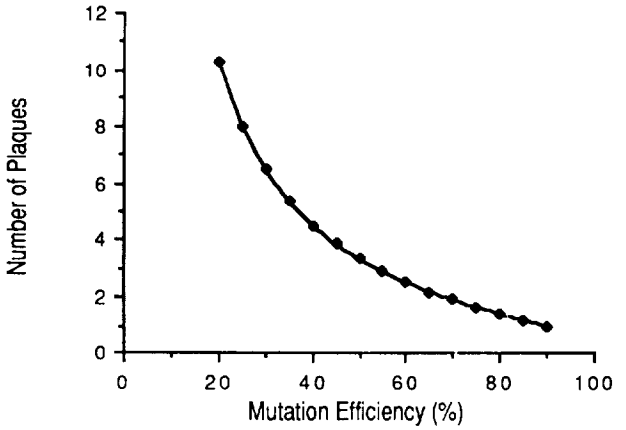

FIGURE 5

Relationship between the number of phage plaques that must be screened to have a 90% probability of identifying a mutant and the efficiency of the mutagenesis method used. (Adapted from Kramer et al. 1982. Copyright 1982, IRL Press, Ltd.)

Most recently, my coworkers and I in Fritz Eckstein's laboratory have developed a very efficient method for oligonucleotide-directed mutagenesis that takes the alternative approach of eliminating the original (+)-strand sequence opposite the mismatched primer in the heteroduplex DNA, before it is used to transform competent cells (Taylor et al. 1985a; Nakamaye and Eckstein 1986; Sayers and Eckstein 1987). In this approach, the mismatched oligonucleotide primer is annealed to the (+)-strand of the M13 vector DNA with its cloned insert in the normal wa y, and then the *in vitro* synthesis of the complementary (-)-strand is performed with the dCTP in the reaction mixture entirely substituted by its α·-thiophosphate analogue, dCTP-α-S. The a-thiophosphate analogues of the natural dNTPs are diastereomeric and all of the DNA polymerases that have been investigated to date readily accept the Sp diastereomer of each dNTP-α-S as a substrate, incorporating it into DNA with inversion of configuration about the phosphorus atoms almost as efficiently and accurately as for the unmodified dNTPs (Burgers and Eckstein 1979). The product of this *in vitro* synthesis is, therefore, heteroduplex covalently closed circular DNA that contains in its (-)-strand the desired mutant sequence, as well as hosphorothioate internucleotidic linkages of the Rp configuration on the 5' side of each dCMP base residue (figure 4b). Phosphorothioate-substituted Ml 3 DNA has similar physicochemical characteristics to the natural DNA and retains its infectivity upon transformation of competent host cells, but also has the property of being resistant to linearization by restriction enzymes, particularly those which cleave their recognition sequences on the 5' side of the base type that was substituted by its a-thiophosphate analogue in the synthesis. Indeed, for about one-third of the restriction enzymes that we have tested, linearization that requires cleavage of a phosphorothioate linkage appears to be completely blocked (Taylor et al. 1985b). However, these enzymes are still able to hydrolyse the unsubstituted (+)-strand of the phosphorothioate DNA quite efficiently in the normal way, to produce double-stranded DNA with a single "nick" placed specifically in the (+)-strand at the restriction enzyme cleavage site. We have exploited this property of the Nci I restriction enzyme in order to specifically hydrolyse the (+)-strand of the heteroduplex DNA containing dCMPS-substitutions in the (-)-strand at recognition sites of the type shown below.

(+)-strand: 5'-CC/GGG-3'
(-)-strand: 3'-GGC/CC-5'

Nci I normally cleaves the internucleotidic linkage on the 5'side of the central base residue in both strands of such sequences, as indicated, but in this case cleavage of the (-)-strand is blocked. The nicks that are generated in the (+)-strand can then be used as starting points for its digestion in the 3' to 5' direction, using the enzyme exonuclease III. This digestion occurs at a reproducible rate of about 100 bases per minute in all of the DNA sample simultaneously, provided that the correct buffer conditions and excess of the exonuclease are used. When digestion has proceeded to a point safely beyond the desired point of mutagenesis (opposite the mismatched primer oligonucleotide), the gapped DNA can be repaired by polymerization with dNTPs, using the mutated sequence of the (-)-strand as the template to produce phosphorothioate DNA having the desired mutant sequence in both strands (figure 4b). In a number of trial base-substitution mutagenesis experiments, where mutant a 13 DNA prepared in this way was used to transform competent cells, 80% - 90% of the plaques obtained were consistently found to contain the desired mutant phage uncontaminated by the unmutated form (Nakamaye and Eckstein 1986).

Based on the above results, the phosphorothioate DNA approach promises to be the most efficient of the enhanced oligonucleotide-directed mutagenesis methods, and it requires only a few hours of additional manipulations over the basic protocol and has no special requirements for unusual host cells or vectors. The dNTP-α-S analogues are commercially available and give satisfactory results when used as the racemic mixtures directly. Furthermore, all of the potential limitations of this approach appear to present no additional difficulties and it should be applicable to every mutagenesis problem. For example, other restriction enzymes besides Nci I may be employed for the nicking reaction (in conjunction with the appropriate α-thiophosphate nucleotide analogue); the exonuclease III digestion and subsequent repair reactions are efficient over a range of several thousand bases; and gapped DNA may also be prepared by digestion from a nick in the opposite, 5' to 3' direction, using either λ exonuclease or the T7 gene 6 exonuclease (Sayers and Eckstein 1987). Thus, one or more appropriate restriction enzyme recognition site present at any position in the vector or cloned gene is probably a sufficient prerequisite for the method and one that is easily satisfied. In the case of Nci I, the recognition sequence described above is present in all of the M13 vectors and the alternative Nci I site, where the (+)-strand sequence is 5-CCCGG-3', does not seem to interfere when it is also present, because its cleavage in the (-)-strand is also blocked by the dCMPS substitution (Nakamaye and Eckstein 1986). Initial experiments (Sayers and Eckstein 1987) indicate that deletion mutagenesis is also just as efficient using these methods, despite the expected difficulty of digesting the loop of unpaired bases in the (+)-strand opposite the mismatched primer during the gapping step. (The third alternative of insertion mutagenesis, in which the heteroduplex phosphorothioate DNA will have an unpaired loop in its (-)-strand, is expected to be straightforward.) Finally, we have failed to detect any unexpected mutations in addition to those that were directed by the mismatched oligonucleotides during our extensive characterization by sequence analysis of the mutants generated by these procedures. This indicates that the phosphorothioate DNA in the (-)-strand is a suitable template for accurate DNA synthesis by DNA polymerase I during repair of the gapped DNA in the final step of the *in vitro* manipulations.

The high efficiency of the phosphorothioate DNA method for mutagenesis suggests that new approaches to the rapid preparation of large numbers of mutants of a given peptide or protein might be possible. Nearly all of the DNA that is prepared *in vitro* contains the mutant sequence that is determined by the mismatched primer oligonucleotide. Therefore, the use of oligonucleotides that contain degenerate positions would allow the effi-

cient single-step preparation of, for example, mutants of an enzyme in which an active site residue is replaced by all other possible naturally occurring amino-acid residues. The subsequent identification and characterization of these mutants would be very rapid, because it could be performed directly by DNA sequencing. Alternatively, multiple specific mutations at many different sites could be generated very rapidly by combining this mutagenesis method and direct sequence analysis with the paper disk approach of Helmut Blocker to synthesize the large number of mismatched oligonucleotides that would be required. This should be extremely useful in the characterization of structure-activity relationships in the larger peptide hormones that we are considering here, particularly when methods are available for the efficient expression and purification of the mutants generated.

EXPRESSION AND PURIFICATION

The most important aspects of the expression of a synthetic gene in *E. coli* cells and the purification of the protein product are the requirements for efficient transcription of the gene and subsequent translation of the mRNA; the location, structure, and stability of the peptide product; and the possible methods by which it, and any mutants of interest, might be separated from the other protein constituents of the host cells. These subjects have been reviewed extensively elsewhere (Gold et al. 1981; Wetzel and Goeddel 1983; Marston 1986) and the present discussion will focus on the particular problems that are associated with the preparation of peptide hormones and their analogues, and the approaches to solving those problems that have been adopted to date.

Various factors are known to affect the efficiency of transcription and translation, sometimes dramatically. These include the strength of the promoter, the structure of the ribosome-binding site and its position relative to the translation start codon (usually AUG) on the mRNA, the stability of the mRNA, and the presence of transcription attenuators near the end of the open reading frame of the message. There are also strong correlations between codon usage in *E. coli* genes and the levels to which the corresponding proteins are expressed, which suggest that expression levels are determined by the codon-anticodon interaction energies and by tRNA abundancies (Grosjean and Fiers 1982). Unfortunately, there appear to be no absolute rules governing the precise choices that must be made in order to optimize these many factors for efficient expression. The approach that most researchers have adopted has been to employ the best natural systems, either directly or through the design of consensus structures. Thus, *trp, lac, lpp,* λP_L and *pho* A promoters and ribosome-binding sites have all been employed directly, or in synthetic combinations such as the tac hybrid derived from *trp* and *lac* (De Boer et al. 1982) or the rac hybrid derived from *rrnB* and *lac* (Boros et al. 1986) which appear to be stronger than their parent structures. The design of hybrid or consensus promoters and ribosome-binding sites does not, however, always lead to better expression systems (see, for example, Deuschle et al. 1986). Most of the promoters that are commonly employed also offer the advantage of being inducible, for example by temperature shift (λP_L) or addition of a metabolite (systems based on trp and lac). This allows gene expression to be switched on at the most advantageous time, which is particularly important in cases where the protein produced is detrimental to the cell.

The codons chosen to design a synthetic gene are usually taken from tabulations of the most abundant tRNAs (Ikemura 1981) or the most abundant codons in highly expressed genes (Yarus and Folley 1985) although exceptions are often made so that convenient restriction enzyme recognition sites can be built in. The importance of this consideration was highlighted by the use of a synthetic gene that had been optimized in this manner to produce bovine growth hormone (Seeburg et al. 1983). The synthetic gene was expressed at much higher efficiency than the natural mammalian gene had been. Additional empirical rules governing codon choice have also been suggested (Yarus and Folley 1985).

Unfortunately, it is possible that the choices made at this stage of the design of an expression system may result in quite unpredictable problems that are difficult to identify or solve: if certain DNA sequences cause the RNA polymerase to slow or stop, this may result in premature dissociation of the transcription complex and truncation of the message; or, if protein folding occurs concomitant with translation, other sequences may disrupt this process through their effects on the rate of translation of the mRNA on the ribosome, and incorrectly folded products may result. Even less information is available regarding mRNA stability and how it can be enhanced. Although stability might be related to secondary structure formation throughout the message, as well as, possibly, its overall length, experiments suggest that the structure of the 5' end is critical. Again, the approach to this problem has been to employ natural sequences in the 5' untranslated region that are taken from stable systems. In the case of the T4 gene 32 message, for example, the 5' sequence has been shown to confer stability on unstable sequences that are fused downstream to it. This property has been exploited in the design of a plasmid vector employing the promoter region and start codon of that gene and requiring phage T4 infection for expression (Duvoisin et al. 1986).

In the expression of foreign proteins in *E. coli* cells, the stabilit y of the product within the cells is an especially important consideration. Such expression has been shown to turn on the production of the Ion protease and other gene products regulated by the htp R gene product associated with the heat shock response, leading to much higher rates of proteolytic degradation than normal (Goff and Goldberg 1985), and the use of Ion- and htp R mutant host strains is certainly helpful in this regard (Buell et al. 1985). Whether these responses are triggered by particular "foreign" amino acid sequences, or by incorrectly folded structures, or some other mechanism is responsible is not presently known. In addition, the half-lives of proteins that are endogenous to the cells vary themselves over a wide range, indicating that there are mechanisms for targetting proteins for degradation that are normally in effect and might act on an overexpressed foreign protein also. *E. coli* has a large number of proteolytic enzymes that might participate in these processes, and are vital to the normal functioning of the cell (Swamy and Goldberg 1981). Empirical correlations suggest a strong relationship between the half-life of a protein in *E. coli* and its N-terminal amino acid sequence. In particular, the "PEST" hypothesis suggests that sequences rich in Pro, Glu, Ser, and Thr will be most highly susceptible to degradation (Rogers et al. 1986). Alternatively, procaryotic cells may have a mechanism for protein turnover that is similar to the ubiquitin-associated mechanism that appears to operate in eucaryots, in which case an important determinant of the half-life will be the N-terminal residue itself, with Arg, Lys, Asp, Leu, and Phe directing the most rapid degradation, and Met, Ser, Ala, Thr, Val, and Gly dictating the greatest resistance to degradation (Bachmair et al. 1986).

Proteolytic degradation of the desired product is, perhaps, the most difficult problem attendant to the production of smaller peptides such as the peptide hormones. Their direct expression is usually followed by their rapid degradation, so that large quantities can never accumulate. This is almost certainly because these peptides are too small to fold into a stable globular structure, and their extended flexible conformations can readily fit into the typical active-site groove of a broad-specificity protease. The first successful solution to this problem, and the approach that is still most commonly applied, was to express the peptides as part of a much larger fusion protein by splicing the corresponding synthetic gene in frame to the end of the gene coding for a highly expressed protein that could act as an intracellular carrier. In this way, somatostatin, β-endorphin) and both the A and B chains of insulin were separately produced, each fused to a point near the C terminus of β-galactosidase (Itakura et al. 1977; Shine et al. 1980; Goeddel et al. 1979). Expression was directed under the control of the strong inducible *lac* promoter and the peptides were recovered from the purified fusion protein by proteolytic (β-endorphin) or

chemical (somatostatin and the insulin chains) cleavage at specific amino-acid residues engineered into the structure for this purpose.

The fusion protein approach solves a number of the problems discussed above simultaneously, provided that the carrier protein is normally expressed at a high level in *E. coli* and it is being used as the N-terminal portion of the hybrid. At the RNA level, the structure around the ribosome-binding site should already be optimized for transcription, translation, and mRNA stability, and codon usage for most of the rest of the message should also be correct. After translation, the N-terminal amino acids should be consistent with the requirements in that region for a stable protein *in vivo.* There are additional advantages in relation to the purification procedures, First, the production of a larger protein aids in its direct identification by polyacrylamide gel electrophoresis, since the identification and visualization of small peptides by these techniques is difficult. This usually eliminates the need for antibodies directed against the desired peptide in order to follow its expression and purification. If enzymatic carrier proteins such as β-galactosidase or alkaline phosphatase are used, activity can often be assayed directly on agar plates by calorimetric methods. Second, affinity columns can often be designed that target the carrier portion of the fusion protein during purification. Such affinity columns are usually constructed using antibodies to the carrier protein or, if it is an enzyme, inhibitors may be employed more conveniently. Affinity chromatography based on the carrier is a particularly important asset when multiple mutants of the peptide portion are being prepared, since the purification procedures should be identical in each case. Finally, procaryotic proteins are synthesized *in vivo* with N-formyl-Met at the N terminus which is usually cleaved from the mature protein during posttranslational processing. However, overexpressed foreign proteins are often produced with the N-terminal Met deformylated but still attached or incompletely removed, and the factors that govern the processing are not understood. The fusion protein strategy, with the carrier portion at the N terminus, therefore eliminates this potential problem also.

To date, many small peptides in addition to those mentioned above have been expressed using the gene fusion strategy. These include antigenic determinants from hepatitis B fused to β-galactosidase and expressed under control of the *lac* promoter (Charnay et al. 1980) or fused to β-lactamase (trp promoter; Edman et al. 1981); influenza virus antigens fused to β-galactosidase *(lac* promoter; Davis et al. 1981); hirudin fused to β-galactosidase *(lac* or λP_L promoters; Bergmann et al. 1986); TGF-α fused to 17 residues of the *trp* LE protein and promoter (Winkler et al. 1986); calcitonin fused to interferon-γ (λP_L promoter; Ivanov et al. 1987); and 27-desamidosecretin fused to β-galactosidase (lac promoter; Sumi et al. 1984). Frequently, these fusion proteins are insoluble within the cellular environment, even when produced at low levels, possibly as a result of incorrect folding of the carrier. This results in the formation of intracellular aggregates or inclusion bodies consisting predominantly of the fusion protein. These structures can often be isolated by centrifugation, and subsequently dispersed under denaturing conditions, as reviewed recently by Marston (1986), with considerable advantages in the purification strategy.

In a number of cases, the fusion approach is unsatisfactory, either because recovery from the inclusion bodies is difficult, because a soluble fusion protein is desired (when the peptide is to be used directly as an antigen) or, very commonly, because proteolyic degradation of the peptide component still occurs, leading to impure products and a low yield. In these cases, proteins such as alkaline phosphatase, Omp A or β-lactamase, which have N-terminal signal- or leader-peptide extensions that direct their translocation across the cytoplasmic membrane and subsequent release into the periplasmic space, have been useful as alternative carriers. When correct transport and processing by signal peptidase occurs, large quantities of the fusion protein can accumulate in the periplasm, where precipitation has not generally been encountered, although proteolytic

degradation can still be a problem. Since relatively few *E. coli* proteins are present in the periplasm, and its contents can usually be selectively released by osmotic shock, secretion to the periplasm is also advantageous in terms of purification (Marston 1986). Examples of peptides that have been prepared in this way include human proinsulin, which was fused to N-terminal fragments of β-lactamase (Chan et al. 1981) and the Met^{27} analogue of human GRF, which was fused to the phosphate-binding protein, pho S (Anba et al. 1987). However, when α-neoendorphin was fused to alkaline phosphatase near its C terminus, transport or release into the periplasm was unexpectedly blocked (Ohsuye et al. 1983). On the other hand, it is often possible to dispense with the mature carrier protein entirely and simply connect the synthetic peptide directly to a signal peptide sequence with its signal peptidase recognition site intact. Thus, for example, human EGF and hirudin have both been expressed, transported across the cytoplasmic membrane and released into the periplasm after correct signal peptidase cleavage, through their fusion to the alkaline phosphatase signal peptide (Oka et al. 1985; Dodt et al. 1986); human growth hormone was similarly processed under the direction of the alkaline phosphatase signal peptide or its own (eukaryotic) signal peptide (Gray et al. 1985); and proinsulin was secreted into the periplasm by fusion to the β-lactamase signal peptide (Chan et al. 1981) or through the correct transport and processing of the preproinsulin gene product (Talmadge et al. 1980). In a small number of cases, the processed peptides have been found predominantly in the extracellular medium, indicating that they have passed through the outer cell membrane also. These include β-endorphin, which had been fused to the Omp F signal peptide and an additional 12 amino-acid residues, and was subject to proteolytic degradation at its C terminus (Nagahari et al. 1985) and human growth hormone (Kato et al. 1987) and α-fibrinogen (Lord 1985) each of which had been fused to β-lactamase signal peptides. In the human growth hormone study, passage across the outer membrane was promoted by additional weak expression of the kil gene, which makes the outer cell wall permeable without cell lysis, suggesting that passive diffusion across this membrane occurred. The large size of α-fibrinogen (molecular weight 67,000) would seem to preclude passive diffusion, indicating that other mechanisms of translocation across the outer membrane may also be important. However, extensive degradation of this protein was demonstrated, and the method of detection employed was dependent only on the presence of a short peptide fragment at its N terminus, so that passive diffusion across the relatively porous outer membrane might explain the extracellular presence of all three of these peptides. Whatever the mechanism, transport to the extracellular medium appears to yield smaller quantities of these peptides than can be obtained from intracellular fractions, and is not necessarily advantageous in terms of purification.

Unless an endogenous proteolytic cleavage system such as the signal peptide/signal peptidase system described above is accurately exploited, the fusion protein strategy to peptide production requires that a specific cleavage reaction must be devised for the release of the target peptide from the fusion protein. This step is performed after cell lysis and, usually, after some initial purification. Several site-specific methods for such peptide backbone cleavages have been employed, all of them requiring that a "recognition site" consisting of one or more residues be engineered into the fusion protein on the N-terminal side of the scissile peptide bond. In most cases, the structural requirements for these sites are limited to the N-terminal side of that bond only. Since many biologically active peptides do not tolerate N- or C-terminal modifications to their natural structures, and given the additional possibility that peptides may be produced in *E. coli* with an additional Met at their N termini (see above), this situation is ideal for recovery of a precise peptide structure if that peptide is positioned at the C terminus of a fusion construct.

The cleavage methods can be divided into three categories: chemical, enzymatic at a single specific residue, and enzymatic at sites defined by a specific sequence of residues. Three chemical methods have been employed. Cleavage on the C-terminal side of Met

residues using cyanogen bromide has often been used to release peptides that contain no additional Met residues from fusion protein products (Itakura et al. 1977; Goeddel et al. 1979). Occasionally, the target peptide sequence has been modified in order to meet this requirement, as in the case of [Thr^{59}] insulin-like growth factor I fused to an eight residue leader peptide (Peters et al. 1985). The cyanogen bromide reaction is usually carried out in 70% formic acid, and is therefore often useful for cleavages that must be performed under denaturing conditions such as those involving insoluble products in inclusion bodies, although additional denaturating agents such as urea or guanidinium thiocyanate are often added. A second chemical method, used to cleave Trp E-bovine growth hormone fusion proteins at Asp-Pro bonds, is acid pH treatment (Szoka et al. 1986). Again, the conditions require 70% formic acid, and often a denaturing agent is added, but elevated temperatures are also required. Although this method has the advantage of greater selectivity because it requires a two-residue site, it results in a cleaved peptide with an N-terminal Pro and may not always be efficient or specific for the Asp-Pro bond. Furthermore, there are reports that some Asp-Pro bonds are quite resistant to acidolysis, even under forcing conditions where extensive side reactions are occurring (see, for example, Allen et al. 1985). The third chemical method is similar to the second, consisting of cleavage at Asn-Gly bonds by hydroxylamine treatment at pH 9 and elevated temperature. This method was recently used to release human insulin-like growth factor from the C-terminal end of an IgG-binding domain of staphylococcal protein A, after the intact fusion protein had been purified on an IgG-Sepharose affinity column (Moks et al. 1987). Its general applicability remains to be determined.

Enzymatic cleavages at single-residue sites include the reported use of clostripain to cleave C-terminal to Arg in an a-chloramphenicol acetyltransferase-calcitonin fusion protein (see Marston 1986) and trypsin cleavage of β-endorphin from β-galactosidase, also C-terminal to Arg, after the fusion protein had been treated with citraconic anhydride to provide temporary protection of the Lys residues in β-endorphin from cleavage (Shine et al. 1980). In this category, the Staphylococcus aureus V8 protease is also potentially useful in that cleavage by this enzyme can be limited to sites C-terminal to Glu residues, even in the presence of Asp residues, under specific buffer conditions (Houmard and Drapeau 1972). This specificity has not yet been exploited, but might well be applicable to the production of a wide variety of active hormone analogues that have been engineered to replace any internal Glu residues with Asp. Such substitutions represent very minor changes in peptide structure, and they might often have little effect on pharmacological activities.

There are, at present, only two enzymes having recognition sites comprised of several amino-acid residues that have been exploited for the recovery of peptides from a fusion product. Collagenase cleaves collagen at multiple sites having a consensus structure -Pro-Xxx-Gly-Pro-Yyy-, with cleavage occurring on the C-terminal side of both of the unspecified residues. In one example of the early application of this cleavage reaction, Germino and Bastia (1984) designed a plasmid to express a fusion protein consisting of β-galactosidase at the N terminus, connected to the plasmid R6K initiator protein *via* a 60 residue segment of chicken proα-2 collagen containing several potential collagenase cleavage sites. They were then able to purify this protein by affinity chromatography based on the β-galactosidase segment and recover the R6K initiator, which is normally rapidly degraded in *E. coli,* after collagenase-catalyzed hydrolysis of the linker at multiple sites. The final product, however, was probably extended from the N terminus of the R6K initiator by about 10 residues of the linker, and may have been heterogeneous. More satisfactory results have been obtained by Nagai and Thøgersen (1984) to recover human β-globin from a hybrid with λcII protein, and by Steven Benner's group (Nambiar et al. 1987) to cleave β-galactosidase-linked ribonuclease A, using the action of blood coagulation factor X_a to cleave at the C-terminal end of its consensus linker -Ile-Glu-Gly-Arg-. In this case, the enzyme does not appear to require the three-dimensional structure

of its normal substrate for efficient cleavage, which probably contrasts with the requirements of collagenase, and the products obtained appeared to be homogeneous and devoid of unwanted N-terminal extensions, although in neither case was any sequence characterization performed. As one final note, it is worth pointing out that the potential utility of any enzymatic method is limited by the purity of the enzyme preparation, and contaminating proteolytic activities are often a problem.

The utility of fusion constructs for enhancing the stability of overexpressed peptides in vivo and for simplifying their purification has stimulated a number of researchers to design novel systems for these purposes that do not involve natural carrier proteins. In several cases, the use of multiple fused copies of the target peptide has been shown to enhance its stability and recovered yield. For example, three copies of the proinsulin gene were found to be optimal for resistance to proteolysis when they were fused directly to the tac promoter, and two copies were optimal when fusion to the lac promoter plus a small fragment of the β-galactosidase gene was tested (Shen 1984). The repeated proinsulin gene segments were connected by linkers coding for the amino acid sequence -Arg-Arg-Asn-Ser-Met-, with the expectation that the correct proinsulin peptide could be recovered by cyanogen bromide cleavage followed by proteolytic digestion with trypsin and carboxypeptidase B. A similar strategy has been employed for the production of Met-enkephalin (Hostomsky et al. 1985). This opioid peptide was released from a fusion product consisting of 11 copies of the peptide connected by -Arg-Arg- linkers and fused to part of the SV40 small-t antigen, after trypsin and carboxypeptidase B digestion, as indicated by RIA and guinea-pig ileal assays. A substance P analogue having a C-terminal homoserine amide was also successfully prepared from overexpressed fusion proteins containing 4, 16 and 64 copies of the corresponding C-terminal Met analogue (Kempe et al. 1985). These were fused together between part of the λ cro repressor on the N-terminal side and ß-galactosidase on the C-terminal side. In this case, the substance P analogue was obtained by cyanogen bromide cleavage to release multiple copies of the C-terminal homoserine lactone analogue, which were converted to the desired [HSe^{11}]substance P with a C-terminal amide, as in the natural hormone, by treatment with 30% ammonium hydroxide. This last approach is likely to prove useful for the preparation of many other peptide hormone analogues where a C-terminal amide is required for activity.

Another approach taken by Sung et al. (1986) to optimize the production of proinsulin was the addition of different homo-oligomeric peptides at its N terminus. As discussed above, this tactic is likely to affect the efficiency of expression as a result of modifications to the 5' end of the mRNA structure, in addition to affecting the lifetime of the peptide *in vivo* as a result of the effects of the N-terminal amino-acid residues on endogenous mechanisms for targetting protein degradation. In this investigation, hexamers of Ala, Asn, Cys, Gln, His, Ser, or Thr were found to produce the highest yields, and the yields for the$(Ala)_6$- and $(Ser)_6$-modified constructs were indeed found to be strongly dependent on the specific choice of codons used, indicating the importance of the structure of the ribosome-binding site on the mRNA. The use of poly-Arg fused to the C terminus of an overexpressed protein has also been explored as an aid to purification (Smith et al. 1984). This method was applied to the production in *E. coli* of human growth hormone (urogastrone) as a fused protein with 14 residues of the trp E protein at its N terminus and a C-terminal tail of 5 Arg residues. After expression, the fusion protein was purified from the cell lysate by cation-exchange chromatography, where it eluted after the bulk of the bacterial proteins on a salt gradient. The poly-Arg tail could be removed by carboxypeptidase B digestion.

In our laboratory, we are attempting to exploit the potential amphiphilic nature of many peptide hormones and other biologically active peptides, and the general propensity of these structures to form organized aggregates in aqueous solution (Taylor and Kaiser

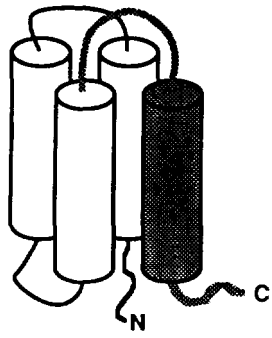

FIGURE 6

Schematic diagram of the structure of a helix bundle protein consisting of four amphiphilic a helices. Fusion proteins of this type, designed to incorporate amphiphilic peptide hormones at their C termini (shaded segment), might be resistant to proteolysis in E. coli.

1986) in order to generate fusion proteins that are specifically designed to include the peptide hormone as an integral part of their globular structure. For example, many of the intermediate-sized flexible peptide hormones can form amphiphilic α-helical segments (Taylor and Kaiser 1986). These peptides might be incorporated into globular fusion protein structures consisting of multiple near-parallel helices similar to the four-helix bundles commonly found in natural proteins (Weber and Salemme 1980) if they were connected to repeated segments of peptide sequences designed to form idealized amphiphilic helices (figure 6). Such tailor-made structures are expected to be soluble and more resistant to proteolytic degradation than the randomly connected fusion proteins usually are. Furthermore, they could be specifically engineered to allow convenient purification of the peptide hormone by, for example, ion-exchange chromatography. Eventually, these simple modifications of the fusion protein approach are likely to result in very efficient systems for the production of peptide hormones in bacterial cells in high yield. Combined with the rapid, multiple oligonucleotide synthesis methods of Blocker's group, and the high-efficiency mutagenesis methods that we have developed in the Eckstein laboratory, the exploration of structure-activity relationships in peptide hormones through the expression of multiple mutant genes in *E. coli* should often be competitive with the direct chemical synthesis approach, and may have advantages in terms of time, cost of reagents, and product purity.

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X-Ray Diffraction Studies of Enkephalins and Opiates

Jane F. Griffin, Ph.D., and G. David Smith, Ph.D.

The conformation or conformations of the enkephalins responsible for biological activity, that is, the conformation assumed at the receptor and resulting in the transduction of a message, has been of enormous interest since the enkephalins were first isolated by Hughes and Kosterlitz (1975). These pentapeptides with sequence Tyr-Gly-Gly-Phe-Leu(Met) have many degrees of conformational freedom due to peptide chain flexibility, centered in the four ϕ and five ψ torsional rotations, and side-chain flexibility from the χ rotations of the Tyr^1, Phe^4 and Leu^5 or Met^5 residues. How does the opioid receptor(s) recognize a flexible linear pentapeptide, the relatively inflexible fused ring morphine-like compounds and the other families of drugs with which it interacts? What are the chemical moieties recognized by the receptor(s) and how are they arranged in three dimensions? Do the different opioid receptor subtypes bind different low energy conformations of the endogenous compounds or induce them into an "active" conformation?

Since 1975 the conformations of enkephalin have been studied by every available spectroscopic technique in a variety of solvents and in the solid state, by more traditional biochemical structure/activity studies, by theoretical methods and by single crystal X-ray diffraction techniques. These studies have been reviewed in an excellent chapter by Schiller (1984). A more detailed review of the crystallographic literature appears in a previous monograph in this series (Camerman and Camerman 1986). This review will concentrate on the enkephalin structures determined by X-ray diffraction techniques since that paper was published, and on certain aspects of the structures which were not highlighted in the previous review. The results of X-ray diffraction studies on some morphine analogues with interesting opioid activity profiles will also be discussed.

INFORMATION OBTAINED FROM X-RAY DIFFRACTION STUDIES

The results of an X-ray diffraction study are a set of atomic coordinates, unit cell constants and space group. Calculations made from the above information provide not only geometric details on the compound under

study, precise bond distances, valency angles and torsion angles, but also intramolecular and intermolecular hydrogen bond geometry. The interactions that represent the forces holding molecular crystals together are exactly the same forces that are responsible for substrate-receptor binding: electrostatic interactions between charged groups, hydrogen bonds between functional groups, hydrophobic interactions and dipolar interactions. Thus the bioactive compound in the crystal lattice is a model of the substrate in a set of complementary surroundings. Determination of different crystalline forms, polymorphs and solvates, of the same material show different gross intermolecular interactions that can be related back to interactions in solution. Stable patterns of aggregation and even solvation present in solution persist in the crystalline form. Thus, multiple determinations of the same compound (either more than one molecule in the asymmetric unit or different crystalline forms) give information on conformational flexibility, stable aggregates, patterns of solvation, and intermolecular interactions.

Main-Chain Conformation

There have been three X-ray crystallographic studies of [Leu^{5}] enkephalin and two studies of the same crystalline form of (Met^{5}]enkephalin. The crystal data on these structures is given in table 1. The structures labelled LE_{E2}, ME_{E2} (Griffin et al., 1985, 1986a), ME_{E2} and $MEBr_{E2}$ the [(4-bromo-Phe^{4}] analogue of [Met^{5}]enkephalin (Doi et al., 1987b) have been determined since the previous review. Structures ME_{E2} and ME_{E2} are two independent studies on crystals that appear to vary only in the amount of water present (see table 1). Previously, the crystal structures of the [(4-bromo-Phe^{4}] analogue of [Leu^{5}]enkephalin and a few other analogues ues with individual amino acid replacements have also been determined. These compounds and the main-chain conformations observed in each of these studies are given in table 2. Two conformations predominate, a β turn centered on Gly^{2}Gly^{3} and a family of extended conformations.

Solution spectra have for the most part been interpreted as showing that there are both extended and folded conformations in solution; the folded conformation most frequently found by interpretation of spectra is a β turn centered on Gly^{3} Phe^{4}, a conformation not yet observed in the solid state. Khaled (1986) has pointed out some of the limitations in magnetic resonance studies of peptides: (1) assignments are complicated in a molecule containing a recurring amino acid residue such as in enkephalin with two adjacent glycine residues; (2) a controversy exists regarding the interpretation of the temperature dependence of NH protons. These assignments are used to distinguish inter- and intramolecular hydrogen bonds. The presence of the type I' β-turn conformation of enkephalin, centered on Gly^{2}Gly^{3} and characterized by a Phe^{4} NH to O=C Tyr^{1} hydrogen bond, observed in the solid state studies of [Leu^{5}]enkephalin and analogues (table 2) has been reported in only one spectroscopic study, enkephalin amides complexed with 18-crown-6-ether in chloroform (Beretta et al. 1984).

TABLE 1

Crystal data on enkephalins.

	[Leu^5]enkephalin			[Met^5]enkephalin		
	LE(B)[a]	LE(E4)[b]	LE(E2)[c]	LE(E2)[c]	ME(E2')[d,e]	MEBr(E2)[d]
Space Group	C2	$P2_1$	$P2_1$	$P2_1$	$P2_1$	P1
Z	16	8	4	4	4	2
No. independent mol./ asym. unit	4	4	2	2	2	2
solvent/ asym. unit	6 H_2O	8H_2O, 8C_3H_7NO, 0.5H_2O		10.6H_2O	10 H_2O	4.5 H_2O
Vol. (A^3)	13501	8459	2999	3448	3413	1730
No. observations	8869	10942	3180	5056	6037	5908
No. obs. > 2 sigma	5430	5965	3004	4725	4407	5270
a (A)	24.861	18.720	11.549	11.607	11.592	11.619
b (A)	17.084	24.732	15.587	17.987	17.871	11.609
c (A)	31.937	20.311	16.673	16.519	16.480	12.943
alpha (°)						93.92
beta (°)	95.54	115.9	92.19	91.24	91.23	96.10
gama (°)						86.99

[a] Smith and Griffin 1978; Blundell et al. 1979.
[b] Karle et al. 1983; Camerman et al. 1983.
[c] Griffin et al. 1985, 1986a. Subsequent to this report of structures LE(E2) and ME(E2), Hastropaolo et al. (1986, 1987) reported the independent structure determination of the same structures using the same X-ray diffraction data of Blundell. The report that the [Met^5]enkephalin crystals were grown from ethanol/water (Hastropaolo et al. 1986) is incorrect. The experimental details of crystallization and data collection from Blundell's laboratory are given in Griffin et al. 1986a.
[d] Doi et al. 1987b.
[e] ME(E2) and ME(E2') are two independent studies on crystals that appear to vary only in the amount of water present. Note the almost identical cell parameters, differing by 34 A^3 in unit cell volume.

TABLE 2

Conformations of enkephalin and enkephalin analogues from X-ray crystallographic determinations.

Peptide	Conformation	Intramolec. H-bonds
Tyr-Gly-Gly-Phe-Leu- $2H_2O$ (Smith and Griffin, 1978)	type I' ß-bend (Gly-Gly)	N_{Phe} to O_{Tyr} N_{Tyr} to O_{Phe}
Tyr-Gly-Gly-Phe-Leu-$2H_2O$, $2C_3H_7NO$,X (Karle et al. 1983)	Extended antiparallel ß-sheets	None
Tyr-Gly-Gly-Phe-Leu•0.5H2O (Griffin et al. 1986a)	Extended antiparallel ß-sheets	None
Tyr-Gl-Gly-Phe-Met•5.3H2O (Griffin et al. 1986a)	Extended antiparallel ß-sheets	None
Tyr-Gly-Gly-Phe-Met•5.0H2O (Doi et al. 1987b)	Extended antiparallel ß-sheets	None
Tyr-Gly-Gly-(4Br)Phe-Leu•$2.5H_2O$ (Ishida et al. 1984)	type I' ß-bend (Gly-Gly)	N_{Phe} to O_{Tyr} N_{Tyr} to O_{Phe}
Tyr-Gly-Gly-(4Br)Phe-Met•$2.3H_2O$ (Doi et al. 1987b)	Extended	None
Tyr-D-Nle-Gly-Phe-NleS•2.5H2O, C_2H_5OH (Stezowski et al. 1985)	type II' ß-bend (D-Nle-Gly)	N_{Phe} to O_{Tyr} N_{Tyr} to O_{Phe}
Tyr-Gly-Gly-Phe•DMSO,2H2O (Foumie-Zaluskie et al. 1977)	type I' ß-bend (Gly-Gly)	N_{Phe} to O_{Tyr}
Gly-Gly-Phe-Leu•H2O (Foumie-Zaluskie et al. 1977)	Extended	None
BOC-Tyr-Gly-Gly-(4Br)Phe-MetOH (Doi et al. 1984)	Extended antiparallel **β**-sheets	None
BOC-Aib-Aib-Phe-MetNH_2•DMSO (Prasad et al. 1983)	3_{10} Helix	N_{Phe} to O_{BOC} N_{Met} to $_{Aib}$
BOC-Gly-Gly-Phe Ethyl Ester (Ishida et al. 1983)	type I' **β**-bend (Gly-Gly)	None

Extended Main-Chain Conformations

The six independent observations of [Leu^5]enkephalin, 4 molecules in LE_{E4} and 2 molecules in LE_{E2}, in the extended conformation are superimposed in figure la. The two independent molecules of [Met^5]enkephalin (ME_{E2}) are superimposed in figure lb. The peptide chains observed in LE_{E2}, but particularly in the three (Met^5) structures are unusually planar. This is apparent by examination of the Φ,Ψ torsion angles for the [Me^5]enkephalin structures and [Leu^5]enkephalin (LE_{E2}) versus the extended conformations seen in LE_{E4} (see table 3). The main-chain torsion angles of the dimer of [4-bromo-Phe^4,Met^5]enkephalin ($MEBr_{E2}$) are remarkably similar to those in ME_{E2} and ME_{E2}, even though the space group and the amount of solvent water in the crystal are different. The Φ/Ψ torsion angles for Gly^2 and Gly^3 in the three [Met^5] structures indicate an unusually extended conformation, all the values lie between ± 160° and ± 180°, The Φ/Ψ values for the Phe^4 residue in the [Met^5]enkephalin structures (six observations) are almost invariant, -154° ± 4°/155° ± 4°, and lie in the ideal β-sheet region; the values in the [Leu^5]enkephalin structures show greater variability, especially in LE_{E4} The Met residues are in two slightly different conformations, -138°/157° and -155°/170°. These results could be interpreted to indicate less flexibility in the main chain of [Met5]- versus [Leu^5]enkephalin, but the small size of the data base imposes caution in drawing this conclusion.

Side-Chain Conformation

In addition to the ϕ,ψ torsion angle values, which define main-chain conformation, table 3 contains the χ torsion angles, which describe the Tyr, Phe, Leu, and Met side-chain conformations in the β-sheet enkephalin structures. The χ^1 values for the Tyr and Phe side chains in the (Met^5]enkephalin structures are almost invariant. both close to +60° (six observations of each). This is in contrast to the values observed in the [Leu^5]enkephalin structures: Tyr ≃ +60° (three observations), 180° (three); Phe ≃ -60° (five), 180° (one).

If the side-chain conformation observed in the extended structures is compared with that observed in the type I' β-turn [Leu^5] structures, LE_β, and [(4-bromo)Phe^4 ,Leu^5]enkephalin, the major difference is in χ^1 of Tyr: ≃ -60° in the β-turn forms and ≃ +60° and 180° in the extended forms; χ^1 of Phe is ≃ -60° in the β turn structures, similar to the major conformer in the β-sheet [Leu^5]enkephalin structures.

In summary, in this admittedly small sample, the side-chain conformations in [Leu^5]- versus (Met^5]enkephalin are distinguished by mutually exclusive Phe χ^1 values. The [Leu^5] structures have χ^1 of Phe ≃ -60°, 180°, while the [Met^5] structures have χ^1 of Phe values≃ +60°. The extended versus β-turn structures have mutually exclusive values for Tyr χ^1, -60° (β turn), and + 60°, 180° (extended),

INTERMOLECULAR INTERACTIONS

The [Met^5]enkephalin structures consist of dimers forming infinite anti-

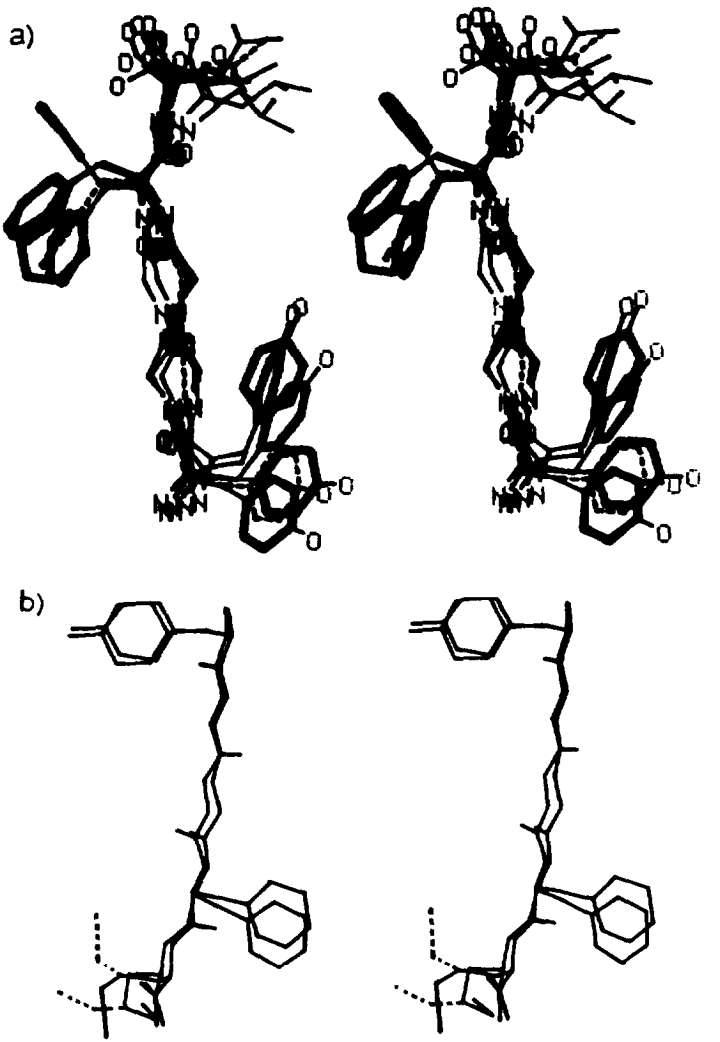


FIGURE 1

(a) A stereo view of a least-squares superposition of the six crystallographically observed extended conformations of [Leu^5]enkephalin. The 21 main chain atoms were fit. (b) A stereo view of a least-squares superposition of the two crystallographically observed extended conformations of [Met^5]enkephahn in ME_{E2}. Each methionine residue is observed in two distinct conformations; one is dashed.

parallel β-sheets with varying amounts of water molecules per dimer. The two crystallographically independent molecules of the dimer are remarkably similar with respect to both main-chain and tyrosine and phenylalanine side-chain conformations (table 3). In both $ME_{E2,}$ and ME_{E2} each methionine side chain is disordered, and the disordered conformations are different in the two independent molecules (see table 3). The disorder appears to be the result of close contacts between methionine side chains in adjacent independent molecules. The amount of solvent in the unit cell appears to affect the methionine side-chain conformation. In the dimer the nitrogen and carbonyl of GIy^2 and Phe^4 of one molecule are hydrogen bonded to the carbonyl and nitrogen of Phe^4 and Gly^2, respectively, of the other molecule (figure 2). Additional hydrogen bonds connect adjacent dimers and form head-to-tail (N_{Tyr} to O_{Met}) connections, which loin crystallographically equivalent molecules related by translation.

The [Leu^5]enkephaIin structure LE_{E2} is made up of planar β-sheets arranged similarly to those in the [Met^5] structures. However, the side chains are oriented differently in the two independent molecules (see table 3). There is only one molecule of water per dimer of [Leu^5]enkephalin versus 10.6 and 10.0 in ME_{E2} and ME_{E2} , respectively. The packing is more compact than is observed in the other two [Leu^5] forms. The one water molecule is tetrahedrally coordinated; it donates two hydrogen bonds to the two independent leucyl carboxyl groups and accepts two hydrogen bonds from the hydroxyl groups of two independent tyrosine side chains.

In the [Met^5]enkephalin structures ME_{E2} and ME_{E2}, the distance between β-sheets is approximately 9.0 Å (one-half the b axis), while the value observed in the [Leu^5]enkephahlin structure is 7.8 Å. As a result of this increase in interplanar spacing, the unit cell of [Met^5]enkephalin accommodates additional water molecules.

Comparison of the β-Sheet Forms

The asymmetric unit, that is, repeating unit, is a dimer in structures $LE_{E2,}$ ME_{E2}, ME_{E2} and $MEBr_{E2}$ and a tetramer in LE_{E4} The dimers are similar in all four structures; two independent molecules form an antiparallel β-sheet with four hydrogen bonds $N(Gly^2)_A$ to $O(Phe^4)_B$ $N(Phe')_B$ to $O(Gly^2)_A$, $N(Gly^2)_B$ to $O(Phe^4)_A$, $N(Phe^4)_A$ to $O(Gly^2)_B$, where A and B refer to the two independant molecules. The dimers are joined in head-to-tail fashion, with identical dimers related by translation forming infinite ribbons. These ribbons are joined to adjacent ribbons by antiparallel hydrogen bonds translated by approximately 1/2 the length of the peptide. Therefore, dimers adjacent in the plane of the β-sheet hydrogen bond across the head-to-tail connections (see figure 2). The β-sheets thus formed are not pleated in the normal direction, that is, along the peptide chain. Instead the chains are very flat and extended, and the pleat is perpendicular to the direction of the peptide chain.

The asymmetric unit in LE_{E4} is a tetramer; two of the four molecules, A and B, form a dimer similar to the one described above for the dimeric structures with respect to hydrogen bonding. The other two molecules of the tetramer. C and D, are rotated 180º in the plane of the β-pleated sheet and form

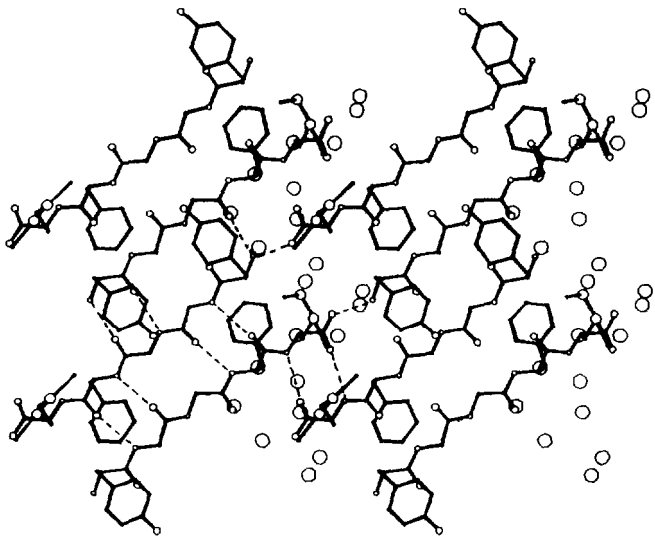


FIGURE 2

Packing pattern of [Met^5]enkephalin ME_{E2_5} showing the β-sheet structure. This pattern is common to the three [Met^5] structures. Water is shown as large circles. The water structure is not common to the three structures.

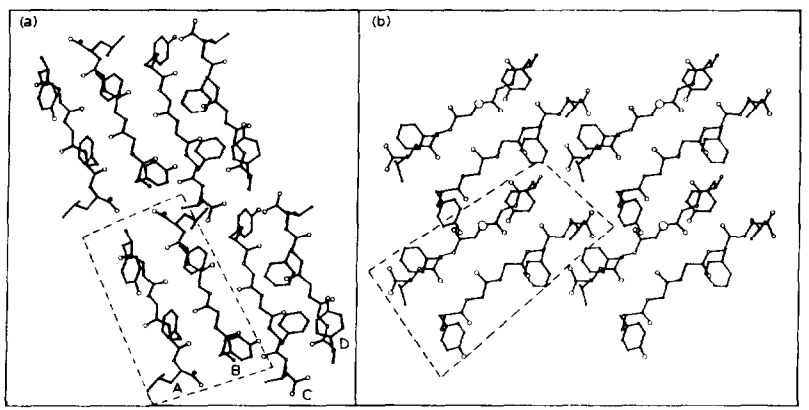


FIGURE 3

Comparison of the β-sheet formed in [Leu^5]enkephalin, LE_{E4}, left, and LE_{E2}, right. The dimer common to both structures is outlined.

hydrogen bonds with one another and with B and the translationally related A molecules through the peptide C=O and NH groups. The tetramers form a continuous β-pleated sheet along **a.** These sheets are connected to translationally related sheets along **c**, forming an infinite β sheet in the **ac** plane. The sheets are separated from one another by solvent, much of it disordered. The distance between the sheets is 12.4 Å (1/2 **b**) and there is no interaction between the side-chain residues of adjacent sheets.

The side-chain residues pack quite differently in the space between adjacent β sheets in the two [Leu^5] structures, LE_{E4} and LE_{E2} (see figure 3). In LE_{E4} there is a layer of solvent molecules separating the side-chains of the β-sheets adjacent along **b.** The structure is a sandwich layered along b, the bottom slice is a β-sheet. followed by a side-chain layer forming hydrogen bonds (Tyr) and hydrophobic interactions (Leu and Phe) to the next layer, solvent, which in turn forms hydrogen bonds and hydrophobic interactions to the side-chain layer from the top β-sheet, the final slice. Because in LE_{E4}, the dimer formed by molecules C and D is rotated 180° in the plane of the sheet with respect to the AB dimer. all three side-chain residues from the plane at $y \simeq 0.25$ and three from the plane at $y \simeq 0.75$ pack in the space between sheets separated by **b**/2. In LE_{E2}, on the other hand, the residues between the planes consist of tyrosyl and leucyl side-chains from one sheet and phenylalanyl residues from the sheet separated by **b**/2. These form relatively tight hydrophobic interactions connecting the B-sheets along **b.**

Packing of the β-Turn Form

The asymmetric unit in the β-turn form LE is a tetramer (see figure 4). The two central molecules form a hydrogen-bonded dimer, the N-terminus of each is hydrogen-bonded to the tyrosyl hydroxyl of the other. Molecules one and three are connected by three water molecules forming bridging hydrogen bonds between them. Molecules two and four are almost identical to one and three. These tetrameric units are repeated in three dimensions by the symmetry operations of the space group. The interactions between separate tetramers are mainly hydrophobic; the two-fold axis is surrounded by Phe and Leu side-chains (see figure 4b).

In the β-turn structure there are two intramolecular hydrogen bonds that connect the Tyr and Phe groups of each of the four independent molecules, N(Tyr) to O(Phe) 2.79-2.84 Å and N(Phe) to O(Tyr), 2.99-3.10 Å. The six water molecules take part in a variety of interactions. Two water molecules form a bridge between the Phe^4 carbonyl oxygen and the Leu^5 carboxy oxygen of one molecule. In each of the four independent molecules, both the Gly^2 nitrogen and the Gly^3 carbonyl oxygen are hydrogen bonded to water molecules.

There are patterns of solvation common to more than one of the enkephalin crystal structures; that is, water molecules bind to the same atoms even though the packing in the structures may vary. The waters common to more than one structure are given in table 4.

TABLE 3

Main-chain and side-chain torsion angles of extended forms

	[Leu^5]enkephalin						[Met^5]enkephalin					
	LE(E4)[a]				LE(E2)		ME(E2)		ME(E2′)		MEBr(E2)	
	A	B	C	D	A	B	A	B	A	B	A	B
[Tyr^1]	135	154	155	137	140	162	164	165	165	165	159	166
[Gly^2]	-144	151	141	-131	-147	172	-179	-171	-166	178	-170	172
	114	-155	-157	142	156	-176	-174	167	164	-174	166	-167
[Gly^3]	-122	154	174	-144	-154	-167	171	-164	-163	170	-160	-165
	132	-151	-170	131	162	165	-175	163	161	-173	165	-170
[Phe^4]	-122	-128	-119	-147	-160	-145	-157	-152	-150	-157	-150	-158
	139	130	149	152	134	152	159	153	153	157	150	157
[Leu^5]	-79	-72	-141	-141	-141	-143 [Met^5]	-138	-155	-154	-138	-158	-137
	176	167	137	151	143	150	154	172	172	157	169	160
[Tyr^1]	177	70	53	169	175	62	71	63	63	69	58	68
	-86,93	-86,99	-85,101	71,-102	83,-92	-84,95	-89,87	-88,89	-89	-89	-90	-86
[Phe^4]	-63	-55	-71	-68	-169	-69	69	62	63	68	53	65
	87,-95	87,-88	-82,98	-91,93	66,-112	-75,104	-83,97	-87,97	96[b]	-83	96[b]	-81
[Leu^5]	-64	-62	-171	-80	170	67 [Met^5]	-175	69	65	129	64	-55
	173	165	-168	179	-173	154	168	168	160	-158	-152	-160
	-67	-69	68	63	64	-93	58[c]	-152[c]	-155[c]	130[c]		
							53	-67	-58	125	82	142
							89[c]	90[c]	68[c]	-47[c]		

[a] Refer to table 1 for crystallographic form and references. A, B, C, D refers to independent molecules in the crystallographic asymmetric unit, tetramer in LE(E4), dimer in LE(E2) and ME(E2) forms.
[b] Values reported in Doi et al. (1987b). Convention requires the smaller number to be reported, which must be $\tilde{=}$ 85, but lacking published coordinates it is impossible to calculate these values.
[c] Disordered methionine side chains; C(γ), S(δ) and C(ε) are disordered in ME(E2), A and B, and ME(E2′), A and B.

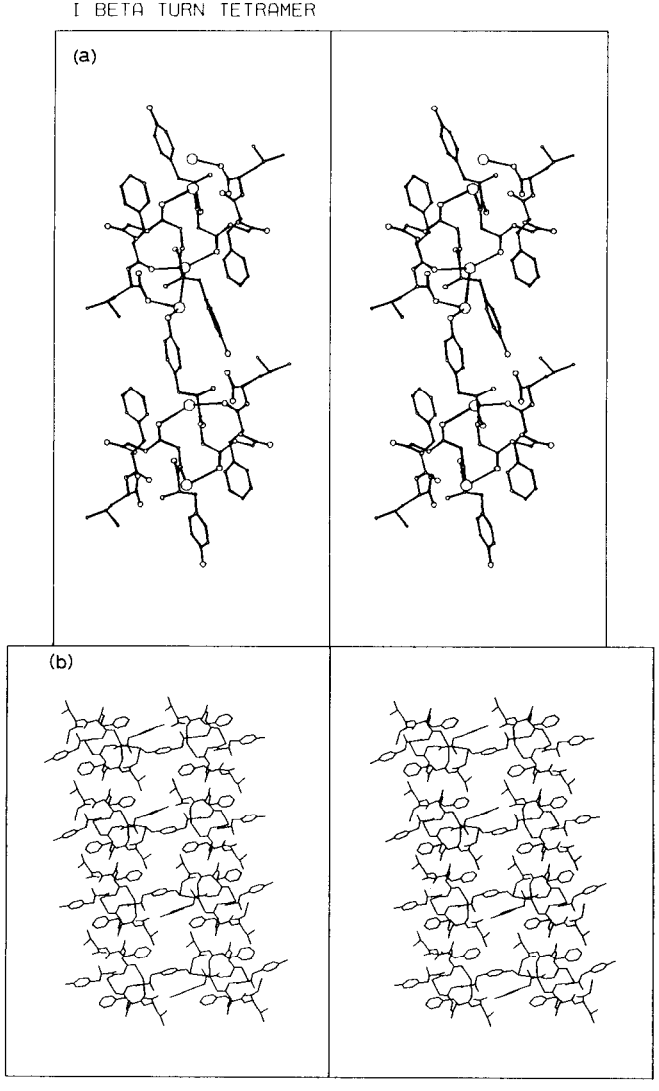


FIGURE 4

(a) Stereo view of the tetramer that makes up the asymmetric unit in LE$_\beta$. Large circles are water molecules. (b) Stereo view of the packing of a unit cell, 16 molecules, in LE$_\beta$. Note the hydrophobic holes formed by tyrosyl, phenylalanyl, and leucyl residues.

TABLE 4

Common patterns of solvation in enkephalin crystal structures: atoms of enkephalin molecules hydrogen-bonded to water.

LE_{β}	LE_{E4}	LE_{E2}	ME_{E2}*
$CO^-(leu^5)$	$CO^-(leu^5)$	$CO^-(leu^5)$	$CO^-(met^5)$
$OH(tyr^1)$	$OH(tyr^1)$	$OH(tyr^1)$	$OH(tyr^1)$
	$N^+(tyr^1)$		$N^+(tyr^1)$
$C=O(gly^2)$			$C=O(gly^2)$
$C=O(phe^4)$	$C=O(phe^4)$		$C=O(phe^4)$

*The authors do not have the crystallographic coordinates for ME_{E2}, and $MEBr_{E2}$, so the solvent structure cannot be calculated.

Conformation of Enkephalin at the μ and δ Receptor

Since the demonstration of the existence of multiple forms of opioid receptors, arguments have been made that the μ and δ subtypes bind different conformations of the endogenous opioids. Soos et al. (1980) suggested this on the basis of CD spectra on the enkephalins and enkephalin analogues. Raman spectra was interpreted as showing more folded conformations of [Leu^5]enkephalin than [Met^5]enkephalin in aqueous solution and DMSO (Renugopalakrishnan et al. 1985). The observed solid state conformations led to the proposal that [Leu^5]enkephalin binds to the μ receptor in a folded conformation and [Met^5]enkephalin to the s in an extended conformation (lshida et al. 1984). Recently Doi et al. (1987b) suggested that a pair of β-bend molecules of enkephalin can mimic the β-sheet dimer, placing similar functional groups in the same regions of three-dimensional space, and bind to the δ receptor. In a subsequent paper the same group (Doi et al. 1987a) suggested that the β-turn monomer and the extended dimer could each bind to both μ and δ subtypes of the opioid receptor. They based this conclusion on computer graphics and empirical energy studies of the two conformations, adjusting the tyrosyl and phenylalanyl side chains on both conformations to superimpose the analogous residues in the same regions of three-dimensional space. This hypothesis requires further testing by biochemical techniques.

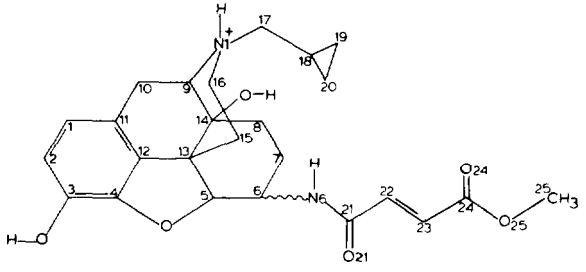
The β-turn structure of [Leu^5]enkephalin was initially proposed to be the biologically active conformation (Smith and Griffin 1978). The conclusion was based on a least-squares superposition of the β-turn conformation of enkephalin with the structures of morphine, etorphine and PET, 7-(1-phenyl-3-hydroxybutyl-3]-endoethenotetrahydrothebaine, the latter two superactive agonists. In addition, the fact that the ϕ,ψ torsion angles of Gly^2 place the conformation in a region of conformational space allowed for D-amino acids, and D-Ala^2 substituted enkephalins had been shown to be very active, supported the proposal. These arguments were made before the existence of opioid receptor subtypes was demonstrated and the selectivity of the

individual subtypes shown experimentally. The activity of etorphine and the tetrahydrothebaines at both u and s opioid receptor subtypes, however, has not been accounted for in the suggestion that u and s subtypes bind different conformations of the enkephalins.

The fully extended conformation of a small peptide can form the maximum number of intermolecular hydrogen bonds. In going from the extended form to the type I' β-turn conformation centered on Gly^2Gly^3, the enkephalin molecule decreases the number of hydro en bonding groups available for receptor interactions and increases the surface hydrophobicity. This may be relevant to the conformation assumed at the receptor(s), since the morphine- and tetrahydrothebaine-like molecules are more hydrophobic than enkephalin.

OPIATE DRUGS

Naltrexone Derivatives



FUNALTREXAMINE

α- and β-Funaltrexamine (α- and β-FNA) are naltrexone derivatives differing only in chirality at C6. Both compounds bind to the μ opioid receptor in mouse vas deferens (MVD) and guinea pig ileum (GPI) preparations, but only the β-epimer binds irreversibly, presumably by forming a covalent bond to the receptor. For this reason, β-FNA has been used to irreversibly block u receptors in order to isolate the binding characteristics of δ receptors in GPI and MVD preparations, and δ and κ Sites in brain homogenate preparations. A two-step recognition process had been proposed to account for the different binding characteristics of α- and β-FNA for the μ receptor; both bind in the first recognition step but only the β-epimer is in the proper orientation for the second recognition step which results in alkylation. The crystal structures of α-- and β-l-FNA suggested a possible explanation for the observed differences (Griffin et al. 1986b). The two compounds have almost identical conformations in the fused rings with the exception of the C ring; the β-epimer has a chair conformation and the α-epimer a twist-boat conformation, resulting in the fumaramate side chain being equatorial to the C ring in both cases. Analysis of the superposition of the two structures

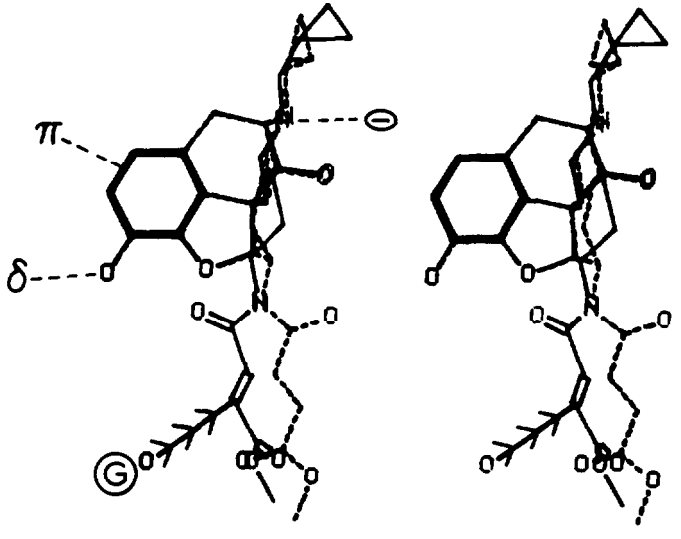


FIGURE 5

Stereo view of the superposition of the crystallographically observed structures of α- and β-funaltrexamine, with the proposed sites of receptor interaction. The first recognition step involves the phenol ring. the phenol hydroxyl, and the charged nitrogen. The second recognition step involves the fumaramate group and possibly the amino nitrogen at C6 (from Griffin et al., 1986b, Copyright 1986, American Chemical Society).

revealed that the fumaramate groups occupied the same region with respect to the naltrexone frame in both structures, but the conjugated fumaramate roups were oriented orthogonal to one another. A close oxygen contact (03 from a symmetry related molecule) to the double bond carbon (C23) observed in the crystal structure was used to model this second recognition site and explain the failure of the α-epimer to irreversibly bind to the receptor (see figure 5).

The conformation of the C ring of naltrexone derivatives appears to be sensitive to substitution at C6. The crystal structure of the 6 -amino derivative of naltrexone, 6α-oxymorphamine (Lever et al. 1985) confirmed their conclusions based on ^{1}H NMR that the C ring in the 6α-amino epimer is in a twist-boat conformation and in the 6β-epimer is a chair. As in the FNA structures this places the 6 substituent in the equatorial position in both epimers. Comparison of the C ring torsion angles in α-FNA and 6α-oxymorphamine shows they have almost identical conformations.

Acylmorphinans

Although the phenolic hydroxyl group is considered essential to activity in morphine and structurally related analogues, its presence results in rapid inactivation when given by oral route. Mohacsi et al. (1985) have reported the crystal structure of a C3-acyl morphinan that retains morphine-like analgesic activity even when administered orally. The 03 atom could be serving as a hydrogen bond acceptor, since the phenolic oxygen in morphine can act as both donor and acceptor of hydrogen bonds. The oxygen to nitrogen distance in the acylmorphinan structure is 7.94 Å, whereas in the 30 crystal structures containing the morphine five fused-ring system in the Cambridge Structural Database (Allen et al. 1979). the value of the distance ranges from 6.77 to 7.16 Å, average 7.03(11) Å

Conformations of the Morphine Fused-Ring System

Brown et al. (1983) reported that the conformation of morphine in the crystal is different from that in aqueous solution, and that the latter is the one "available for receptor binding," although no evidence or explanation for this assertion was included in the report. Their study was based on a comparison of the high resolution ^{13}C NMR spectra of solid morphine sulfate versus solid morphine free base, solid morphine sulfate versus morphine sulfate dissolved in D_2O, and morphine sulfate dissolved in D_2O at 22°C and 70°C. Their data were interpreted as showing a minor component of the nitrogen invertoisomer in solution, the nitrogen methyl went from equatorial to axial, and the inversion was associated with a "greater freedom of motion in the piperidine ring than previously believed." They speculated that the downfield chemical shifts of the spectral lines assigned to C2, C7, C11, C15, and C16 on going from solution to solid were due to interactions in the solid state between these atoms and the sulfate group. The authors did not identify the crystalline polymorph they were examining. Solid state spectral studies should always verify the identity of the crystals studied by means of

powder or single crystal X-ray diffraction to determine cell constants and space group. Without this information, no meaningful comparison can be made between the spectral studies and crystallographic results. The change in chemical shifts are difficult to explain from the crystal structure of morphine, 0.5 SO_4, 2.5 H_2O (Wongweichintana et al. 1984); although C2, Cl5, and Cl6 show close contacts to sulfate oxygens in the crystal (3.39 to 3.49**Å),** C7 and C11 do not, while C3 and the N-methyl carbon also show short contacts.

The Cambridge Structural Database (Allen et al. 1979) contains 30 crystal structure determinations of compounds that contain the morphine five fused-ring framework. In this data base the methyl substituent on the piperidyl nitrogen is always observed equatorial and there is little evidence for great freedom of motion in the piperidine ring especially at C15 and C16. The piperidyl ring is always a chair, and the torsion angle C13-C15-C16-N only varies from -47° to -57° in the entire sample, average 51.2 (2.0)°. The main region of flexibility is ring C, and the changes in ring C conformation can be correlated with changes in chemical constitution, either a double bond at C6. C7 or C7, C8. or the substitution of a nitrogen on C6, as discussed above. There are also changes in the three carbon-nitrogen bonds of the piperidyl nitrogen correlated with whether the structure is the free base or salt.

SUMMARY

Information on intramolecular geometry, low energy conformations, hydrogen bonds, both intramolecular and intermolecular, and preferred intermolecular interactions is obtained from single crystal X-ray diffraction experiments. Structure determinations of crystals with more than one molecule in the asymmetric unit and of different crystalline forms provide information on conformational flexibility and stable aggregation states.

The single crystal X-ray diffraction studies of enkephalin have demonstrated a number of minimum energy conformations of the main chain and side chains, a type I' β turn centered on Gly^2Gly^3 and a number of extended main-chain conformations. In the studies of native enkephalins, dimers and tetramers form repeating units, and some patterns of solvation are observed in more than one form. Certain conformations and interactions proposed on the basis of solution spectroscopic studies have yet to be observed in the solid state. Efforts should be made to grow different crystalline forms of the enkephalins and to determine the solid state structure of enkephalin analogues that exhibit high selectivity for a particular opioid receptor subtype.

Crystallographic studies of opiate drugs with selective activity profiles at different receptor subtypes can give infomration on the features responsible for active site differenttation between the subtypes of opioid receptor.

Analysis of the crystallographic data base of 30 compounds containing the five fused-ring morphine moiety indicate that: (1) the methyl group on the

piperidyl nitrogen prefers the equatorial position, (2) the conformation of the piperidyl ring is a relatively invariant chair conformation, (3) C-ring conformation depends on the chemical constitution of the C-ring and substitution at C6.

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Conformational Analysis of Cyclic Opioid Peptide Analogs

Peter W. Schiller, Ph.D., and Brian C. Wilkes, Ph.D.

INTRODUCTION

Extensive conformational studies of the enkephalins [H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH] by various spectroscopic techniques and by X-ray diffraction analysis revealed that these linear pentapeptides are highly flexible molecules capable of assuming a number of both folded and extended conformations of comparably low energy (Schiller 1984). In fact, it has been demonstrated quite convincingly that in solution enkephalin exists in a conformational equilibrium (Fischman et al. 1978). It has, therefore, become clear that conformational studies of these small opioid peptides and of many of their analogs are unlikely to provide any insight into their bioactive (receptor-bound) conformation(s). Furthermore, the conformational flexibility of the enkephalins is most likely the reason for their lack of specificity toward the different opioid receptor classes (μ, δ, κ) because conformational adaptation to the various receptor topographies is possible. Thus, even though the enkephalins preferentially bind to the δ-receptor, they also have quite good affinity for the μ-receptor and, therefore, are only moderately δ-receptor selective.

CONFORMATIONALLY RESTRICTED ANALOGS OF OPIOID PEPTIDES

The conformational flexibility of opioid peptides can be reduced through incorporation of conformational constraints. In recent years conformationally restricted analogs of enkephalin have been synthesized with two goals in mind. First, such analogs are more amenable to a meaningful conformational analysis since because of their relatively rigid structure they are unlikely to undergo major conformational changes upon binding to the receptor and, therefore, Information about the bioactive conformation at the various opioid receptors can be obtained. Second, the introduction of conformational constraints may result in improved receptor selectivity, because the conformationally restricted analog may have good affinity for one receptor type but may no longer be able to undergo a conformational change necessary to

bind to another receptor class. Local conformational restrictions in enkephalins have been achieved either at particular peptide backbone positions (e.g., through N^{α}- or C^{α}-methylation or through incorporation of small ring structures) or in selected side chains (e.g., through substitution of a dehydroamino acid) (for a review, see Schiller 1984). However, the most drastic restriction of the overall conformational space available to the peptide has been realized through the design and synthesis of cyclic enkephalin analogs.

Three families of biologically active cyclic opioid peptide analogs have been synthesized to date. A cyclic enkephalin analog was first prepared through substitution of D-α, γ-diaminobutyric acid (A_2bu) in position 2 of the peptide sequence followed by cyclization between the side chain amino group of A_2bu and the C-terminal carboxyl group (DiMaio and Schiller 1980). The resulting analog, H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (figure 1, compound 2), containing a fairly rigid 14-membered ring structure, turned out to be moderately μ-receptor selective (Schiller and DiMaio 1982). Pharmacologic comparison of 2 with a corresponding open-chain analog revealed that its μ-receptor preference is a direct consequence of the conformational restriction introduced through ring closure and, furthermore, permitted the fundamental conclusion that μ- and δ-opioid receptors differ indeed from one another in their conformational requirements toward peptide ligands (Schiller and DiMaio 1982). Homologs of 2 (compounds 1, 3, and 4) were obtained through variation of the side chain length in position 2 and also were found to display moderate preference for μ-receptors over s-receptors (DiMaio et al. 1982). On the other hand, a diastereomer of cyclic peptide 2, H-Tyr-cyclo[-D-A_2bu-Gly-Phe-D-Leu-], was non selective (Mierke et al. 1987). Several partial retro-inverso analogs of 2 were found to be very potent and μ-selective (figure 1, structures 2b and 2c), whereas both diasteromers of 2d showed very weak activity (Berman et al. 1983; Richman et al. 1985), presumably because the Gly^3 -Phe^4 peptide bond is important for binding to the receptor.

A second class of cyclic enkephalin analogs was obtained through side chain-to-side chain cyclization between a D-Cys and a D- or L-cys residue substituted in positions 2 and 5 of the peptide sequence. respectively (Schiller et al. 1981). The resulting cystine-containing analogs H-Tyr-D-Cys-Gly-Phe-L(or D)-Cys-X (5) were highly potent and showed either no receptor selectivity (X = NH_2) or moderate δ-receptor selectivity (X = OH) (Schiller et al. 1985a). The Trp^4 analog H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2 (6) displayed an activity profile similar to that of the corresponding Phe^4 parent compound (Schiller 1983). Replacement of the half-cystine residues in positions 2 and/or 5 with penicillamine residues resulted in compounds (7-7c) with markedly improved δ-receptor selectivity (Mosberg et al 1983).

The configurational requirements of cyclic analogs 4 and 5 at the residues in positions 1, 2, 4, and 5 of the peptide-sequence were

H-Tyr-D-A_2pr-Gly-Phe-Leu (1)

H-Tyr-D-A_2bu-Gly-Phe-Leu (2)

H-Tyr-D-A_2bu-Gly-Phe-D-Leu (2a)

H-Tyr-D-Glu-Gly-Phe-gLeu (2b)

H-Tyr-D-A_2bu-Gly-gPhe-L(or D)-mLeu (2c)

H-Tyr-D-A_2bu-gGly-L(or D)-mPhe-Leu (2d)

H-Tyr-D-Orn-Gly-Phe-Leu (3)

H-Tyr-D-Lys-Gly-Phe-Leu (4)

H-Tyr-D-Cys-Gly-Phe-L(or D)-Cys-X (5)

H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2 (6)

H-Tyr-D-Pen-Gly-Phe-L(or D)-Cys-NH_2 (7)

H-Tyr-D-Pen-Gly-Phe-L(or D)-Cys-OH (7a)

H-Tyr-D-Cys-Gly-Phe-L(or D)-Pen-OH (7b)

H-Tyr-D-Pen-Gly-Phe-L(or D)-Pen-OH (7c)

H-Tyr-D-Orn-Phe-Asp-NH_2 (8)

H-Tyr-D-Orn-Phe-D-Asp-NH_2 (9)

H-Tyr-D-Asp-Phe-Orn-NH_2 (10)

H-Tyr-D-Orn-Phg-Asp-NH_2 (11)

H-Tyr-L-Orn-Phe-Asp-NH_2 (12)

H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2 (13)

FIGURE 1

Structural Formulas of Cyclic Opioid Peptide Analogs

found to be the same as those of the corresponding residues in linear enkephalins (Schiller and DiMaio 1983). This finding as well as other structure-activity data obtained with these compounds indicated that corresponding moieties in these cyclic and linear enkephalin analogs interact with the same subsites on the receptor. It can thus be concluded that both H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-] and H-Tyr-D-Cys-Gly-Phe-Cys-NH_2 have the same mode of binding to the receptor as the linear enkephalins.

Another family of side chain-to-side chain cyclized opioid peptide analogs was obtained through amide bond formation between the side chain amino and carboxyl groups of appropriately substituted Orn (or Lys) and Asp (or Glu) residues (Schiller and Nguyen 1984; Schiller et al. 1985b, 1987). Among the various cyclic lactam analogs of this type prepared, H-Tyr-D-Orn-Phe-Asp-NH_2 (8) turned out to be potent and highly μ-selective. The latter analog contains a rather rigid 13-membered ring structure and a Phe residue in the 3-position as it is the case with the dermorphins and the β-casomorphins. Among several prepared analogs of 8, H-Tyr-D-Orn-Phe-D-Asp-NH_2 (9) and H-Tyr-D-Asp-Phe-Orn-NH_2 (10) showed the same high μ-selectivity as the parent compound, whereas H-Tyr-D-Orn-Phg-Asp-NH_2 (11) was less μ-selective due to reduced affinity for the μ-receptor. Analogs H-Tyr-L-Orn-Phe-Asp-NH_2 (11) and H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2 (13) showed very weak activity. Taken together, the structure-activity data obtained with analogs of H-Tyr-D-Orn-Phe-Asp-NH_2 indicated that the mode of binding of the latter peptide identical with that of the dermorphins but different from that of the β-casomorphins (Schiller et al. 1987).

CONFORMATIONAL ANALYSIS OF CYCLIC OPIOID PEPTIDE ANALOGS

Side Chain-to-End Group Cyclized Enkephalin Analogs

The conformational behavior of the cyclic prototype analog H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (2) has been investigated by various groups, using a variety of theoretical and experimental techniques. In a first theoretical conformational analysis, a molecular mechanics approach was used to determine low energy conformers of 2 (Hall and Pavitt 1984a). The lowest energy conformer obtaized was characterized by a Gly^3 -Phe^4 type II' bend, and it was suggested by the authors that the same type of bend might also be present in the receptor-bound conformation of linear enkephalins. Furthermore, some of the low energy conformers found showed a transannular hydrogen bond between the side chain NH of A_2bu and the A_2bu carbonyl group. Another energy minimization study of cyclic analog 2 resulted in a number of low energy conformers that contained either relatively planar ring structures stabilized by intramolecular hydrogen bonds (β-turns or C_7 structures) or twisted ring structures devoid of any hydrogen bonds (Maigret et al. 1986). Furthermore, the authors postulated that the tyramine portion of Tyr^1 and the Gly^3 carbonyl group in 2 might correspond to the tyramine portion and the C-ring hydroxyl group contained in morphine. It was then

attempted to impose spatial overlap of these pharmacophoric moieties with those in the rigid morphine molecule through conformational adaptation of each one of the low energy conformers of cyclic peptide 2. This was possible at a relatively low energy expenditure (6 kcal/mol) with only one of the low energy conformers and resulted in a tilted structure that was characterized by intramolecular hydrogen bonds from Leu^5-NH to A_2bu-CO (β-bend) and from Gly^3-NH to Tyr^1-CO (C_7 structure). It was suggested that the latter conformation might represent the bioactive one at the μ-receptor, since the 6 kcal/moL increase in energy as a consequence of the imposed fit could certainly be compensated for by favorable binding interactions at the receptor. It must be realized, however, that this proposed model depends entirely on the authors' somewhat arbitrary choice of the pharmacophore.

In a third study, both computer simulations and ^{1}HNMR spectroscopy were employed to determine the conformation(s) of cyclic analog 2 (Mammi et al. 1985). The performed molecular dynamics study showed that the ring structure of 2 is not entirely rigid but fluctuates about a few equilibrium conformations. The most stable ring conformations were found to be stabilized by transannular hydrogen bonds implicated in the formation of C_7 structures. The carried out energy minimization study resulted in one lowest energy conformer characterized by hydrogen bonds from A_2bu(side chain)-NH to A_2bu-CO, from Leu^5 -NH to Gly^3-CO and from Gly^3-NH to Tyr^1 -CO. The ring structure of another obtained low energy conformer also showed a Leu^5 NH → OC- Gly^3 hydrogen bond, whereas the A_2bu side chain NH was hydrogen-bonded to the Phe^4-CO rather than to the A_2bu-CO. Determination of the temperature dependence of the amide proton chemical shifts ($d\delta/dT$) in the ^{1}HNMR study performed with cyclic analog 2 in [$^2H_6$]DMSO revealed that both the Leu^5 NH and the side chain NH of A^2bu may be involved in hydrogen bond formation. These results are consistent with the hydrogen bonding patterns obtained in the energy minimization and suggest that the solution conformation of 2 may indeed be stabilized by the transannular hydrogen bonds observed in the calculated lowest energy conformers.

It is obvious that the results of these various conformational studies on cyclic analog 2 have not yet led to a consensus concerning a possible unique bioactive conformation at the receptor. Clearly, the most important finding of these endeavors is the realization that the ring structure in 2 still retains some flexibility and that the various intramolecular hydrogen bonds observed are constantly formed, broken, and re-formed again, as shown most conclusively in the molecular dynamics study.

In a recent study, the conformational behavior of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (2) was compared with that of its diastereomer with Leu^5 in the D-configuration (analog 2a), using again a combination of computer simulations and ^{1}HNMR in [$^2H_6$]DMSO (Mierke et al. 1987). The obtained results showed that, in

contrast to the L-Leu[5] peptide, the conformation of the D-Leu[5] analog is not stabilized by any intramolecular hydrogen bonds. Furthermore, the NMR data (T_1 relaxation time measurements) and molecular dynamics study indicated that both the peptide backbone and the side chains in 2a are considerably more flexible than in 2. In particular, the computer simulation carried out for 20 picoseconds revealed that the important intramolecular distance between the Tyr[1] and Phe[4] aromatic rings remains constant (~20 Å) in the case of the L-Leu[5] analog, whereas considerable variation of that same distance with time (5-16 Å) is seen in the case of the D-Leu[5] analog. It was concluded that the more rigid conformation and the relatively large and fixed distance between the Tyr and Phe[4] aromatic rings of analog 2 may be responsible for its μ-receptor preference, whereas the Lack of receptor selectivity shown by 2a may be due to its comparatively higher structural flexibility The same approach was also used to investigate the conformational behavior of partial retro-inverso analogs of 2, characterized by a reversed amide bond in three different positions of the ring structure (figure 1, compounds 2b, 2c, and 2d). Various transannular hydrogen bonds, giving rise to C_7 structures or also C_6 and C_8 structures, were detected in the computer simulation study and where found to be compatible with the results of the NMR temperature perturbation study, which permitted the identification of hydrogen-bonded amide protons. No β-turns were observed, as the conformational restriction introduced through ring closure renders the formation of a 4 → 1 hydrogen bond energetically unfavorable. In all analogs the side chains of the Phe[4] and Leu[5] residues were found to be very flexible, whereas the Tyr side chain appeared to exist primarily in the trans conformation ($\chi_1 \sim 180°$), which was favored by more than 3 kcal/mol over gauche conformations.

Hall and Pavitt (1985) compared the Low energy conformations of H-Tyr-cycLo[-D-A_2pr-Gly-Phe-Leu-] (1) and H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] (3) with those of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (2), using the same molecular mechanics approach as in their original study of 2 (Hall and Pavitt 1984a). This investigation resulted in a number of low energy ring conformations common to all three analogs, which differed from one another primarily in the orientation of a particular amide bond. Several of these conformations contained a type II' bend centered on Gly[3] -Phe[4] , and it was speculated that one of these might represent the bioactive conformation at the receptor. Kessler et al. (1985) performed a ^{1}H NMR study in [$^2H_6$]DMSO with H-Tyr-cyclor-D-Orn-GLy-Phe-Leu-] (3) and H-Tyr-cyglo[-D-Lys-Gly-Phe-Leu-] (4). The obtained NMR data indicated that cyclic analog 3 has- a relatively rigid ring conformation, and the determination of the temperature dependence of the amide proton chemical shifts ($d\delta/dT$)suggested the existence of two transannular hydrogen bonds (Leu[5]-NH → OC-Gly[3] (γ-turn) and Orn[2]-$N^{\delta}H$ → OC-Orn[2]). The analogous γ-turn and A_2bu-$N^{\gamma}H$ → OC-A_2bu hydrogen bond had also been observed with cyclic analog 2 in the conformational study described above (Mammi et al. 1985). In comparison with 3, the ring conformation in cyclic analog 4 was found to be considerably

more flexible. The $d\delta/dT$ values and coupling constants determined with 4 suggested the existence of a β-turn centered on Phe^4-Leu^5 and stabilized by a hydrogen bond between the $N^{\varepsilon}H$ group of $D\text{-}Lys^2$ and the carbonyl group of Gly^3. The ring conformation of analog 3 defined by the two hydrogen bonds detected in this study formed the basis for a recent computer-modeling study aimed at determining topological similarities between this cyclic peptide and 7-α-[(1R)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-endo-ethenotetrahydrooripavine (PEO) (DiMaio et al. 1986).

Side Chain-to-Side Chain Cyclized Enkephalin Analogs

Using the molecular mechanics approach, Hall and Pavitt (1984b) performed a theoretical conformational analysis of the potent, non selective enkephalin analogs H-Tyr-D-Cys-Gly-Phe-D(or L)-Cys-NH_2 (5). A Gly^3-Phe^4 type II' bend was indicated by the obtained results, as it had been the case with the H-Tyr-cyclo[-D-Xxx-Gly-Phe-Leu-] analogs analyzed in the same manner by these authors (see above). However, comparison of the 14-membered ring structures in these two cyclic peptides with the 14-membered ring of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (2) indicated a higher structural flexibility in the case of the cystine-containing analogs, which may explain their lack of receptor preference. In a conformational study by fluorescence techniques, the tyrosine fluorescence quantum yield determined with H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 was found to be 2.5 times lower than that of the linear analog H-Tyr-D-Ala-Gly-Phe-Met-OH (Schiller 1983), presumably due to the quenching effect of the adjacent disulfide group, which is known to occur at distances of less than 6 Å between the Tyr phenol ring and the -S-S moiety. This observation suggested the existence of an energetically favorable intramolecular complex between the phenol ring and the disulfide group, which would result in an interaction energy of about 1 kcal/mol at an optimal distance of 3.5 Å between the plane of the aromatic ring and one of the sulfur atoms. On the basis of these findings and arguments, a close interaction between the tyrosine side chain and the disulfide bridge in H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 was proposed. Singlet-singlet energy transfer between the phenol ring of Tyr^1 and the indole moiety of Trp^4 was measured in the analog H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2 (6). Evaluation of the determined fluorescence parameters on the basis of Förster's equation resulted in an average intramolecular distance of 9.7 ± 0.2 Å between the two aromatic rings contained in 6. This mean distance is nearly identical with the average Tyr^1-Trp^4 distance (9.5 ± 0.3 Å) in the linear enkephalin analog H-Try-D-Ala-GLy-Trp-Met-OH, which was determined by the same technique.

The conformations of the two non selective cystine-containing analogs H-Tyr-D-Cys-Gly-Phe-L-Cys-NH_2 and H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 (structures 5) were compared with those of the related s-selective Pen^2-containing analogs H-Tyr-D-Pen-Gly-Phe-L-Cys-NH_2 and H-Tyr-D-Pen-Gly-Phe-D-Cys-NH_2 (structures 7) in a ^{1}HNMR study carried out in D_2O (Mosberg and Schiller 1984), Similar chemical

shifts, $d\delta/dT$ values, and coupling constants were observed for corresponding penicillamine and cysteine containing analogs, indicating similar overall conformations. However, the obtained NMR data suggested that, in comparison with the corresponding CYS[2] analogs, the Pen[2] analogs show higher structural rigidity in the C-terminal part of the molecule, which may explain their δ-receptor selectivity. The determined temperature dependences of the amide proton chemical shifts did not indicate the existence of intramolecular hydrogen bonds in any of these four cyclic analogs. The large chemical shift difference observed for the two penicillamine methyl resonances in both Pen[2] analogs are indicative of a ring current effect caused by the tyrosyl aromatic moiety and, as in the case of H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2, (see above), suggest a close proximity between the Tyr[1] aromatic ring and the disulfide moiety.

The same type of NMR analysis was then performed with six penicillamine-containing cyclic enkephalin analogs (structures 7a, 7b, and 7c), all of which display δ-receptor selectivity (Mosberg 1987), Some variation in various NMR parameters was observed even between compounds that displayed similar potency and receptor selectivity. These findings were interpreted to indicate that these analogs must contain the crucial pharmacophoric elements in a similar spatial disposition, presumably as a consequence of the observed conformational flexibility of the Gly[3] residue, which allows for conformational compensation. Measurement of the temperature dependence of the amide proton chemical shifts indicated that most amide protons in these analogs were fully exposed to the solvent. The exceptions were the D-Pen[5] amide protons in [D-Cys[2], D-Pen[5]]enkephalin and [D-Pen[2], D-Pen[5]]enkephalin, which showed low $d\delta/dT$ values, indicating a possible involvement in an intramolecular hydrogen bond. Interestingly, very good agreement of all NMR parameters was Observed between [D-Cys[2], D-Pen[5]]enkephalin and [D-Pen[2], D-Pen[5]]enkephalin, suggesting very similar conformations of these two compounds. The latter analog is about six times more δ-receptor selective than the former as a consequence of its poor affinity for the μ-receptor. These results indicate that the improved δ-receptos selectivity of [D-Pen[2], D-Pen[5]]enkephalin as compared to [D-Cys[2], D-Pen[5]]enkephalin is not due to a different conformational behavior but rather to the presence of the gem dimethyl group of the Pen[2] residue, which causes more severe steric interference at the μ-receptor than at the δ-receptor.

Belleney et al. (1987) performed a comparative ^{1}HNMR study with H-Tyr-D-Pen-Gly-Phe-Pen-OH and H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET)in[$^2H_6$]DMSO In contrast to the data obtained by Mosberg (1987) with H-Tyr-D-Pen-Gly-Phe-Pen-OH in D_2O, low $d\delta/dT$ values were observed for the amide proton chemical shifts of both L-Pen[3] and Gly[3] in the case of the cyclic peptide. These data were interpreted to indicate that the conformation(s) of H-Tyr-D-Pen-Gly-Phe-L-Pen-OH_4in [$^2H_6$]DMSO might contain two turns either a γ-turn around Phe or a β II' turn centered on Gly[3]-Phe[4] in the C-terminal region and a γ-turn centered on Pen[2] in the N-terminal

region. In the case of DTLET, the obtained $d\delta/dT$ values also suggested the existence of a γ-turn and a β-turn in the N- and C-terminal region of the peptide, respectively, and, thus, H-Tyr-D-Pen-Gly-Phe-Pen-OH and DTLET appear to have similar backbone conformations in DMSO. This type of backbone conformation together with the similar arrangement of the Tyr^1 and Phe^4 side chains in both the cyclic and the linear peptide was suggested to be responsible for the observed preferential interaction with the δ-receptor. Furthermore, the authors proposed on the basis of these models that the two threonine methyl groups in DTLET might play the same role in the interaction with the receptor as the gem dimethyl groups of the two Pen residues in the cyclic peptide.

Cyclic Lactam Analogs Containing the Phenylalanine Residue in the 3-Position

Recently, Wilkes and Schiller (1987) applied a systematic procedure for the determination of the allowed low energy conformations of the highly μ-receptor selective cyclic analog H-Tyr-D-Orn-Phe-Asp--NH_2 (8), using the software package SYBYL (Tripos Associates, St. Louis, MO). A comprehensive grid search of the 13-membered ring structure of 8 lacking the exocyclic Tyr^1 residue and the Phe^3 side chain resulted in only four low energy conformers. These four conformations showed considerable similarity, being all fairly round and flat. The three amide bonds within the ring were generally either perpendicular to the plane of the ring or slightly tilted toward the ring center, but no linear transannular hydrogen bonds were observed. The exocylic Tyr^1 residue and Phe^3 side chain were then added to these four low energy conformers and an extensive energy minimization was carried out with each on of them. The obtained results indicated that the Tyr^1 and Phe^3 side chains en joy considerable orientational freedom but nevertheless only a limited number of low energy side chain configurations were found. The lowest energy conformer obtained by this approach was characterized by a tilted stacking arrangement of the two aromatic rings (figure 2, structure 8). However, several other conformers with different side chain configurations were found to be only slightly higher in energy (1 kcal/mol or less above the energy minimum).

This conformational analysis has recently been extended to several 13-membered ring cyclic analogs related to 8 which show considerable diversity in their μ-receptor affinity and selectivity (figure 1, compounds 9-13) (Wilkes and Schiller 1988). Again, the analyses performed with the bare ring structures resulted in no more than four low energy conformers (within 2 kcal/mol of the lowest energy structure) in all cases. The gross topological features of all the low energy ring conformations observed with these analogs were similar to those of the low energy conformers of the cyclic parent peptide 8. Thus, in all cases no transannular hydrogen bonds were found. Only two low energy ring conformations were obtained in the case of compound 11, indicating that the additional conformational

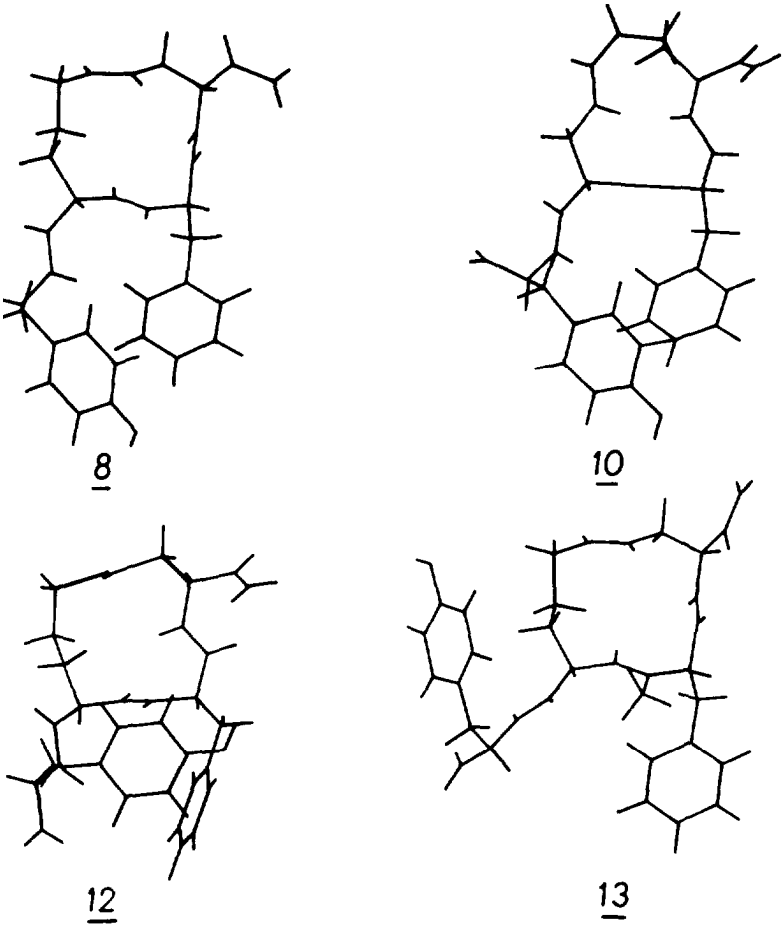

FIGURE 2

Lowest Energy Conformers of Cyclic Opioid Peptide Analogs 8, 10, 12 and 13.

constraint introduced by N-methylation of the Phe[3] residue led to a further increase in structural rigidity. After addition of the exocyclic Tyr[1] residue and the Phe[3] side chain to the low energy ring conformations of analogs 9-13 low energy side chain configurations were again determined by extensive energy minimization (figure 2). The two potent and μ-selective analogs 9 and 10 showed a tilted stacking arrangement of the two aromatic rings in their lowest energy conformations similar to that observed in the lowest energy conformer of 8. Among the analogs with reduced μ-receptor affinity, compound 11 showed a lowest energy conformation characterized by a fully stacked parallel arrangement of the two aromatic rings rather than a tilted stacking interaction. Analysis of the weak μ-agonist 12 revealed that chiral inversion at the 2-position precludes a low energy stacking configuration. The lack of stacking of the aromatic rings observed in the low energy conformers of the weakly active analog 13 appears to be due to steric interference of the bulky N-methyl group at the 3-position residue. Taken together, these results suggest that a specific tilted stacking interaction of the aromatic rings in the 1- and 3-position of cyclic opioid peptide 8 and its analogs may represent an important structural requirement for binding at the μ-receptor. However, it should be kept in mind that other side chain configurations were found to be only slightly higher in energy and that a change in side chain conformations could occur upon binding to the receptor at an energy expense, which could be compensated for by part of the binding energy.

CONCLUSION

Conformational studies of three families of cyclic opioid peptide analogs carried out to date have revealed that the ring structures contained in these compounds are not entirely rigid but, depending on the type and size of the ring, undergo more or less extensive conformational fluctuations. Nevertheless, the results of the performed molecular mechanics and molecular dynamics studies indicate that these structural fluctuations are relatively minor in the case of 13- or 14-membered rings and that the various Low energy ring conformers observed for a given cyclic analog do not differ very much in their overall shape from one another. As expected, the results various conformationa analyses indicate that the exocyclic Tyr[1] residue and the Phe[3] side chain still enjoy considerable orientational freedom. In order to determine the distinct conformational requirements of the μ - and the δ-receptor, it will be necessary to prepare and characterize cyclic opioid peptide analogs in which the conformational freedom of these important pharmacophoric moieties is restricted in various ways.

FOOTNOTES

[1]Abbreviations: A_2bu, α, γ-diaminobutyric acid; A_2pr, α, β-diaminopropionic acid; DTLET, H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH; NMR, nuclear magnetic resonance; Pen, penicillamine; Phe(NMe), N^{α}-methylphenylalanine; Phg, phenylglycine.

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Conformational Studies of Dermorphin

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Dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, a D-Ala-containing amidated heptapeptide, is a potent μ-receptor agonist. It was originally isolated from the skin of a South American frog of the genus Phyllomedusae by Broccardo et al. (1981) (for a review, see Feuerstein 1986). and it was subsequently synthesized (de Castiglione et al. 1981; Montecucchi et al. 1981). Its occurrence in mammalian tissue has not been demonstrated to date (Negri et al. 1981). Dermorphin is similar to the other opioid peptides, especially the enkephalin and dynorphin family of peptides, in having Tyr residue in the first position of the sequence. The presence of D-Ala residue in the second position and the occurrence of Phe and Tyr residues in the third and fifth positions should be expected to confer unique conformational preferences and thus dermorphin serves as an excellent model system for investigating conformation-receptor site selectivity relationships. We have investigated the conformations of dermorphin using vibrational spectroscopic studies (which are ideal for peptides with chromophoric residues possessing unique electronic transitions), 1D and 2D NMR studies (both at room temperature and at elevated temperatures), molecular mechanics-dynamics approach, and CD studies. CD studies of dermorphin in H_20, trifluroethanol (TFE) and methanol, and the temperature effects in the above solvents provide insight into the conformation of dermorphin, although chromophoric residues present in dermorphin complicate the observed CD spectra.

The studies reported here represent our continuing investigations (Bhatnagar et al. 1985; Pattabiraman et al. 1986), and similar studies from other laboratories are also discussed. Aqueous solutions of dermorphin were used in all the spectroscopic studies, whereas CD studies were performed in H_20, trifluroethanol (TFE), and methanol. While the earlier 1D and 2D NMR studies had utilized dimethyl sulfoxide (DMSO) (Salvadori et al. 1983; Arlandini et al. 1985; Pastore et al. 1985; Toma et al. 1985) as the solvent, we employed 90 per cent H_20-10 per cent D_20 in our 1D and 2D NMR studies, as we believe aqueous solutions are more relevant than organic solvents to deduce physiologically relevant conformation(s).

Modern spectroscopic techniques utilized here offer the advantage of suppression of H_20 signals, a problem that plagued especially IR and NMR studies in the 1960s and 1970s.

EXPERIMENTAL STUDIES

CD, FT-IR, and Raman studies were performed as described earlier (Renugopalakrishnan et al. 1985, 1986; Prescott et al. 1986; Rapaka et al. 1987). 1D and 2D ^1H NMR studies of polypeptides were performed in 90 per cent H_20-10 per cent D_20 on a 500 MHz Bruker AM-500 spectrometer (Renugopalakrishnan et al. 1987, 1988; Huang et al. 1988). Typical concentrations were in the range of 10-12.4 mM. Proton resonances were assigned based on homonuclear decoupling experiments and chemical shift data (Wuthrich 1986). Chemical shifts are reported in ppm relative to the shift of HDO at 4.8 ppm as an internal reference. In 2D NOESY experiments, H_20 resonance was irradiated at all times except during the data acquisition (Huang et al. 1988).

THEORETICAL STUDIES

Molecular mechanics studies employed the program AMBER developed in the laboratory of Peter Kollman, and the conformations were displayed visually using the program MIDAS (Weiner and Kollman 1981). The details of the calculations have been reported previously (Pattabiraman et al. 1986). Recently, we have begun a reinvestigation of the conformation of dermorphin using the program CHARMM developed in the laboratory of Martin Karplus (Karplus and McCammon 1981). The molecular dynamics calculations have been recently extended up to 15 picoseconds, and the results will be reported elsewhere (Prabhakaran et al. submitted for publication).

In the molecular mechanics calculations, we considered two B-turn conformations and an extended conformation to restrict the search in conformational space. A distance-dependent dielectric constant, ε, simulated qualitatively the effect of solvent, but we did not explicitly include solvation shells in the computations.

GENERAL DISCUSSION OF THE CONFORMATIONAL MODEL OF DERMORPHIN DERIVED FROM EXPERIMENTAL AND THEORETICAL STUDIES

An energetically stable conformation of dermorphin derived from molecular mechanics calculations is shown in figure 1. Dermorphin assumes a type III′ B-turn conformation at the N-terminal segment, Tyr-D-Ala_2-Phe_3-Gly_4, and a type I B-turn conformation at the C-terminal segment, Tyr_5-Pro_6-Ser_7-NH_2. Total energies of the ß-turn conformations, (III′-I), (II′-III), and (II′-I), and a fully extended conformation are presented in table 1. The fully extended conformation is less stable than the folded conformations. The three folded conformations, however, differ by 1 kcal/mole from one another and therefore can interconvert on

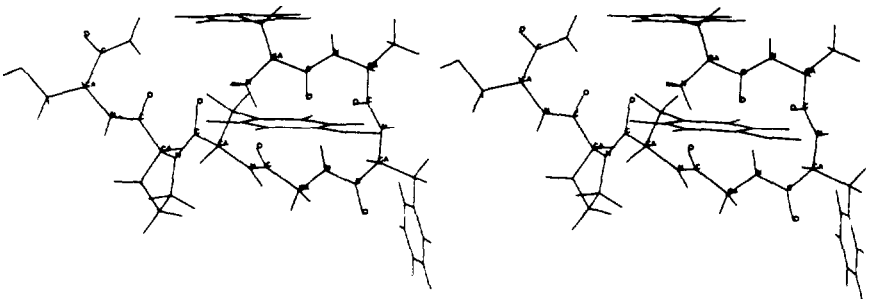

FIGURE 1

Conformation of dermorphin from molecular mechanics calculations

conventional spectroscopic time scale, although time-resolved FT-IR and Raman may be able to detect them. The Tyr residues, which stack with a skew and therefore are not superimposable, probably provide the major driving force for the stabilization of the folded conformations. The stability of the folded conformation is, however, not surprising, considering the presence of D-Ala_2 and Pro_6 residues, which are compulsive B-turn promoting residues (Chou and Fasman 1978). The stacking of Tyr_1-Tyr_5 side chains can be observed from figure 1.

The folded conformation is consistent with the bulk of the experimental spectroscopic data in aqueous solution. Temperature dependence of CD spectra of dermorphin in TFE solution (not shown here) manifests an unusual CD pattern with a broad positive band at λ=226nm (band II) and somewhat less broad positive band at λ=198nm (band I). As the temperature is increased to 45°C, a dramatic shift in the CD bands occurs. A broad negative band begins to appear at λ=218nm, which persists at 60°C with a shift of the negative trough toward lower wave length region. The above observation qualitatively indicates that the chromophore responsible for band II is disrupted by heating. The CD spectra of dermorphin in TFE, H_20, and methanol, representing solvents of varying polarity, suggest that dermorphin manifests discrete conformational states in solution phase. The observed CD spectra are difficult to interpret in terms of the secondary structure but probably are the composite of two ß-turns (Renugopalakrishnan et al. submitted for publication).

Raman spectrum of dermorphin in H_20 shows an amide I band at 1681 cm^{-1} and a doublet amide III band at 1253 cm-l and 1265 cm^{-1}. Raman amide I bands beyond the 1668-1678 cm^{-1} are

TABLE 1 Conformational Energies of the seven models of dermorphine

Model	β-Turn-I	β-Turn-II	Total Energy in kcal/mol
Ill′-I	III′	I	-70.7
II′-III	II'	III	-69.9
II'-I	II'	I	-69.6
III'-III	II′	III	-57.2
V'-I	V′	I	-56.9
V'-III	V′	III	-46.4
Extended	Trans	Trans	-46.3

believed to originate from ß-turns (Bandekar and Krimm 1979; Tu 1986). The doublet amide III band at 1253 cm^{-1} and 1265 cm^{-1} is at best representative of extended/"random"/ß-turn structures. The alternative explanation for the amide III doublet can be advanced from the side chain Raman vibrations of the aromatic residues. To decide between the alternatives, deuterium exchange studies have been carried out to observe the shift of amide III bands resulting from exchange of labile hydrogen atoms. After the dissolution and equilibration of the sample in D_20 solution, one of the amide III bands, the band at 1253 cm^{-1} is lost, whereas the 1265 cm-l band shifts toward lower wave numbers to 1261 cm^{-1}. Therefore the 1253 cm-l band is assigned to the peptide backbone vibrations, and the above frequency is probably a mixed mode originating from R-turn and probably extended structures. The amide I' band occurs at 1661 cm-l. Although Raman results are largely Indicative of R-turn or folded conformation, an admixture of extended structure cannot be ruled out.

500 MHz 1D ^1H NMR spectrum of dermorphin in H_20 is shown in figure 2. Ramachandran angles, ϕ_1, for Tyrl, D-Ala_2, Phe_3, Gly_4, Tyr_5 and Ser_7 were derived from a Karplus-like equation, using the values of the coefficients A, B, and C derived by Bystrov et al. (1973). From the ϕ angles derived, it is concluded that a fully extended conformation is not compatible with the observed spin coupling constants, J. From the calculated Ramachandran angles, ϕ, one is led to conclude that either type II′ or III′ ß-turn occurs at the N-terminal segment, whereas the C-terminal ß-turn may contain any one of three types of ß-turns--types I, II, and III. It is difficult to discriminate between them based only on spin coupling constant data. The variation of aromatic proton resonances as the temperature is increased is indicative of the unfolding of the dermorphin from a folded conformation to an unfolded state in which Tyr residues are no longer stacked. From the combined results, one cannot but conclude that dermorphin probably assumes a manifold of folded conformations, which interconvert in the spectroscopic time scale. It is possible the heptapeptide contains a partially extended

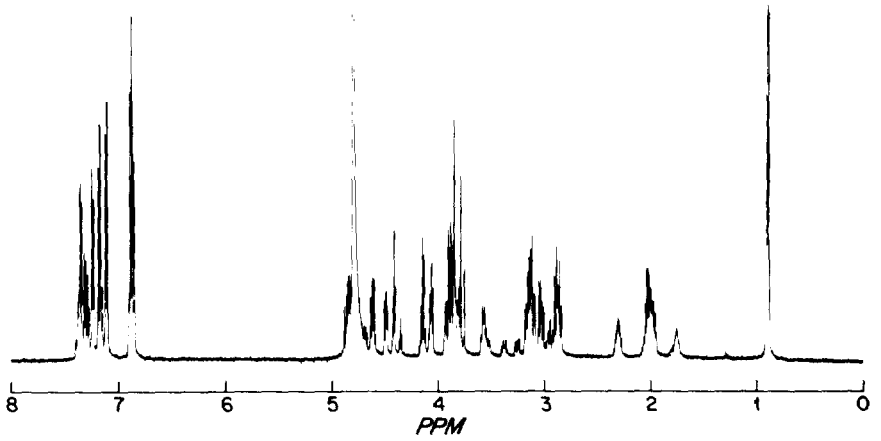

FIGURE 2

500 MHz ^{1}H NMR spectrum of dermorphin in H_20

segment at the N-terminal end, but, in any event, such a conformational state has a low probability of occurrence in the aqueous solution.

Most of the previous spectroscopic studies of dermorphin were exclusively performed in dimethyl sulfoxide (Salvadori et al. 1983; Arlandini et al. 1985; Pastore et al. 1985; Toma et al. 1985), and NMR studies were utilized to derive the secondary structure of dermorphin. Toma et al. (1985) investigated the solution conformation of a series of [Ala^{n}] substituted dermorphin analogs by 500 MHz ^{1}H NMR spectroscopy. Preferred conformations were calculated using a semi-empirical method. They reached the conclusion that none of the calculated conformations were able to satisfy the experimental data. In contrast, both experimental and theoretical studies could at best be reconciled with a type I B-turn at the C-terminus. Nevertheless, no consensus could be reached at the N-terminus, and the question of any preferred conformation at the N-terminus could not be resolved. However, Pastore et al. (1985) have reported 1D and 2D-NMR study of dermorphin in DMSO and reached the conclusion that the heptapeptide assumes an essentially extended structure. The above investigators assumed corrections for ring current effects in dermorphin. The question of interaction between amide protons and DMSO was thought to be a major factor in the observed conformation in DMSO. An extended structure of dermorphin in DMSO may be a special

situation that is probably not valid in aqueous solutions of dermorphin. We believe that dermorphin assumes a manifold of folded conformations in aqueous solution, although an extended conformation at the N-terminus should also be considered. Nevertheless, in a llpophilic environment, it is likely that dermorphin assumes a folded conformation, which is probably the most relevant at the μ-receptor site (Alford et al. in preparation). Therefore, two different perspectives of dermorphin conformation relevant to DMSO and aqueous solution exist in the literature currently. Due to the relatively small size of dermorphin, which exhibits rapid molecular tumbling, it is difficult to provide a clear-cut resolution of the existing conformational populations.

Numerous μ- and δ-receptor selective opioid peptide analogs have been synthesized, and conformational studies have been conducted on a number of analogs. ß-turns, ß-sheets, and extended structures have been hypothesized as some of the recognition features for the opioid receptor types (see Rapaka 1986 for a review). However, conformation-receptor selectivity relationships have not yet been established. It is expected that with the multifaceted research techniques employed, the large number of analogs available, and a greater understanding of the biochemistry of the opioid receptors, the critical factors for conformation-receptor selectivity relationships will be better understood, paving the way for design of highly receptor selective analogs and safe and potent analogs devoid of undesirable side effects.

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Use of Molecular Biological Methods to Study Neuropeptides

Michael J. Brownstein, M.D., Ph.D.

INTRODUCTION

Armed with molecular biological techniques, neurobiologists interested in biologically active peptides have begun to work on problems that they could only dream of attacking 10 years ago. The structures of several peptide precursors have been determined by isolating and sequencing DNAs complementary to their respective mRNAs, and similarities among peptide precursors have been cataloged. Based on the structures of the precursors, inferences about their processing were generated and a number of processing enzymes have been identified. Two of these have been purified, and one has itself been cloned.

The availability of cDNA probes that specifically hybridize to mRNAs that encode peptide precursors and processing enzymes allows the levels of these mRNAs to be measured and—by means of in situ hybridization histochemistry—to be visualized in discrete cells. In addition, cDNA probes can be used to screen genomic libraries to isolate genes that encode peptide mRNAs (or to determine the cause of genetic defects in peptide production). The regulatory elements of the genes can ultimately be identified and the transactivating molecules that mediate tissue-specific expression of gene products and regulate mRNA levels can be identified. Examples of the latter include the recently characterized intracellular receptors for steroids and thyroid hormones.

To date only two neuropeptide receptors have been purified and sequenced. Advances in expression cloning technology may soon contribute to pushing aside our ignorance of this class of binding protein.

CLONING PEPTIDE PRECURSORS: THE CASE OF GALANIN

Galanin, a 29 amino acid peptide, was isolated from the upper small intestine of pigs by Tatemoto, Mutt, and their colleagues (1983). Like so many other peptides, it has a C-terminal amide. This structural feature led to its discovery. Afterwards, galanin was found to be present in the enteric nervous system of the gastrointestinal tract (Melander et al. 1985; Rökaeus et al. 1984), the urogenital tract (Bauer et al. 1986a), the pancreas (Dunning et al. 1986), the adrenal medulla (Bauer et al. 1986b), and the brain (Melander et al. 1986). It has several actions, including inhibition of phasic activity of the small intestine (Fox et al. 1986), production of hypoglycemia (McDonald et al. 1985), suppression of insulin release, and central stimulation of growth hormone (Ottlecz et al. 1986).

With few exceptions, DNAs encoding peptide precursors have been isolated from cDNA libraries by screening them with mixtures of synthetic oligonucleotides, the compositions of which were based on the amino acid sequence of the peptides. It was in this way that we isolated cDNA. encoding the galanin precursor from a pig adrenal medullary library (Rokaeus and Bownstein 1986). The precursor (preprogalanin, preproGAL) is ruher typical of this class of proteins. It is 123 amino acids long and is comprised of a leader (signal) sequence. the 29 amino acids of galanin, and a 59 amino acid sequence (galanin message associated protein, GMAP) (see figure 1). Unlike proopiomelanocortin, for example, preproGAL may encode only one active peptide, galanin itself. The galanin sequence is flanked on both sides by pairs of lysine and arginine residues. The C-terminal lysine and arginine are separated from the final alanine in galanin by a glycine, the donor of the amide moiety.

1

Met Pro Arg Gly Cys Ala Leu Leu Leu Ala
Ser Leu Leu Leu Ala Ser Ala Leu Ser Ala
Thr Leu Gly Leu Gly Ser Pro Val Lys Glu
Lys Arg Gly Trp Thr Leu Asn Ser Ala Gly
Tyr Leu Leu Gly Pro His Als Ile Asp Asn
His Arg Ser Phe His Asp Lys Tyr Gly Leu
Ala Gly Lys Arg. . . Ser

FIGURE 1. Partial sequence of preprogalanin. A very hydrophobic N-terminal signal sequence (beginning with residue 1, Met) precedes the (underlined) sequence of galanin (residues 33-61). Pairs of basic amino acids (Lys-Arg) bracket the galanin sequence. A glycine residue, the donor of the C-terminal amide, is found in position 62. The 59-residue sequence of as yet unknown function comprises the amino-terminal half of the precursor.

It is clear that precursors like preproGAL are cleaved to yield their active products by a series of enzymes. The first of these, located in the cisternum of the rough endoplasmic reticulum, removes the signal peptide from the prepropeptide converting it to a propeptide. Subsequent processing seems to occur principally in the secretory granule after the propeptide has traversed the Golgi apparatus. First the propeptide is split by an endopeptidase. More than one such endopeptidase may exist. A 70,000 dalton paired basic residue specific aspartyl protease (Chang and Loh 1984; Loh et al. 1985) has been shown to be involved in the processing of proopiomelanocortin and provasopressin. The pH optimum of this enzyme is low (four-five) allowing it to function in the acid milieu of the secretory vesicle. It cleaves precursors between their paired bases or C-terminal to them.

In addition to the paired basic residue specific cleavage enzyme, there are single basic residue cleaving enzymes. Such an enzyme has been found associated with rat brain membranes (Devi and Goldstein 1984). It cleaves

dynorphin B_{1-29} between Thr_{13} and Arg_{14} to yield dynorphin B_{1-13}. It seems to be a neutral thiol protease.

Peptides liberated from their precursors by the action of one of the above endopeptidases may have basic residues attached to their C- or N-termini. These need to be trimmed off. The first trimming enzyme to be discovered (Hook et al. 1982) and purified (Fricker and Snyder 1983) was a carboxypeptidase. Complementary DNA encoding this enzyme has recently been isolated. There is also an aminopeptidase that removes basic residues from peptides' N-termini when cleavage by the paired base specific endopeptidase occurs between two basic residues leaving one behind (Gainer et al. 1984). This enzyme appears to be a metalloprotease stimulated by Co^{+2} and Zn^{+2}.

The enzyme responsible for carboxyl-amidation of peptides was first described by Bradbury et al. (1982). This copper dependent enzyme uses a C-terminal glycine as the donor of the amide group. Other functional groups are physically added to peptides by enzymes, notably N-acetyltransferases, kinases, and sulfate transferases.

CLONING PEPTIDE "PRECURSORS": THE CASE OF VALOSIN

The major structural features of a typical peptide precursor and its mode of processing were outlined above. Comparison of the structure of a protein containing the sequence of valosin to other precursors illustrates the power of cDNA cloning to help in identifying peptides as strong or weak candidates for biological functions.

Valosin is a 25 amino acid peptide purified from side fractions of an earlier preparation of porcine peptide HI and secretin (Schmidt et al. 1984). Following its isolation, valosin was found to release gastrin, to augment pentagastrin-induced gastric secretion, to stimulate pancreatic protein secretion, and to suppress migrating myoelectric complexes of the small bowel (Schmidt et al. 1985).

On screening a porcine adrenal medullary cDNA library with an oligonucleotide pool constructed on the basis of the amino acid sequence of

valosin, we detected several cDNAs (Koller and Brownstein 1987). The longest of these had a 2,466 base pair open reading frame that encoded a ~ 88,660 dalton protein containing the amino acid sequence of valosin (see figure 2). This protein is unlike other peptide precursors characterized to date: (1) It has no obvious N-terminal or internal signal sequences and seems, in fact, to reside in the cytoplasm of cells. (2) Its message is found in many tissues, neuronal and nonneuronal ones alike. (3) The valosin sequence is not bracketed by single or paired basic residues. In fact, it appears to have been released from the valosin-containing protein by the action of a chymotrypsin-like enzyme. It seems likely, then, that valosin is not a physiologically active molecule but rather an artifactual product of a (hitherto unknown) and rather ubiquitous cytoplasmic protein.

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...Gly Leu Glu Asp Val Lys Arg Glu Leu
Gln Glu Leu Val Gln Tyr Pro Val Glu
His Pro Asp Lys Phe Leu Lys Phe Gly
Met Thr Pro Ser Lys Gly Val Leu Phe
Tyr Gly Pro Pro Gly Cys Gly Lys Thr
Leu Leu Ala ...

FIGURE 2. The portion of the valosin-containing protein (VCP) that contains the valosin sequence (underlined). Note that valosin is not neighbored by canonical processing signals. While VCP does have pairs of basic residues scattered throughout it, many other proteins that are not peptide precursors do too. Furthermore, analysis of its entire sequence shows that VCP has no obvious N-terminal or internal signal sequences.

PUTTING CLONING TO WORK

Cloning a cDNA and sequencing it are not ends in themselves. A cDNA and the structural information derived from it should be used to forge tools that can be used for biological studies (see table 1).

TABLE 1. Examples of uses to which cloning data can be put.

A. Uses of cDNAs

1. Screening genomic libraries allows specific genes or gene families to be isolated and sequenced. Putative regulatory elements can be fused to marker genes for studies of gene expression. New genes can be introduced into the cells of transgenic animals. Genes from animals with genetic defects can be analyzed.

2. Full-length cDNAs inserted into appropriate vectors can be used to manufacture large amounts of proteins for biochemical studies. (In the case of peptide precursors, substrates for processing enzyme assays can be prepared this way.)

3. cDNAs, cRNAs, or synthetic oligonucleotides can be used to measure specific mRNAs in tissues or to visualize mRNAs in discrete cells by means of Northern blotting and in situ hybridization histochemistry, respectively.

4. Mutant cDNAs can be expressed in cells in order to determine the function of the altered protein domains.

B. Uses of peptides synthesized on the basis of cDNA sequences

1. Novel candidates for roles as neuropeptide transmitters can be synthesized and their biological activities explored.

2. Antibodies against peptides can be used for immuno-cytochemistry or immunoptecipitation (following pulse-chase experiments, for example).

A WORD ABOUT RECEPTOR CHARACTERIZATION

In principle, there are three ways to clone cDNAs that encode receptors. One might isolate the receptor, partially sequence it, and screen a cDNA library with oligonucleotide probes based on the amino acid sequence. To purify enough of a protein to sequence—50-100 pmoles perhaps—is nontrivial, and receptors are frequently N-terminally blocked. Consequently close to a nmole of material might be required, the pure receptor has to be fragmented and the fragments themselves purified and sequenced.

Antireceptor antibodies can be used to screen bacterial expression libraries. Raising such antibodies and proving that they are specific for a particular receptor is difficult. however, and if one employs an antibody that does not recognize the receptor of interest or one that is promiscuous, one's work will be for naught.

Finally, it should be possible to transfect a negative mammalian cell line with a eucaryotic expression library and to identify clones of cells that begin to make surface receptors. This method has not yet been employed successfully for neurotransmitter receptors, but recent advances in vector design and transfection procedures should lead to its wider adoption.

CONCLUDING REMARKS

Numerous biologically active peptides have been discovered in the past decade. Unfortunately, more is often known about the anatomy and neurochemistry of peptidergic neurons than about their function. It is to be hoped that use of molecular biological methods will contribute to overcoming this problem.

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Three Technical Approaches for Cloning Opioid Receptors

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INTRODUCTION

A basic tenet of modern pharmacology is that the biological activity of a drug is the result of chemical events triggered by the drug's interaction with specific receptors on responsive cells. Although the chemical structures of drugs have been extensively studied, very little information has been obtained about the molecular structures of drug receptors. The structural analysis of receptors is fraught with difficulties, which are in part due to limitations of the techniques used to manipulate integral membrane proteins. Most membrane proteins are hydrophobic, and &he removal of the lipid moiety severely limits or abolishes the solubility of the protein in aqueous media. Although the techniques of nonaqueous protein chemistry have advanced over the past few years, certain experimental constraints often make a direct attack on a protein‘s structure difficult to manage. In addition, the removal of the membrane lipid can disrupt the oligomeric structure of the membrane proteins and can obscure the involvement of associated receptor subunits. Limiting receptor abundance presents an additional problem. Depending on the nature of the signal transduction, a specific receptor can constitute from less than 0.01 percent to as much as 0.5 percent of the plasma membrane protein mass. Pharmacologically important receptors generally fall into the lower end of the abundance spectrum. Thus, attempts to purify receptors are compromised by the need for large amounts of starting material. This problem is often exacerbated by the need to use a tissue that is scarce or difficult to obtain. In addition, receptor preparations are often very unstable. Both native and purified receptor preparations can be sensitive to assorted cations, oxidizing or reducing agents, and a variety of other materials and methods commonly used for their preparation and storage. The loss of receptor activity compromises the ability to assess the integrity and purity of the receptor preparation. For these reasons, it is likely that the determination of the primary amino acid structure of many low

abundance neuroreceptors will be inferred from the analysis of their cloned nucleic acid sequences.

This review will discuss the strategies that can be applied to the molecular cloning of one specific family of neuroreceptors, the opioid receptors.

THE OPIOID RECEPTORS

The analgesic and euphoric responses to opiates are the result of a cascade of biochemical events that are triggered by the interaction of the opiates with specific receptors found on the cell membranes of nervous system tissues (West and Miller 1983; Paterson et al. 1984; Holaday 1985). These opioid receptors not only recognize exogeneous alkaloids such as morphine, but moreover interact with their endogeneous ligands, the opioid peptides. The opioid receptors are clearly involved in physiological phenomena such as pain perception, addiction, and withdrawal, and are implicated in hormone secretion, response to injury, and gastrointestinal motility (Holaday 1985). The molecular basis of the participation of opioid receptors in these phenomena is unknown.

Martin and coworkers (Martin et al. 1976; Gilbert and Martin 1976) were the first to postulate the existence of different types of opioid receptors based on differences in the pharmacological profiles of the different opiates. Although the precise number of opioid receptor types is still a matter of conjecture, -it is generally accepted that the opioid receptors can be subdivided into three major types, μ, δ, and κ (Pasternak et al. 1983; Paterson et al. 1984). These three receptor types are distinguished on the basis of four criteria: (1) patterns in ligand selectivity, (2) anatomical distribution, (3) physiologica:. and behavioral profiles, and (4) differences in the cellular response to activation (Dole et al. 1975; Paterson et al. 1984; Holaday 1985).

Three models can account for the heterogeneity of the opioid receptors. First, the three opioid receptors are structurally different and are encoded by different genes; second, the opioid receptors are encoded by different but homologous genes; or third, the opioid receptors are encoded by identical genes but differ in posttranslational modifications or in their interactions with second messengers. At the present time, there is no definitive evidence to favor one model over the others.

Using Schwyzer's concept (Schwyzer et al. 1980), one can formulate the existence of at least two domains in the active site of the opioid receptor: the message domain, which recognizes the common amino terminal sequence of the enkephalins, Tyr-Gly-Gly-Phe, and the address domain recognizing the remainder of the opioid peptide sequence. This model suggests that at least one region of the opioid receptor is common to the different receptor types and that the opioid receptors must therefore be homologous.

Furthermore, in view of the recently discovered homology between the β-adrenergic receptor, muscarinic acetylcholine receptor, and opsin genes (Kubo et al. 1986; Dixon et al. 1986; Bonner et al. 1987; Hall 1987), the possibility that the opioid receptors may also be encoded by homologous if not identical genes is very attractive.

Knowledge about the physical characteristics of the opioid receptor has been obtained directly from receptor purification experiments. Attempts at opioid receptor purification have been hampered by the receptor's sensitivity to denaturation and low abundance (Zulkin and Maneckjee 1986). These receptors constitute less than 0.005 percent of the total rat brain membrane proteins. In spite of the extreme difficulties in purifying opioid receptors, several laboratory groups (Bidlack et al. 1981; Newman and Barnard 1984; Gioannini et al. 1985; Simonds et al. 1985; Cho et al. 1986) have succeeded in purifying opioid receptor proteins to homogeneity. The two common characteristics established for the ligand binding subunit of the opioid receptor are (1) an apparent Mr range of 55,000-65,000 and (2) its glycosylated nature. These two properties, plus ligand selectivity, represent the important criteria for judging the authenticity of a cloned opioid receptor.

APPROACHES TO THE MOLECULAR CLONING OF THE OPIOID RECEPTOR

Our hope in understanding the molecular structure and activation of the opioid receptors lies with the use of recombinant DNA technology. The cloning of the opioid receptors will allow detailed examination of receptor structure-function relationships and receptor gene expression. This approach will therefore help explain the molecular mechanisms of receptor activation and its resulting cellular responses.

The opioid receptors are macromolecular components of plasma membranes, which specifically bind opioid ligands according to three criteria: (1) high affinity for opioids and opiates, (2) saturable binding, and (3) binding that is stereospecific and naloxone reversible (Dole et al. 1975). These three criteria constitute the accepted functional definition of the opioid receptor. Therefore, an authentic molecular clone of the opioid receptor must be able to code for a product that fulfills all three criteria.

In addition, an opioid receptor clone must encode a product with the expected characteristics of a membrane protein. Two structural characteristics of membrane proteins are the presence of a signal sequence that facilitates the insertion of the receptor in the membrane, and the presence of hydrophobic sequences that correspond to potential transmembrane domains of the receptor, The signal peptide is not an absolute requirement, since some membrane proteins do not have signal sequences.

Since the distribution of the opioid receptor in the central nervous system and in peripheral organs is well documented (Pert

and Synder 1373; Pfeiffer et al. 1982; Atweh and Kuhar 1983; Paterson et al. 1984; Mansour et al. 1987), in situ hybridization analysis using an opioid receptor clone as probe should correlate sites of receptor mRNA synthesis with sites of receptor protein localization. However, the distinction between sites of mRNA synthesis and receptor protein localization may be difficult to evaluate precisely because of differences in assay sensitivity. Alternatively, an opioid receptor clone may be used to express receptor protein in sufficient quantity to serve as an antigen in the preparation of antibodies. These antibodies can then be used in immunocytochemical analyses to precisely correlate the distribution of the receptor encoded by the molecular clone to the known distribution of the opioid receptor. Finally, based on physical criteria obtained during receptor purification experiments, the opioid receptor clone should encode a glycoprotein with an apparent Mr range of 55,000-65,000.

Three different strategies can be employed in the molecular cloning of the opioid receptor. The first approach begins with the isolatior of the receptor in sufficient quantity to permit amino acid sequencing. This sequence would be reverse translated into a DNA oligonucleotide probe and this probe would then be used in the isolation of an opioid receptor cDNA or gene clone. The second strategy proposes to clone the opioid receptor through gene expression without prior purification of the receptor protein. The third strategy assumes some sequence similarity between opioid receptors and other neuroreceptors and exploits this feature in hybridization experiments designed to identify potential opioid receptor clones.

CLONING THE OPIOID RECEPTOR WITH THE USE OF RECEPTOR AMINO ACID SEQUENCE INFORMATION

The traditional cloning strategy consists of purifying and sequencing the opioid receptor protein. The amino acid sequence information would then be used to identify a corresponding receptor cDNA clone.

This approach requires large-scale protein purification in order to prepare a sufficient quantity of homogeneous opioid receptor to permit partial amino acid sequencing. Several laboratories have succeeded in purifying the opioid receptor to homogeneity (Bidlack et al. 1981; Newman and Barnard 1984; Gioannini et al. 1985; Simonds et al. 1985; Cho et al. 1986). The objectives of these laboratories were to purify the ligand binding domain of the opioid receptor. Two approaches have been employed to purify opioid receptors: the labeled ligand binding approach and the affinity chromatography approach.

In the labeled ligand binding approach, radioactive opioid ligands are bound and covalently cross-linked to opioid receptors in the membrane; the label serves as a marker in subsequent purification steps. Since binding of the ligand is performed with native receptor-membrane complexes, artifacts due to

aberrant protein interactions are minimized. One drawback, however, is that the ligand binding site is irreversibly occupied, making binding assays or reconstitution experiments impossible.

The purification of solubilized opioid receptors by affinity chromatography is dependent on the receptor's ability to recognize immobilized opioid ligands. The receptor population isolated by this technique can be tested for binding ability and can be obtained in sufficient quantity to sequence. The major drawback to this approach is the difficulty in preserving biological activity during subsequent purification steps.

Pure receptors are subjected to partial proteolytic digestion. Peptide fragments are separated by high-performance liquid chromatography and analyzed using automated high-sensitivity gas phase amino acid sequencing. Currently, automated peptide sequencing requires as little as 10 picomoles of purified peptide. The sequences of at least two peptide fragments are then reverse translated into their corresponding DNA sequences and oligonucleotides complementary to these sequences are then chemically synthesized. The choice of oligonucleotide length and sequence is critical for successful cloning and has been discussed (Anderson and Young 1987). In general, oligomers of at least 17 bases in length containing the least amount of degeneracy are synthesized. The radiolabeled synthetic oligomers then serve as hybridization probes to identify receptor cDNAs (or genes) in appropriate libraries. This isolated clone is then used as a template for determining the entire sequence of the opioid receptor.

CLONING THE OPIOID RECEPTOR BY GENE TRANSFER IN EUKARYOTIC CELLS

Gene transfer systems have been used successfully in the molecular cloning of eukaryotic genes, including receptor genes (Kuhn et al. 1984; Chao et al. 1986). This approach is based on the expression of cell surface receptors on eukaryotic cells and does not rely on the availability of receptor amino acid sequence information. In this approach, a large population of cDNAs (or large fragments of genomic DNA) from tissues known to express opioid receptors are transfected into receptor-deficient eukaryotic cells. Transfectants that have incorporated exogeneous DNA are selected according to traditional techniques (Southern and Berg 1982) and are tested for their ability to express opioid receptor by binding analyses. In transfections using genomic DNA fragments, the fragments are purified by conducting secondary transfections. This step consists of repeating the transfection procedure using the DNA of the primary transfectant as the donor. If pools of cDNAs are transfected into cells, the opioid receptor cDNA can be isolated by gradually decreasing the size of the analyzed pool to unity. Several different variations of this procedure can be envisioned; in particular, cDNA libraries enriched in opioid receptor sequences can be constructed through mRNA subtraction (Hedrick 1984).

There is also the possibility of expressing an opioid receptor cDNA in prokaryotic cells. However, in view of the requirements necessary to maintain the biological activity of the receptor, it seems unlikely that such an expression system would be successful.

The gene transfer and expression approach was adapted by our laboratory as one strategy to attempt to clone the human opioid receptor gene. This strategy is shown in figure 1. Large fragments of human genomic DNA are cotransfected with plasmid DNA (pRSV*neo*), which confers neomycin resistance to opioid receptor-deficient mouse L cells. Drug resistant colonies are selected by growth in neomycin analogue G418 and receptor-bearing colonies are identified using an opioid receptor detection assay. If receptor antibodies are available, these would be used in fluorescent-activated cell sorting or *in situ* rosetting assays to identify receptor-bearing transfectants. The unavailability of opioid receptor antibodies that recognize extracellular antigenic determinants has precluded the use of these conventional detection assays and prompted the development of new techniques that allow screening of transfectants with radioactive ligands (see next section for details). Once receptor-bearing transfectants have been identified, DNA from these cells is transferred into new recipient mouse L cells. DNA from secondary transfectants containing the opioid receptor gene is then used to prepare a genomic library in either a lambda or cosmid cloning vector. The genomic library is then screened by filter hybridization using nick-translated human repetitive sequence DNA to detect sequences contained in the original human donor, but not recipient, mouse cell lines. Positive clones containing human donor DNA are mapped with restriction endonucleases, and then reintroduced by transfection into recipient L cells to assay for opioid receptor gene expression.

Development of Tools for the Detection of Neuroreceptor Expression in Eukaryotic Cells

Eukaryotic cells are ideal gene transfer recipients for the expression of neurohormone and neurotransmitter receptors. The eukaryotic cell membrane environment and posttranslational machinery are necessary for correct neuroreceptor conformation and are critical determinants in the successful detection of the receptor by ligand binding. In spite of the ideal host environment, oukaryotic expression libraries have not been widely used for the isolation of clones expressing neuroreceptors. One major obstacle that has hindered the use of eukaryotic expression libraries has been the lack of rapid and reliable screening procedures.

To facilitate the screening of large numbers of transfectant colonies, our laboratory has recently adapted a replica copy technique originally used for identifying somatic cell mutants (Raetz et al. 1982). The transfer membrane is a polyester nylon cloth that permits colony replication with high fidelity and

Gene Transfer and Molecular Cloning of The Human Opioid Receptor Gene

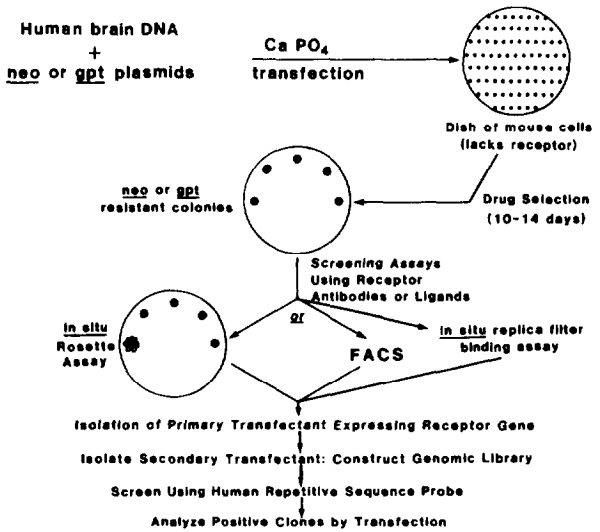

FIGURE 1

Molecular cloning of the human opioid receptor by gene transfer and expression in eukaryotic cells.

superior resolution (see fig. 2). Following drug selection of stable transfectants, one or more sheets of sterilized polyester nylon are placed on top of the cell colonies and overlaid with a monolayer of glass beads. The beads retain the filter uniformly against the bottom of the dish and permit the exchange of growth medium to the cells without changing the colony pattern. After 5-7 days of growth, the medium is aspirated, the beads are discarded, and the nylon membranes are removed with sterile tweezers. Up to five multiple copies of the transfectant population have been made from a single dish.

The multiple copies allow simultaneous screening of transfectant colonies with different radioiodinated neurohormone ligands in either the presence or the absence of nonradioactive blockers (see fig. 3). Positive signals that appear in duplicate using the radioactive ligand alone are identified by autoradiography. The specificity of the binding can be determined with the use of nonradioactive blockers. If desired, cell colonies that express neuroreceptocs can then be purified from the master plate of transfectant;.

To test the level of sensitivity of the in situ binding assay in detecting opioid receptors, the assay was standardized by using the mouse neuroblastoma N4TG1, a cell line that expresses approximately 50,000 δ opioid receptors per cell (Amano et al. 1972). As shown in fig. 4, 10^6,10^5, and 10^4 N4TG1 or opioid receptor-negative mouse L cells were mechanically spotted on nylon cloth; the filters were then subjected to binding with (3-[^{125}I]iodotyrosyl^{27}) ß··endorphin (0.15 nM in 50 mM Tris-HCl pH 7.4, 1 percent BSA, 0.05 percent poly-L-lysine, 50 ug/ml bacitracin, 10 ug/ml leupeptin, and 10 ug/ml trypsin inhibitor, for 1 hr at room temperature) in the absence or presence of opioid receptor antagonist, naloxone (100uM). The filters were then washed with ice cold buff'er, dried briefly, and placed under X-ray film. Figure 4 shows the results of an 18 hr autoradiographic exposure of a typical experiment. Clear signals emanating from as few as 10,000 opioic receptor-positive N4TG1 cells were observed; very little signal was observed for mouse L cells or for N4TG1 cells whose receptor sites were blocked with naloxone. Other experiments have shown that a signal from as few as 1,000 N4TG1 cells can be observed. This represents a detection limit of as few as 5 X 10^7 opioid receptors for this assay.

Screening Expression Libraries for Opioid Receptor Expression

Eukaryotic expression libraries were constructed using high molecular weight human genomic DNA [from the human neuroblastoma SKN-SH cell line, which expresses 50,000 opioid receptors per cell (Yu et al. 1986)] as the donor DNA for transfections. This DNA was cotransfected with neomycin resistance plasmids into opioid receptor-deficient mouse L cells and stable transfectants isolated by selection with G418. Assuming that the human genome contains approximately 3 X 10^9 bases and that each stable transfectant incorporates 10^6 bp of DNA, the entire human genome

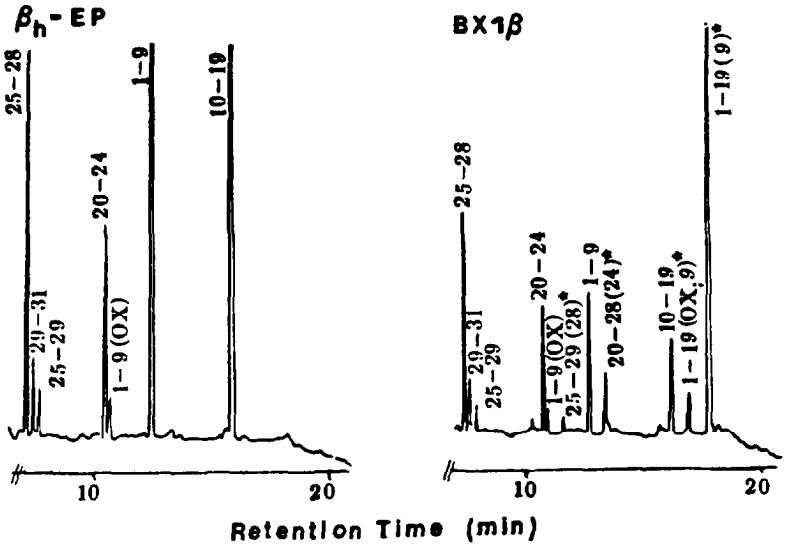

FIGURE 2

Transfectant colonies were grown on polyester nylon filters for 7 days. Replica filters were carefully removed from the master dish and colonies fixed with 10 percent trichloroacetic acid (10 minutes) and stained with 0.05 percent Coomassie blue in methanol:water:acetic acid (45:45:10; 10 minutes). Excess stain was removed by washing with methanol:water:acetic acid.

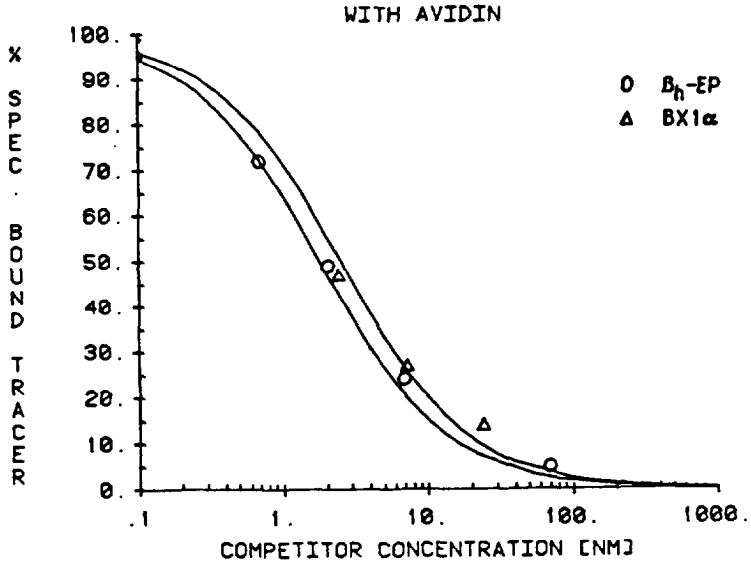

FIGURE 3

In situ replica filter binding assay for detection of transfectants expressing neuroreceptors.

DOT ASSAY FOR OPIOID RECEPTOR EXPRESSION

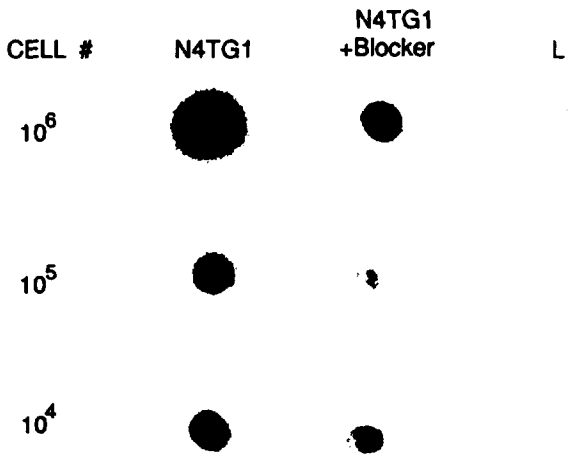

FIGURE 4

N4TG1 or L cells were spotted on nylon cloth and subjected to binding with (3-[^{125}I]iodotyrosyl^{27}) ß-endorphin (0.15 nM, room temperature, 1 hr.) in the absence or presence of the opioid antagonist, naloxone (100 uM). The filters were then washed with ice cold buffer, dried briefly, and placed under X-ray film. Clear signals emanating from as few as 10,000 opioid receptor-expressing N4TG1 cells are observed; very little signal is observed for L cells or for N4TG1 cells whose receptor sites are blocked with naloxone. The aberrant signal emanating from 10,000 N4TG1 cells in the presence of blocker is not reproducible and is believed to be an artifact.

can be easily represented by approximately 3,000 independent transfectant colonies (Kuhn et al. 1984). We have prepared several transfection libraries and screened these libraries with (3-[^{125}I]iodotyrosyl^{27}) **ß-**endorphin using the detection technique described in the previous section. Thus far, we have been unsuccessful in detecting opioid receptor-positive clones. Our results with the opioid receptor-rich N4TG1 cell line indicate that an opioid receptor-bearing transfectant colony (1,000-5,000 cells per colony) can be detected if the receptor gene is expressed at a level of at least 10,000 receptors per cell. If the human opioid receptor promoter is weak in the heterologous mouse cell system, our screening assay may not be sensitive enough to detect transfectants expressing receptor at low levels.

CLONING THE OPIOID RECEPTOR BY THE HOMOLOGY APPROACH

An emerging concept in the field of molecular neurobiology is that transmembrane signalling neuroreceptors involve three distinct components. First, there is a specific receptor exposed on the external surface of cell membranes that recognizes and interacts with ligands, such as hormones or drugs, or responds to a sensory stimulus such as light. Second, exposed, at the cytoplasmic surface are effector enzymes such as adenylate cyclase that either generate the second messenger cAMP or in some other way effect an intracellular response. Third, interposed both functionally and physically between the receptor and its effectors are transducing or coupling proteins that bind GTP (so-called G-proteins). The available evidence suggests that signalling systems of this type demonstrate a high degree of structural, functional, and regulatory homology (Dolhman et al. 1987).

To date, seven pharmacologically or functionally related G-protein receptors have been cloned and their primary sequences shown to be similar. These are the ß-adrenergic receptor from human (Kobilka et al. 1987), hamster (Dixon et al. 1986), and turkey (Yarden et al. 1986), the muscarinic acetylcholine receptor from porcine brain and heart (Kubo et al. 1986a, b), the human and bovine opsin protein (Nathans and Hogness 1984, 1983), as well as the visual pigments from <u>drosophila</u> (Zuker et al. 1985), the human mas-oncogene (Young et al. 1986), and the yeast mating factor receptor (Burkholder and Hartwell 1985). All these proteins display a certain degree of sequence similarity at the amino acid level, display similar hydropathic profiles, and have conserved functionally important amino acids or glycosylation sites. The opioid receptor interacts with the same regulatory G-proteins as these other receptors. It is therefore postulated that the opioid receptor will possess an amino acid structure similar to the above mentioned G-protein related receptors. This postulated relationship is the premise upon which the approach to cloning the opioid receptor by homology is based.

Probe Design

The most often used probes are full-length double-stranded cDNAs, short synthetic oligonucleotides, or mRNA riboprobes that are labeled with ^{32}P, ^{35}S, or biotin. The selection of the probe will depend on the screening strategy. Full-length cDNAs for a related receptor or short oligonucleotides for a consensus region are the most appropriate probes for the screening of recombinant DNA libraries. We shall focus our discussion on these methods. If two nucleic acid sequences are related, the use of a cDNA probe complementary to one should identify the other if the probe is labeled by nick-translation and the regions of similarity are a significant fraction of the nick-translated lengths.

An alternative to using double-stranded cDNAs is to use single-stranded synthetic oligonucleotides, which are complementary to putative consensus regions of the receptor. Oligomers, which typically can range from 14 to 60 or more bases, are easily end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Since the probe is labeled at either its free 5'-phosphate or hydroxyl group, only one radiophosphate is incorporated per strand. Therefore, this type of probe has a lower specific activity than nick-translated cDNAs.

Screening of the Library

Screening of recombinant libraries involves the hybridization of radioactive probes to filter bound nucleic acid targets that constitute the elements of the library. Duplex formation in terms of both the number of positives and the intensity of signals is a function of both the stability of the duplex formed and the rate of hybridization. Nucleic acid hybridization depends on the random collision of two complementary sequences. The time course of the reaction is thus determined by the concentration of the reassociating species and by a second-order rate equation. While the quantitative hybridization of two perfectly matched sequences in solution can be rigorously described mathematically, the hybridization of a nucleic acid strand in solution to a filter-immobilized complementary sequence can only be qualitatively approximated.

An important consideration when identifying new proteins by cross-hybridization involves the discrimination of homologous from heterologous matches. When probing with both single-strand oligomers and denatured double-strand cDNAs, the ratio of the extent of hybridization of homologous sequences to heterologous sequences is not constant but varies with time. Hybridization to similar sequences is maximal early in the reaction and decreases with increasing time of incubation. Therefore, although the homologous reaction is faster and will reach completion earlier, the heterologous reaction will eventually catch up. The discrimination of related sequences is therefore maximal early in the reaction out deteriorates very quickly. Thus, for most cases, maximal discrimination between homologous and heterologous

sequences is best accomplished by keeping the incubation time short.

Nucleic acids hybridize very slowly at low ionic strength. As the ionic strength of the hybridization solution increases, so does the rate of duplex formation. This effect is most dramatic at low salt concentrations (0.15 M Na^+), but remains marked up to 1.5 M Na^+. High salt concentration stabilizes mismatched duplexes; so to detect cross-hybridizing species, the salt concentration of both the hybridization and wash solution must be kept fairly high, generally at 2-6xSSC (1xSSC is 0.15M NaCl, 0.015 NaCitrate, pH 7.0).

The stability of a nucleic acid duplex can be described by its melting temperature, T_m. In general, the rate of reassociation as well as the stability of the duplex is maximal at 20-30°C below the T_m. The optimal temperature for nucleic acid reassociation in aqueous salt solution lies in the range from 60-75°C. However, extended incubation at such temperatures can lead to a considerable amount of thermal strand scission. Hence, it is desirable to reduce the temperature while maintaining the stringency of the nucleic acid interaction. The effective incubation temperature can be lowered by including formamide in the hybridization solution. Formamide acts to destabilize hydrogen-bonding between double-strand nucleic acids (McConoughy et al. 1969). In addition to affecting the T_m, formamide also affects the rates of hybridization. This effect is minimal at formamide concentrations of 30-50 percent; however at concentrations of 20 percent formamide, the rate is decreased by one-third, while at 80 percent the rate can drop as much as threefold for DNA-DNA and as much as twelvefold for DNA-RNA duplexes. Thus, in practice, the inclusion of formamide in the hybridization solution can be used to alter the stringency of the incubation by holding the incubation temperature constant and varying the formamide concentration. The effective incubation temperature can be reduced to as much as 50°C below the T_m for perfect matches. The effects of temperature, base composition, and formamide can be approximated by an equation that estimates the T_m of the duplex;

$$T_m = 69.3 + 0.41(G+C) \text{ percent} - 650/L$$

where (G+C) percent is the percentage of G and C residues in the duplex and L is the average length of the probe (Marmur and Doty 1962; Wetmur and Davidson 1968). Furthermore, taking into consideration possible mismatches, the T_m of the duplex decreases 1°C with every 1 percent increase in the number of mismatches (Bonner et al. 1973). and the T_m at differing ionic strengths (ul and u2) can be related by $(T_m)_{u2} - (T_m)_{u1} = 18.5 \log(u2/u1)$ (Dove and Davidson 1962).

After hybridization, washing is carried out to remove unhybridized probe and to dissociate unstable hybrids. The temperature and salt concentration of the washing solution determine which hybrids will be detected. In general, washing should be done under stringent conditions: at 5-20°C below the

T_m for a well-matched hybrids (65-70°C), and at 12-2O°C below the T_m for cross-reacting species (50-60°C). No absolute rule exists for the duration of the washing period. In general, several short (5-10 minutes) nonstringent washes are conducted, prior to the final stringent washes (15-30 minutes). During the washes, filter-bound background radioactivity can be monitored with the use of a hand-held monitor.

In principle, this strategy can lead to the molecular cloning of any G-protein related receptor. Since the extent of the similarity between opioid receptor sequences and other G-protein related sequences can only be approximated, the successful use of this strategy in the cloning of the opioid receptor will require the careful determination of the appropriate hybridization conditions. This determination, although based on theoretical considerations discussed above, will be primarily empirical. The molecular clone obtained by the homology approach will then be expressed in eukaryotic cells to confirm the expression of authentic opioid receptors.

CONCLUSION

Three different strategies can be employed in the molecular cloning of the opioid receptor. The first strategy consists of purifying and sequencing the opioid receptor and utilizing the amino acid sequence in the isolation of a cDNA clone. This traditional cloning approach has proven to be difficult in the cloning of the opioid receptor because of the receptor's low abundance and high sensitivity to denaturation. The second strategy proposes to clone the opioid receptor through gene expression in eukaryotic cells without prior purification of the receptor protein. The cloning of the opioid receptor by this approach has remained elusive. The third strategy utilizes the possible homology between opioid receptors and other G-protein neuroreceptors as a hybridization tool in identifying potential opioid receptor clones. This approach, while extremely attractive in light of the growing number of G-protein related receptors that have found to be homologous, requires the analyses of many full-length cDNA clones. Regardless of the approach used in the cloning of the opioid receptor, authenticity must be verified by careful examination of the clone's structural and pharmacological properties.

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Effects of Opioid Peptides on Human Neuroblastoma Cells

Wolfgang Sadee, Dr. rer. nat.; Victor C. Yu, Ph.D.; and Günther Hochhaus, Ph.D.

The discovery of multiple opioid receptor types (e.g., μ, δ, κ) makes it necessary to study the molecular mechanisms of opioid action in transformed cell lines that yield homogenous cell populations under well-defined conditions in vitro. Much has been learned from the use of such cell lines, which include the mouse neuroblastoma x rat glioma hybrid NG 108-15 (Gilbert and Richelson 1983). However, this line, as well as other neuroblastomas tested, expresses only the δ receptor. Therefore, we have screened a series of human neuroblastoma cell lines for the presence of different opioid receptor types. Of three lines with 10,000 opioid sites or more per cell, all displayed s receptor sites (Hochhaus et al. 1986); however, one of these human neuroblastomas also expressed abundant μ. receptor sites (SK-N-SH, ~ 50,000 sites per cell) in a ratio of μ/δ = 4.5 (Yu et al. 1986). This cell line has potential as an excellent in vitro model for studying the effects of opiates and opioid peptides. However, in this cell line we found only a 20 percent inhibition of PGE_1-stimulated adenylate cyclase activity following treatment with opioid agonists (Yu et al. 1986). An additional problem is that there are several interconverting phenotypes of SK-N-SH, which include both a strongly substrate adherent nonneuronal form and a neuroblast form that grows more slowly to higher saturation cell densities. Only the latter appears to express opioid receptors.

AN IN VITRO MODEL TO STUDY OPIOID EFFECT

In order to provide more reproducible cell culture conditions, we selected a phenotypically stable neuroblast subclone of SK-N-SH, designated SH-SY5Y (Ross and Biedler 1985). The SH-SY5Y clone carries a similar number of opioid receptors as the parent line, yet opioid inhibition of adenylate cyclase remains slight.

In order to increase the opioid response, we tested the effects of neuronal differentiation on receptor-adenylate cyclase coupling. Three differentiating agents, retinoic acid, nerve growth factor, and dibutyryl

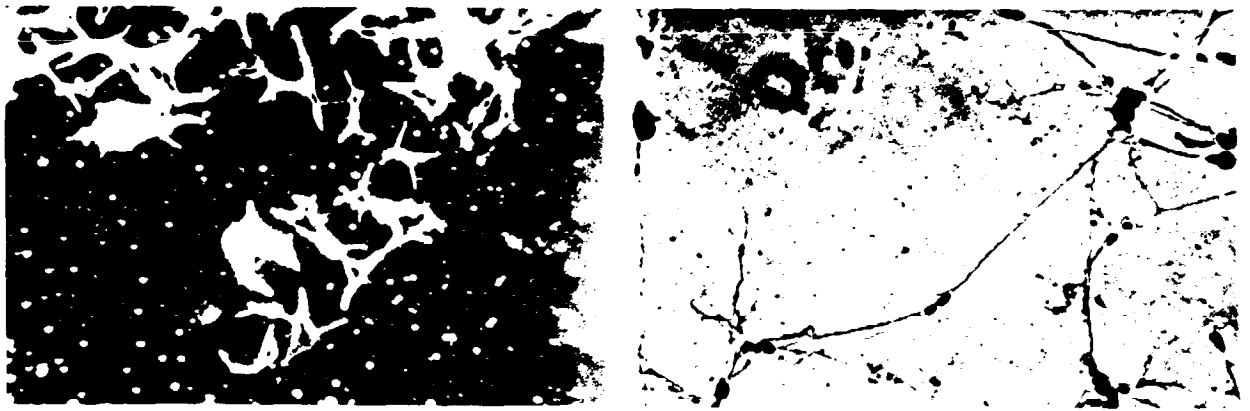

FIGURE 1. Human neuroblastoma cells, clone SH-SY5Y, grown in culture in either the absence (left) or the presence (right) of 10 uM retinoic acid.

cAMP, caused neurite extensions that are characteristic of neuronal maturation (fig. 1). Of these three, only retinoic acid (10 μM maintained over 5-6 days) produced differentiated cells that yield a 10- to 50-fold increase in PGE_1-stimulated adenylate cyclase activity. Moreover, the ability of opioids to inhibit the PGE_1 response was also enhanced by retinoic acid (45 percent inhibition), while nerve growth factor was less effective (- 35 percent inhibition). We also tested the ability of opioids to inhibit stimulation of adenylate cyclase by forskolin (100 μM), which directly acts on the enzyme rather than a stimulatory receptor. Opioids were even more effective in inhibiting the forskolin response (65 percent inhibition in retinoic acid treated cells). This opioid effect was inhibited by naloxone and pretreatment with pertussis toxin, suggesting the involvement of opioid receptors via an N_i coupling protein (fig. 2). Hence, the SH-SY5Y subclone, when differentiated with retinoic acid, represents a system for quantitative studies of u and possibly δ receptor mechanisms that include tolerance and opioid dependence.

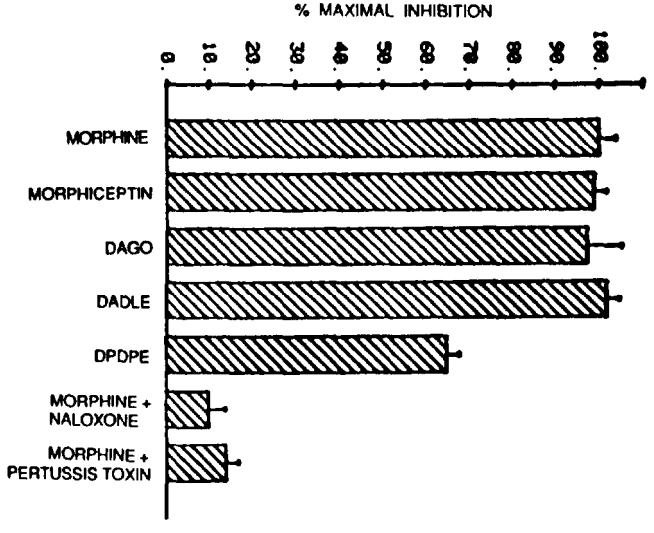

FIGURE 2. Inhibition of stimulated cAMP production in intact cells by opioids. The morphine response was defined as maximum inhibition (i.e., 100 percent). Both PGE_1 (1 μM) and forskolin (100 μM) were employed as the stimulating agents.

EFFECT OF μ AND δ RECEPTOR SELECTIVE OPIOID PEPTIDES

In order to determine which opioid receptor type is responsible for inhibition of the PGE_1- or forskolin–cAMP response, we tested a series of opioids for their efficacy. Both morphine and the potent general agonist etorphine gave similar maximal responses (45 percent of the PGE_1 response and 65 percent of the forskolin response). Since these agonists are not highly selective for either μ or δ sites, we then chose several opioid peptides of high selectivity (fig. 2). It should be noted that the efficacy of any of these ligands at the u sites has not been previously established. Both the μ specific agonist morphiceptin and the highly μ selective agonist DAGO gave the same maximal inhibitory effects as morphine and etorphine. This result strongly suggests that these peptides are full agonists and that the inhibitory response is mediated by the μ sites. Indeed, we confirmed earlier findings that morphiceptin is inactive at the s sites in NG 108-15 cells. In contrast, the potent and highly δ selective DPDPE gave only a small partial response at 30 nM, at which concentration one might expect near maximal activation of δ sites. Maximal response of DPDPE at 10 uM was only ~ 65 percent of that obtained by the other agonists, and it is likely that this partial response is largely mediated by cross-reaction with the u sites. The μ and δ agonist DADLE, on the other hand, gave full inhibitory effects at rather low concentrations (< 1 μM). These results support the view that the opioid receptor inhibition of adenylate cyclase in SH-SY5Y cells is largely mediated by the μ site. Therefore, this subclone is ideally suited for studying the efficacy, tolerance, and dependence of the narcotic analgesics in vitro:

It should be noted that all of the above results were obtained in the presence of the phosphodiesterase inhibitor IBMX. Therefore, the conclusions from these studies are limited to the activity of adenylate cyclase. However, initial experiments in the absence of IBMX suggest that the opioid agonists may also increase the activity of phosphodiesterase. This would indicate a second mechanism by which cellular cAMP levels could be regulated. We are currently investigating this possibility.

BIOTINYLATED β–ENDORPHIN

A better understanding of the molecular events involved in the opioid response in neuroblastoma cells can be attained through the study of opioid receptors on single cells. Such studies would clarify both the distribution of opioid receptors throughout a cell population and their location on the cell. A method for rapid isolation and quantitation of receptors is desirable. Therefore, we intend to utilize the avidin-biotin system for opioid receptor analysis (Korpela 1984). Typically, a suitable ligand is labelled with biotin and allowed to bind to the receptor site. Next, the biotinyl-tracer receptor complex is incubated with avidin.

Avidin has an extremely high binding affinity for biotin ($K_d \cong 10^{-15}$M). The avidin molecule is then labelled with fluorescent dyes, colloidal gold, or enzymes. It is important to employ a biotinylated tracer that retains high receptor affinity in the presence of avidin. β_h–(1-31)-endorphin reacting with biotinyl N-hydroxysuccinamide, with or without an intervening ε-aminocaproyl spacer arm, yields a series of biotinylated products with one or more substitutions per molecule. Tyr-1 and Lys 9, 19, 24, 28, 29 represent possible reaction sites, as shown in figure 3.

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-**Lys**-Ser-
1 10

Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe- **Lys**-Asn-
11 20

Ala-Ile-Ile-**Lys**-Asn-Ala-Tyr-**Lys**-**Lys**-Gly-Glu
21 30

FIGURE 3. Human β–endorphin.

These derivatives were purified by HPLC and characterized by FAB-mass spectrometry and trypsin digestion followed by HPLC (fig. 4) and again FAB-MS. Biotinylation of a lysine residue eliminates one tryptic cleavage site, which allows one to determine the site of biotinylation. The Tyr-1 terminal NH_2 group was not affected in any of the products isolated, perhaps because, in the tertiary structure, it could have an internal location and/or it could be involved in hydrogen binding. Monobiotinylated fractions with substitution at either Lys-9 (fig. 4), Lys-19, or Lys-24,28,29 (Bx1α) were analyzed for binding to the μ and δ opioid receptors in rat brain homogenates and neuroblastomas. All derivatives retained significant opioid receptor affinity, even in the presence of avidin. The highest affinity among the derivatives was found with Bx1α, which was equally potent to native β_h–endorphin, even in the presence of avidin (fig. 5). Therefore, this derivative fulfills all criteria for application of the biotin-avidin system to the analysis of the opioid receptor.

The biotinylated β_h–endorphin derivative Bx1α also displayed high affinity to a β_h–endorphin specific antibody (Peninsula Labs). We therefore developed an ELISA method for the quantitation of β_h–endorphin, using 21Bx1α as the tracer and an avidin-alkaline phosphatase complex as the enzyme indicator. Optimal incubation conditions yielded a sensitivity of 0.5 femtomole ßh-endorphin per sample, equivalent to the most sensitive ^{125}I-RIA currently available. Current applications of the biotin-avidin system to opioid molecular pharmacology are in progress.

Replica Transfer of Transfectant Colonies

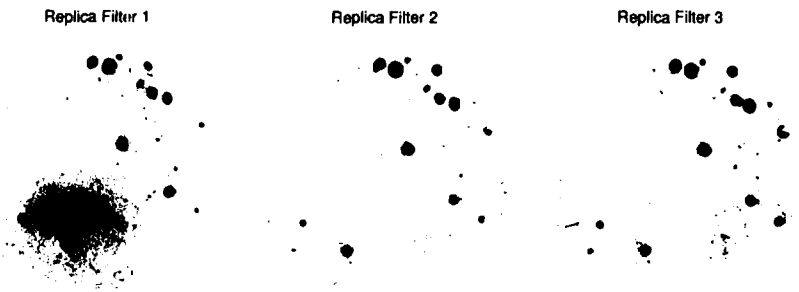

FIGURE 4. HPLC records (reverse phase) of tryptic digests of β_h–endorphin and Bx1β with biotinylation predominantly at Lys-19.

Screening Eukaryotic Expression Libraries For Neuropeptide Receptor Genes Using Novel In Situ Replica Filter Binding Assays

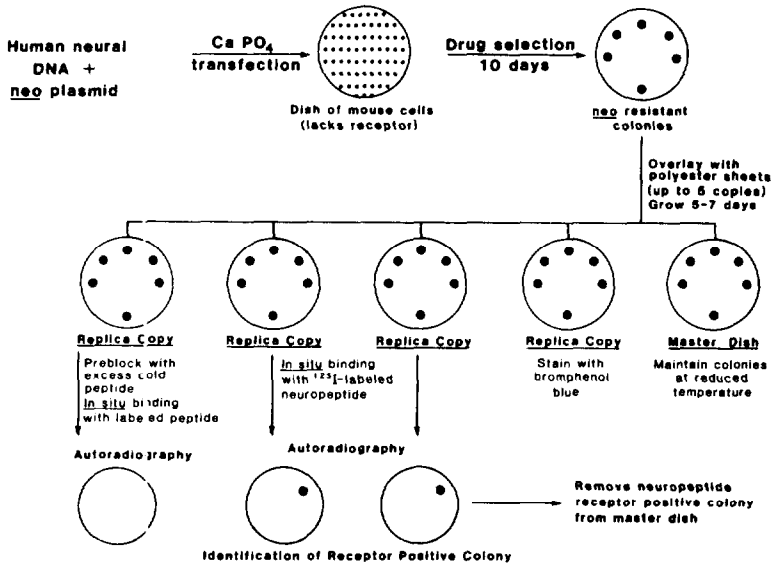

FIGURE 5. Equilibrium binding competition curves between ^{3}H-DAGO (1 rM) and β_h–endorphin or Bx1α in rat brain homogenates in the presence of avidin.

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Analgesia and Neuropeptides

David J. Mayer, Ph.D.

INTRODUCTION

Until quite recently, virtually nothing was known about the neurochemical basis of pain. The past few years have seen an unprecedented explosion of information in the neurosciences, neurochemistry the role of peptides in behavior, and, most important here, the neurobiology of pain and pain modulation.

As statistics about the epidemiology of pain have surfaced, the biomedical importance of pain research has become apparent. Thus pain research in general and research on the involvement of opioid and other eptides in pain in particular have undergone an impressive expansion in the past decade. This chapter will review the extensive literature on the mechanisms of pain modulation by opioid and other neuropeptides.

Commensurate with this increase in knowledge about endogenous peptide involvement in pain modulation has come a search for the environmental stimuli that might normally activate these systems. Much interest has focused on the role of painful and stressful environmental events. This chapter will examine the mvolvement of these types of events in the activation of endogenous peptide pain modulatory systems.

THE ROLE OF OPIATES AND OPIOID PEPTIDES IN PAIN MODULATION

The discovery of the enkephalins (Hughes 1975) and the subsequent discovery of related opioid peptides has led to a great interest in the role of these substances in pain modulation. This research has elucidated an important role for these substances in pain modulation. This section will review both animal and human studies of the role of endogenous opioids in pain modulation.

Historical Perspective

A number of critical discoveries about the neurobiology of opiate action have occurred in the past few years: (1) The demonstration of stereospecific

and saturable binding sites for opiates in the central nervous system (Hiller *et al.* 1973; Pert and Snyder 1973; Terenius 1973). (2) The associated demonstration for multiple binding sites [See Martin, (Martin 1983) For an excellent review on this topic). (3) The discovery of endogenous ligands for opiate receptors (Hughes 1975). (4) The concept of using opiate antagonists to antagonize endogenous behavior repertoirses (Akil *et al.* 1972). Things findings, along with the extensive utilization of the intracerebal microinjection technique to localize site of action of neurochemicals within the central nervous system (Tsou and Jan 1964), have, in turn, had an important impact on theoretical and methodological research strategies utilized for the study of the effects of opiates on behavior.

Methodological Considerations

Before these discoveries the study of the analgesic effect of opiates (as well as other peptides) was primarily phenomenological. The analgestic effects of opiates were catalogued and seen in the light of designing drugs with more therapeutically desirable and fewer therapeutically undesirable effects for application to clinical pathology. Although clinical utility certainly remains an important concern of opiate research, the discoveries described above have resulted in research that is conceptually broader. The current overall view is that opiates often act on endogenous biological substrates for behaviors. Hence the behavioral pharmacology of opiates now addresses the organization of the neurobiological substrates of behavior. Much of the research in this area now proceeds in the following pattern: (1) Can a behavior elicited by opiates be elicited by exogenous environmental manipulations? For example, can analgesia be elicited by transcutaneous nerve stimulation? (2) If so, can the behavior, when initiated by nonpharmacological means, be shown to utilize endogenous opiates? This question is generally approached by examining wheth er the behavior can be antagonized by opiate antagonists and reduce by the induction of tolerance to opiates (that is, does cross-tolerance occur?). Additional support for a role of endogenous opiates is provided by the demonstration of a correlation between release of endogenous opiates and the occurrence of the behavior under study. (3) If the behavior is shown to involve endogenous opiates, the precise neuroanatomical foci underlying the behavior are examined utilizing the microinjection of opiate agonists and antagonists into restricted central nervous system loci. (4) The opiate receptor type involved in the behavior is examined by administering opiate agonists and antagonists at least partially selective for particular receptor subtypes (mu, kappa, delta, etc.). (5) Ideally, steps (3) and (4) are combined to determme the anatomical locus of specific receptor types mvolved in the behavior being studied. (6) An attempt is made to determine the precise endogenous ligand (beta-endorphin, Met-enkephalin, dynorphin, etc.) involved in a particular behavior. Again an attempt is made to determine the anatomical locus of action of the particular ligand.

This is, of course, an idealized outline of the path followed to investigate opioid involvement in a behavior. More often than not the actual course of research is less direct than this, and the final conclusions are not usually simple. Nevertheless, keeping in mind the general approach described above will aid the reader to see the overall progression of the field.

Until the early 1970s, in spite of evidence to the contrary (Tsou and Jang

1964; Dewey *et al.* 1969), the generally accepted theory of opiate analgesic action (Lim 1966) considered opiates to produce analgesia by a central nervous system mechanism analogous to the action of local anesthetics on a peripheral nerve. That is, it was thought that opiates acted by directly inactivating the afferent transmission of pain in the central nervous system. Since then, a major advance in our conception of the neural processing of pain has occured. It has become clear that information about tissue damage is not passively recieved by the nervous system. Rather, it is filtered, even at the first synopse, by complex modulatory systems. The discovery of these central nervous system contains endogenous substances, endorphins, that possess analgesic properties virtually identical to opiates of plant and synthetic origin. In this section, the development of these concepts is examined. The existence of opiate and nonopiate central nervous system pain modulatory mechanisms activated by environmental stimuli such as stress is then discussed.

Development of the Concept of Endogenous Pain Control

It has long been recognized that a simple invariant relationship between stimulus intensity and the magnitude of pain perception is often not present. Two general classes of observations support the complexity of this relationship. The first is the clinical observation that pain is often present without any apparent precipitating pathology. This situation represents the clinical problem of pain treatment. More important for the topic of this section is the common observation that pain may not be experienced in the presence of factors that should produce it; that is, under a variety of circumstances, total or partial analgesia is seen. These observations were explicitly recognized in earlier models of pain perception in spite of the lack of direct evidence supporting the theoretical models (Noordenbos 1959; Melzack and Wall 1965). Thus, the concept that the nervous system possesses intrinsic pain inhibitory mechanisms was recognized when only indirect evidence was available.

The earliest work indicating opiates produces analgesia, at least in part, by activation of endogenous pain inhibitory systems was done by Irwin *et al.* (1951). They demonstrated that morphine was not effective in inhibiting the spinally mediated tail flick response in spinalized rats. They reasoned based on this result that morphine must activate supraspinal neural circuitry which has an output to the spinal cord and modulates the processing of nociceptive information at spinal level. This work was largely ignored until the early 1970's even though it was replicated in the mouse (Dewey *et al.* 1969).

The first impetus for the detailed study of pain-modulatory circuitry resulted from the observation that electrical stimulation of the brain could powerfully suppress the perception of pain (Reynolds 1969; Mayer et al. 1971 Further investigation of stimulation-produced anal esia provided considerable detail about the neural circuitry Involved [see ayer and Watkins (1984) for a detailed review of this topic].

Significantly, at that time, several similarities were recognized between these observations and information emerging from a concomitant resurgence of interest in the mechanisms of opiate analgesia (Mayer et al. 1971 The most important parallel facts revealed by these studies were the fo lowing: (1)

Effective loci for both opiate microinjection analgesia (Tsou and Jang 1964) and stimulation-produced analgesia (Mayer et al. 1971) lie within the periaqueductal and periventricular gray matter of the brain stem. (2) Opiate analgesia and stimulation-produce analgesia are both mediated in part by the activation of a centrifugal control system that exits from the brain and modulates pain transmission at the level of the spinal cord (Dewey *et al.* 1969; Irwin *et al.* 1951). (3) The ultimate inhibition of the transmission of nociceptive information occurs, at least in part, at the initial processing stages in the spinal cord dorsal horn and homologous trigeminal nucleus caudalis by selective inhibition of nociceptive neurons (Satoh and Takagi 1971).

In addition to these correlative observations, studies of stimulation-produced analgesia provided direct evidence indicating that there were mechanisms in the central nervous system that depend upon endogenous opiates: (1) Subanalgesic doses of morphine were shown to synergize with subanalgesic levels of brain stimulation to produce behavioral analgesia (Samanin and Valzelli 1971). (2) Tolerance, a phenomenon invariably associated with repeated administration of opiates, was observed to the analgesic effects of brain stimulation (Mayer and Hayes 1975). (3) Cross-tolerance between the analgesic effects of brain stimulation and opiates was demonstrated (Mayer and Hayes 1975). (4) Stimulation-produce (analgesia could be antagonized by naloxone, a specific narcotic antagonist (Akil *et al.* 1972, 1976a). This last observation, in particular, could be most parsimoniously explained if electrical stimulation resulted in the release of an endogenous opiate-like factor. Indeed, naloxone antagonism of stimulation-produced analgesia was a critical impetus leading to the eventual discovery of such a factor (Hughes 1975).

Coincidental with work on stimulation-produced analgesia, another discovery of critical importance for our current concepts of en ogenous analgesia systems was made. Several laboratories, almost simultaneously, reported the existence of stereospecific binding sites for opiates in the central nervous system (Hiller *et al.* 1973; Pert and Snyder 973; Terenius 1973. These "receptor" sites were subsequently shown to be localized to neuronal synaptic regions (Pert *et al.* 1974) and to overlap anatomically with loci involved in the neural processing of pain (Pert *et al.* 1975). The existence of an opiate receptor again suggested the likelihood of an endogenous compound with opiate properties to occupy it.

In 1974, Hughes (1975) and Kosterlitz reported the isolation from neural tissue of a factor enkephalin) with such properties. An immense amount of subsequent work has characterized this an other neural and extraneural compounds with opiate properties. As with the opiate receptor, the anatomical distribution of endogenous opiate ligands shows overlap with sites involved in pain processing [see Akil *et al.* (1984) for a recent review of these studies].

To summarize these important historical developments, the existence of an endogenous opiate analgesia system is suggested by several lines of evidence. Electrical stimulation of the brain produces analgesia The anatomical structures and neural mechanisms involved in stimulation-produced analgesia parallel those involved in opiate analgesia. The central nervous system contains opiate binding sites and endogenous ligands capable of interacting with those sites. These binding sites and ligands are found

at anatomical loci consistent with sites at which stimulation-produced analgesia and opiate microinjection analgesia are elicited.

Neural Circuitry Involved in Analgesia Resulting from the Administration of Exogenous opiates

This section will review the current data available on the sites and mechanisms involved in the modulation of pain by the administration of exogenous opiates. Primarily, two lines of experimentation will be examined: (1) The locations in the central nervous system of sites at which administration of opiates results in analgesia and the administration of opiate antagonists blocks analgesia. (2) The locations in the nervous system where lesions block the action of exogenously administered opiates.

Following the work of Tsou and Jang (1964), it wasn't until the early 1970's, with one exception (Lotti *et al.* 1965), that opiate microin'ection mapping studies began. Initially these studies concentrated on the periaueductal- eriventricular regions of the mesencephalon and diencephalon (Jacquet and Laitha 1973; Pert and Yaksh 1974; Yaksh *et al.* 1976). Overall, these and other studies confirmed the importance of the periaqueductal-periventricular region in opiate analgesia and provided an impetus for the examination of other brain areas.

A second brain area that has proved to be of considerable importance for opiate action is the anatormcally complex re ion of the ventromedial medulla. This region consists of at least three distinct nuclei: the medially located nucleus raphe magmus (NRM), more laterally situated nucleus reticularis paragigantocelluaris (NRP), and the dorsolaterall located nucleus reticularis gigantocellularis (NRG). Based on retrograde labeling criteria, Watkins *et al.* (1980) have proposed the term nucleus raphe alatus (NRA) for the combined cell groups in NRM and NRP. Takagi *et al.* 1976) were the first group to map this region for analgesia resulting from morphine microinjection. Overall (Takagi 1980), this group found the NRP to be approximately 20 times more sensitive to mo hine than the NRG. They found microinjection of morphine into the NRM to be ineffective in the production of analgesia. This point is controversial, since other groups Azami *et al.* 1982; Zorman *et al.* 1982) have reported analgesia from microinjection into NRM. Axami *et al.* (1982) did f!ind, however, that the NRM was less sensitive than the NRPG.

A number of other brain areas including the amygdala (Rodgers 1977, 1978), the medial lemniscus (VanRee 1977, nucleus medialis dorsalis of the thalamus (VanRee 1977), the mesence halic reticular formation (Haigler and Mittleman 1978; Pert and Yaksh 1974), and the nucleus of the solitary tract (Oley *et al.* 1982) have been reported to produce analgesia when injected with opiates. However, the work on these areas is scant compared with those discussed above, and the relative potency of injections into these areas has not been explored.

A final but crucial point to be made in this section concerns the analgesic effects of microinjections of opiates directly into the intrathecal space of the spinal cord. Although Tsou and Jang (1964) reported no analgesia from Direct spinal application of morphine subsequent work has consistently demonstrated relatively potent effects of intrathecal morphine microinjection

(Yaksh and Rudy 1976, 1977).

From this line of evidence it appears, then, that at least three general areas of the central nervous system are involved in opiate analgesia: the periaqueductal-periventricularray matter, the ventromedial medulla, and the spinal cord. This observation indicates that the analgesic effects of a systemitally administered opiate may produce analgesia by acting at any, all, or some combination of these distinct regions.

The utilization of the microinjection of narcotic antagonists has provided at least a partial answer to this question. A number of early studies concluded that supraspinal sites of opiate action are the effective ones, since analgesia from systemically administered opiates was antagonized by either intracranioventricular or intracerebral microinjection of narcotic antagonists Albus *et al* 1970; Jacquet and Lajtha 1974; Tsou, 1963; Vigouret *et al* 1973). Later work, however, demonstrated that naloxone administered intrathecally could antagonize the analgesia resulting from even relatively high doses of systemically administered opiates (Yaksh and Rudy 1977). Thus, these stud ies lead to the paradoxical conclusion that both supraspinal sites and spinal sites of opiate action are the critical ones involved in analgesia.

This seeming paradox was resolved in a series of complex but unusually important studies by Yeung and Rudy (1980a,b). They demonstrated that by simultaneously administering various doses of morphine intrathecally (into the spinal cord) and intraventricularly (into the brain) a multiplicative dose response function was observed. That is, simultaneous spinal and supraspinal morphine resulted in greater analgesia than the same total dose administered at either location alone. The effect is quite large, with the multiplicative factor being as much as 45 under certain circumstances (Yeung and Rudy 1980a). This type of multiplicative interaction, as will be seen below, may turn out to be a confounding factor in many pharmacological and physiological analyses of the involvement of opiates in various behaviors. Thus, the reader should keep in mind, when evaluating the literature on this and other topics, the potential for complex interactions between the same or various neurotransmitters and/or neuromodulators.

Although the experiments just described elucidate the contribution of spinal vs. supraspinal sites to opiate analgesia, the relative contribution of the various supraspinal sites at which opiates act to produce analgesia is Iess clear. The only work to examine this issue utilizing microinjection of narcotic antagonists was done by Azami *et al.* (1982). They found, as did the work of Takagi's group, that the NRP was more sensitive to morphine than the NRM for the elicitation of analgesia. Surprisingly, however, they found, when analgesia was produced by systemically administered morphine, naloxone injection into the NRM antagonized anal esia more effectively than injection into more lateral medullary regions including the NRP. They concluded that NRP does not make a significant contribution to the analgesia resulting from systemic administration of morphine. The relative contribution of medullary vs. more rostral mesencephalic sites has not been examined.

An approach similar to the one just described for dissectin the neural circuitry participating in opiate analgesia utilizes the selective destruction of

nuclei and pathways suspected of being involved in opiate analgesia. Opiates are administered systemically or at discrete sites in the nervous system and the effects of particular lessons are examined. An overview of this work supports the conclusion reached above utilizing injection of antagonists. It appears that several brain areas including the periaqueductal gray matter, NRM, and NRP need to be intact for the full expression of opiate analgesia.

Environmental Activation of Endogenous Analgesia Systems

The demonstration that opiates activate well-defined neural systems capable of potently blocking pain transmission suggests, but by no means proves, that the function of this stem is to dynamically modulate the perceived intensity of noxious stimuli. If, in fact, this system as such a physiological role, then one might expect that the level of activity within the system would be influenced by impinging environmental stimuli. If environmental situations that produce analgesia could be identified, it would give credibility to the idea that invasive procedures, such as bram stimulation or narcotic drugs, inhibit pain by mimicking the natural activity within these pathways.

TABLE 1

	SYSTEMIC OR IT NALOXONE	DYNORPHIN ANTISERUM	ENKEPHALIN ANTISERUM	CROSS TOLERANCE	CNS ENDORPHIN LEVEL
BRIEF FPFS	15,65,73,10,14 75,80			80	
CCA	37,39,81			81	11
ECS	23,30,50 24,78,79			24,78 79	
DEFEAT	59,60,76			59 18	59
ACUPUNCTURE (LO FREQ)	12,13,17 35,56,86	33	34	33	59
PROLONGED 4PFS	10,16,19,32,62 75,78,82,87				66,67 6,51
IMMOBILIZATION	31,44			31	
15 MIN COLD	69			69	
EXERCISE	43,72				
COND HELPLESS	18,42,53			18	42
PROLONGED TS	71				
SPA (PAG)	1-3,9,45,46,58 70			18,52 54,55	40,41,2,4 64
SPA (NRM)	46,77,89				
KINDLED SEIZURE	22				
TNS (LO FR/HIGH INT)	21,63,83				
VAGINAL PROBING	38				
DNIC	48				
FOOD DEP	57				
PLACEBO	29				
HYPERTENSION	88				
ANXIETY (HUMAN	84,85				
SEX (MALE)					74
PREGNANCY	25				
SPON HYPERTENS	68				
INTER COLD WATER	26,27			26	
20° C SWIM	36				
COLD WATER	7,8,27,61				
WARM WATER(32° C)	61				
HEAT (20° C)	49				
RADIATION	37,77				
HYPERALGESIA	5,16,47				
MILD FOOTSHOCK	28				

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87. Woolf and Wall 1983
88. Zamir *et al.* 1980
89. Zorman *et al.* 1982

TABLE 1 - Summary of representative studies on endogenous opiate analgesia systems. Each number in each cell of the matrix indicates a study utilizing a particular opiate manipulation to implicate endogenous Opiates in various forms of environmentaliy produced analgesia. Citation numbers are matched with authors at the bottom of the table. The various opiate manipulations studied are indicated in the vertical columns and the environmental manipulations in the horizontal rows. Abbreviations: CCA = classically conditioned analgesia; COND = conditioned; CNS = central nervous system; DEP = deprivation;

DNIC = diffuse noxious inhibitory controls; ECS = electroconvulsivehock; FREQ = frequency; FPFS = front paw footshock; INT = intensity; INTER = intermittent; IT = intrathecal; LO = low; MIN = minutes; NRM = region of the nucleus raphe magnus; PAG = periaqueductal gray matter; SPA = stimulation produced analgesia,- SPON = spontaneous; TNS = transcutaneous nerve stimulation; TS = tail shock; 4PFS = four paw footshock

A systematic search for environmental stimuli that activate pain inhibitory systems was begun by Hayes *et al.* (1978ab). They observed that potent analgesia could be produced by such diverse stimuli as brief footshock, centrifugal rotation, and injection of intraperitoneal saline. These effects appeared to be specific to pain perception insofar as normal motor behavior, righting and corneal reflexes, vocalization, startle responses, and response to touch remain unimpaired (Hayes *et al.* 1978b). Two important additional concepts emerged from this work. First was the conclusion that exposure to "stress" was not sufficient to produce analgesia. Although almost all environmental stimuli studied to date that produce analgesia are stressors (cf. table 1), the failure of classical stressors, such as ether vapors and horizontal oscillation, to produce pain inhibition indicated that "stress" was not the critical variable responsible. Second was the rather unexpected finding that the opiate antagonist, naloxone, did not block all environmentally induced analgesias (Hayes *et al.* 1978b). Therefore, it appeared that non-opiate systems must exist in addition to the system activated by opiates described earlier.

Although the stimuli studied by Hayes *et al.* (1978a,b) did not appear to activate an opiate system, subsequent investigations found clues that endogenous opioids might be involved in at least some types of environmentally induced analgesias. Akil *et al.* (1976b) studied the analgesic effects of prolonged footshock. In contrast to the results of Hayes *et al.* (1978a,b), naloxone did partially antagonize the analgesia. This initial indication of opiate involvement led Akil and coworkers to look for biochemical evidence that footshock caused brain opiates to be released. They found that changes in brain opiate levels did indeed parallel the development of footshock-induced analgesia (Akil *et al.,* 1976b). When tolerance developed to the analgesic effects of footshock, brain opiate levels returned to control values.

The controversy over the involvement of opiates in footshock induced analgesia was resolved, in art, by Lewis *et al.* (1980). They noted that the duration of footshock used by Hayes *et al.* (1978b; 1978a) and Akil *et al.* (1976b differed greatly and exammed whether this variable might explain the difference in their results. By comparing the effects of naloxone on analgesia produced by brief (3 min) vs. prolonged (30 min) footshock, Lewis *et al.* (1980) showed that only the latter could be blocked by naloxone. This suggested that different analgesia systems become active as the duration of footshock increases.

Concurrent with this work of Lewis *et al.* (1980), Watkins *et al.(* 1982d) made the observation that brief shock restricted to the front paws produced analgesia that was antagonized by low doses (0.1 mg/kg) of naloxone. In contrast, even high doses (20 mg/kg) of naloxone failed to reduce analgesia produced by hind paw shock. In addition, they showed that animals made tolerant to morphine showed cross-tolerance to front paw but not hind paw

footshock analgesia. Thus it appears that front paw shock activates an endogenous opiate anal gesia system while hind paw shock activates an independent nonopiate analgesia system. In addition, this work showed again that stress is not a sufficient factor to activate opiate analgesia systems, since identical shock parameters were used for hind paw and front paw shock (Watkins *et al.* 1982d).

Additional work has revealed the following important facts about front paw and hind paw footshock-induced analgesias (FSIA): (1) Front aw FSIA is mediated by central nervous system opioids, since elimination of extraneural opiates by hypophysectomy, adrenalectomy, or sympathetic blockade does not block the effects (Watkins *et al.* 1982c). (2) Front paw FSIA involves a neural circuit that ascends to the brain and then descends by way of the dorsolateral funiculus (DLF) to block pain transmission at the spinal level (Watkins *et al.* 1982a). This descending DLF athway originates in the nucleus raphe alatus (NRA)(Watkins *et al.* 1983a). (3) The complete circuitry for the effect is caudal to the mesencephalon since decerebration does not affect the analgesia (Watkins *et al.* 1983b) (4) The critical opiate synapse for the system is situated in the spinal cord (see figure 1) at the segment of nociceptive input, since intrathecal injection of naloxone in the lumbosacral but not thoracic spinal cord blocks the effect (Watkins and Mayer 1982). (5) Once the system is activated, continued opiate release is not needed, since naloxone only blocks the effect when given before footshock but not after (Watkins and Mayer 1982). (6) The integri of spinal cord serotonin is critical for the expression of front paw FSIA (Watkins *et al.* 1984a). (7) Front paw FSIA is blocked by small systemic or intrathecal doses of the peptide CCK-8 (Faris *et al.* 1983) and potentiated by the putative CCK antagonists proglumide and benzotript (Watkins *et al.* 1984b) (see section below on CCK for a more detail discussion of this point).

Hind paw FSIA is also a CNS-mediated phenomenon (Watkins *et al.* 1982c). However, this manipulation activates both intraspinal and suraspinal pain inhibitory systems (Watkins *et al.* 1982a). The brain centers or hind paw FSIA differ from those for front paw FSIA since NRA lesions do not eliminate the analgesia Watkins *et al* 1983a . The neurochemical bases of hind paw FSIA also differ from front paw FSIA: (1) CCK, serotonin, and norepinephrine do not appear to be involved in hind paw FSIA (Watkins *et al.* 1984a). (2) Brain, but not spinal cord, acetylcholine is necessary for the expression of hind paw FSIA but does not appear to be involved in ront paw FS IA (Watkins *et al.* 1984c).

Of considerable interest is that both hind paw and front paw FSIA can be classically conditioned by repeated pairings of a CS with footshock (Watkins *et al.* 1982b). Regardless of whether hind paw or front paw shock is used as involved in front paw FSIA is activated, since conditioned analgesia is eliminated by (1) systemic and intrathecal naloxone, (2) morphine tolerance, (3) DLF lesions, and (4) NRA lesions and is unaffected by hypophysectomy, a enalectomy, and sympathetic blockade (Watkins *et al.* 1982c) . In addition, as would be expected, higher structures are involved in the conditioned analgesia since it is eliminated by decerebration and reduced by periaqueductal gray (PAG) lesions (Watkins *et al.* 1983b).

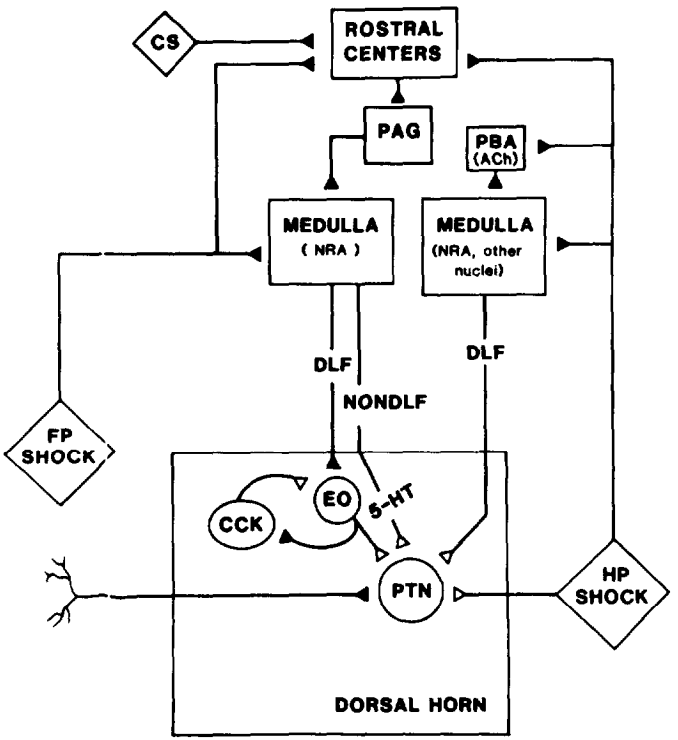

Figure 1. Neural circuitry of opiate and nonopiate analgesia induced by front paw and hind paw shock Front paw shock activates the nucleus raphe alatus (NRA) within the ventral medulla This nucleus sends descending projection through the DLF to the dorsal horn of the spinal cord A serotonergic pathway lying outside of the DLF (non-DLF) is recruited as well In turn, endogenous opioids are released, inhibiting pain transmission neurons (PTN). Activation of endogenous opiates stimulates a negative feedback loop, which utilizes CCK to reduce activity of endogenous opioid systems. Hind paw shock inhibits PTN via two nonopioid pathways: an intraspinal pathway and a descending DLF pathway. The latter originates from the NRA and from some other yet unidentified medullary area(s). Classical conditioned (opioid) analgesia seems to result from activation of the same DLF output path way as from paw (opioid) FSIA. After conditioning trials in which the conditioned stimulus is paired with either front paw or hind paw shock (the unconditioned stimulus), the conditioned stimulus becomes capable of activating rostral centers in the brain which, in turn, activate the periaqueductal gray (PAG) and subsequently the nucleus raphe alatus. This results, via a descending DLF pathway, in the release of endogenous opiods within the dorsal horn, producing analgesia.

A circuit diagram of these systems is shown in figure 1. A point that should be emphasized is that the involvement of other neurotransmitters or neuromodulators at the spinal cord level may be quite complex. For exam le, as shown in figure 1 and discussed in detail below, cholecystokinin (CCK) appears to modulate endogenous opioid systems. Intrathecal application of CCK antagonizes anagesia from application of exogenous opiates as well as analgesia elicited by activation of endogenous opiates (Faris *et al.* 1983). Also, CCK antagonists applied intrathecally potentiate these analgesias as well as reversing opiate tolerance Watkins *et al.* 1984b). These findings suggest that other transmitters and/or modulators may interact with opiates to form complex circuits.

In addition to the work done by Liebeskind's group [see Terman *et al.* (1984) for a review and our own, a number of other laboratories have now demonstrated that numerous environmental variables can be critical in determining the particular pain modulatory circuitry activated. Table 1 summarizes the environmental events now known to influence the transmission of pain utilizing endogenous opioid peptides. It is clear that numerous environmental manipulations result in modulation of pain transmission. The involvement of endogenous opioids in pain modulation is now beyond question. On the other hand, man questions remain unanswered. For example, it is generally not known where a particular endogenous opioid is released by a particular environmental manipulation nor is the endogenous ligand or the receptor type involved usually known. Nevertheless, the techniques and general strategies for answering these questions are available, and progress in this area should be forthcommg. It should also be pointed out that, in addition to systems that modulated nociceptive information utilizing endogenous opioids, there are nonopiate systems as well. For example, Maier's group has shown that inescapable tail shock results in opiate analgesia, but analgesia resulting from tail shocks with identical temporal and intensity characteristics is nonopiate if the shocks are escapable (Maier *et al.* 1982). In sum, a review of the animal data provides strong evidence for the existence of multiple pain modulatory systems. Our knowledge of endogenous opiate analgesia systems probably represents the most detailed description of any opioid behavioral system available. It is, however, less clear what the critical characteristics of the environmental stimuli activating these systems are. Although many of the events activating the systems are clearly stressful, not all are. Also, a number of known stressors are ineffective.

Evidence for Endogenous Opiate Analgesia in Man

At this point, some parallels will be made between the work described above and experimental and clinical studies in humans. This will be done in order to high ight the potential relevance of this work to the very difficult problem of treating pain syndromes in man. Throughout this discussion, it will be important to bear in mind that a number of distinct modulatory systems have been identified under controlled laboratory conditions. In the more naturalistic circumstances of clinical research, it is likely that more than one of these systems may be active at any given time, which may account for the variability and controversy in the clinical literature.

Research on the involvement of endogenous opioids in pain modulation in

man has examined a number of environmental manipulations known to have some degree of efficacy for the reduction of clinical and experimental pain. Most of these procedures were developed before the recent explosion of information about endogenous pain control systems. Indeed, many of them evolved from theoretical approaches that are now outdated or incorrect. Nevertheless, the procedures are efficacious and have inspired a considerable body of research aimed at determining the involvement of endogenous opioids in pain modulation.

There are at least two situations available for study in which endogenous pain modulatory systems may be active in man. The first involves the basal, tonic activity within these systems and allows the experimenter to assess whether pain inhibition occurs continuously, at least to some degree. The second involve:; clinical manipulations that attempt to activate pain inhibitory systems.

Attempts have been made to determine whether pain modulatory systems are tonically active. The assumption made by these studies has been that administration of opiate antagonists should alter the perception of pain if opiate systems are tonically active. This change in pain perception would be recorded either as a decreased pain threshold or an increased level of ongoing pain. In general, naloxone has failed to affect pain thresholds of normal human volunteers (Grevert *et al.* 1978; El-Sobky *et al.* 1976). On the other hand, Buchsbaum *et al.* (1977) found that naloxone lowered the thresholds of subjects with naturally high pain thresholds, yet had no effect in subjects with low pain thresholds. This observation is consistent with reports that the high pain thresholds seen in some cases of congenital insensitivity to pain can lowered by naloxone (Cesselin *et al.* 1984; Dehan *et al.* 1977; Dehen *et al.* 1978. It should be pointed out that demonstrating analgesic or hyperalgesic effects of drugs in human subjects is diffcult. These studies, taken together, suggest that endogenous opiate pain modulatory circuits may not always be tonically active in people. Such a conclusion is supported by the report that naloxone decreases the higher pain thresholds seen in man in the morning as compared with those in the afternoon (Davis *et al.* 1978).

Naloxone a pears to be more consistently effective when delivered to experimental subjects who are experiencing some level of clinical pain. In this regard, these results are consistent with the animal studies described above in which pain was observed to be a powerful activator of endogenous analgesia systems. Thus, Lasagna (1965), Levine *et al.* (1979), and Gracely *et al.* (1983) report that naloxone can increase the reported intensity of posto erative pain. It is important to note that two of these studies reported cmplex dose-response interactions between naloxone and pain. Levine *et al.* found that while naloxone produced hyperalgesia at high doses, low doses produced analgesia. The Lasagna study is somewhat more difficult to interpret because no placebo control group was included, but the results are consistent with a conclusion that naloxone has both analgesic and hyperalgesic effects. These reports, in turn, are consistent with reports in the animal literature (Girardot and Holloway 1984a; Grau 1984; Mcgivem *et al.* 1983) indicating that certain environmental manipulations produce hyperalgesia as well as analgesia, and some of these "hyperalgesia systems" may be opiate in nature since the hyperalgesia can sometimes be antagonized by naloxone. This is a complex but important observation because it indicates that studies

of the effects of naloxone on pain levels must be interpreted with extreme caution. In conclusion, it appears that, under normal circumstances, endogenous opiate pain inhibitory systems have little spontaneous activity. However, when some level of pain is present, these systems seem to be activated.

A second line of research on the involvement of endogenous opioids in pain modulation in man has examined a number of environmental manipulations known to have some degree of efficacy for the reduction of clinical and experimental pain. This research has utilized two primary experimental strategies. The first reasons that if a particular environmental manipulation induces anal esia by utilizing endogenous opioids, that analgesia should be antagonized by a narcotic antagonist (usually naloxone). The second strate reasons that if endogenous opioids are mvolved in an environmentally elicited analgesia, then changes should be observed in the levels of these compounds in plasma or the central nervous system.

Stimulation-produced analgesia in man

Perhaps the most dramatic outcome of the basic science research on endogenous opioids has been the rapid and effective clinical application to the treatment of chronic pain syndromes in man. As early as 1973, Richardson and Akil (1977) reported the use of periventrtcular gray stimulation to treat pain syndromes. Since then there have been more than 20 reports in the literature describing various studies of this technique. In a recent review of this literature Young *et al.* (1984) conclude that "the method is reasonably effective in properly selected patients and, importantly, safe."

Several lines of evidence indicate a likely but not unequivocal role for endogenous opiates in stimulation produce analgesia (SPA) in man. Table 2 summarizes the evidence for the involvement of endogenous opioids in SPA in man. Opiate antagonists are reported to reduce SPA, tolerance develops to SPA, and dependence upon SPA has been reported. A more controversial literature exists with regard to the release of endogenous opioids, primarily Beta-endorphin, by electrical stimulation of the periaqueductal gray matter in man. It can be seen from table 2 that generally increased levels of endogenous opioids have been found to result from this stimulation. However, the possibility that these results are due to an artifact of using a contrast medium for electrode placement has been raised Dionne *et al.* 1984; Fessler *et al.* 1984). This criticism has been convincingly disputed for at least for some circumstances (Akil and Richardson 1985). At this point, it seems likely that endogenous opioids mediate, at least in part, the analgesia elicited by periaqueductal gray stimulation in man. The particular endogenous opioid and its site an mechanism of action have not been established.

Counter-irritation analgesia in man

The belief that an acute painful stimulus can be used to alleviate ongoing pain has been held since antiquity and is known as counter- irritation. This procedure has a great deal in common with acupuncture and TNS. All use the application of somatic stimuli, either noxious or innocuous, to obtain relief from pain. The site of treatment in relation to the painful area is

highly variable, ranging from the painful dermatome itself to a theoretically unpredictable constellation of points in classical Chinese acupuncture. Lastly, the duration of treatment varies from less than a minute to hours. All of these factors, as we have seen, are important determinants of the effects produced by footshock in animals. Thus, the highly variable effects observed in the clinic would be predicted from animal research. Nevertheless, human data suggest the involvement of the same systems described above.

TABLE 2

EVIDENCE FOR THE INVOLMENT OF OPIOID PEFTIDES IN STIMULATION-PRODUCED ANALGESIA IN MAN

NALOXONE TOL/DEP		ENDORPHIN LEVELS	
		B-ENDORPHIN	ENKEPHALIN
		PLASMA CSF	PLASMA CSF
4<	15	2> 5>	4>
10<	14	6= 3>	
13<	8	7= 1l>	
1<	12	9> 15<	

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TABLE 2 - The effect of various opiate manipulations on analgesia and endorphin levels resulting from electrical stimulation of the brain Symbols "< " = attenuates analgesia; "= " = no effect on endorphin level; ">" = increases endorphin levels. Numbers in the table refer to numbered citations listed alphabetically below the table. Abbreviation: CSF = cerebrospinal fluid; TOL/DEP = tolerance or dependence

Twelve studies have measured the effect of naloxone on clinical or experimental analgesia produced by acupuncture. Of these, eight reported that naloxone reduce the analgesia while four found no effect. In two of the studies failing to find analoxone effect (Chapman *et al.* 1980, 1983) the negative interetation of the results has been called into uestion (Miglen and Szekely 985; Mayer and Price 1981). The third of the four negative studies examined lon -term effects of acupuncture on migraine headache (Lenhard and Waite 1983) and thus does not fit into the general paradigm of the other studies addressed here. The final ne ative study (Kenyon *et al* 1983) utilized a dose of naloxone (0.4 mg.), which is on the low end of the

TABLE 3

EVIDENCE FOR THE INVOLVMENT OF OPIOID PEPTIDES IN COUNTERRITATION ANALGESIA IN MAN

A

ACUPUNCTURE ANALGESIA

NALOXONE	ENDORPHIN LEVELS			
	B-ENDORPHIN		ENKEPHALIN	
	PLASMA	CSF	PLASMA	CSF
(9)< (27)<	(24)=	(6)>	(13)>	(9)>
(14)= (1)<	(28)=	(23)>	(6)=	
(12)= (22)<	(25)=			
(4)= (3)<	(13)=			
(29)< (18)<	(26)=			
(5)= (17)<	(16)>			

B

TRANSCUTANEOUS NERVE STIMULATION

NALOXONE		B-ENDORPHIN LEVELS		
HI FREQ	LOW FREQ	HIGH FREQ		LOW FREQ
		PLASMA	CSF	PLASMA
(11)== (15)=	(15)<	(19)=	(11)=	(19)=
(8)≤ (19)=	(19)=	(7)>		(10)>
(29)< (8)=	(2)<	(10)>		
(21)= (2)=	(29)<			
(29)=	(21)=			
(20)=	(22)<			
(22)=				

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1. Boureau *et al.* 1979
2. Casale *et al.* 1983
3. Chapman 1978
4. Chapman *et al.* 1983
5. Chapman *et al.* 1980
6. Clement-Jones *et al.* 1980
7. Facchinetti *et al.* 1984
8. Freeman *et al.* 1983
9. He and Do 1983
10. Hughes *et al.* 1984
11. Johanson *et al.* 1980
12. Kenyon *et al.* 1983
13. Kisher *et al.* 1983
14. Lenhard and Waite 1983
15. Lundberg *et al.* 1985
16. Masala *et al.* 1983
17. Mayer *et al.* 1977
18. Mayer *et al.* 1976
19. O'Brien *et al.* 1984
20. Pertovaara and Kermppainen 1981
21. Pertovaara *et al.* 1982b
22. Sjolund and Eriksson 1979
23. Sjolund *et al.* 1977
24. Szczudlik and Kwasucki 1984
25. Szczudlik and Lypka 1983
26. Szczudlik and Lypka 1983
27. Tsunoda *et al.* 1980
28. Umimo *et al.* 1984
29. Willer *et al.* 1982b

TABLE 3 - The effect of various opiate manipulations on analgesia and endorphin levels resulting from transcutaneous nerve stimulation and acupuncture. Symbols:"<"= attenuates endorphin or analgesia level; "=" = no effect on analgesia or endorphin level; ">" = increase in endorphin levels. Numbers in the table refer to numbered refrences. Abbreations CSF= cerebrospinal fluid

range of effective doses. Thus, it seems clear that naloxone, at least under most circumstances, appears to antagonize acupuncture analgesia.

Table 3-A summarizes the studies that have examined the effect of naloxone on "acupuncture" analgesia as well as those studies which have measured "acupuncture''-induced changes in plasma or CSF Beta-endorphin or enkephalin levels. It is important to note that "acupuncture" is not a well-defined procedure. The only criterion for including a study in this table was that the authors call the procedure "acupuncture." Many of the procedures discussed below under transcutaneous electrical nerve stimulation (TNS) are similar or identical to those defined as "acupuncture" here.

The effects of acupuncture on CSF and plasma endorphin levels resent a somewhat less consistent picture, but this is not surprising considering the complexities of these types of data Considering that one could question the entire concept of plasma endorphin levels, since they are indicative of CNS level in only very indirect ways, a nevertheless somewhat consistent picture emerges. As can be seen in table 3-A, five studies have reported endorphin increases while six have reported no effects. Such results should be interpreted with extreme caution since (1) the meaning of increases in plasma endorphin levels are entirely unclear, and 2) even CSF endorphin levels are likely to be ambiguous since the site of endorphin release probably varies with the particular type of acupuncture stimulation. Nevertheless, an overview of these data is consistent with an involvement of endogenous opioids in at least some forms of acupuncture analgesia.

The literature concerning the involvement of endogenous opioids in TNS analgesia is considerably more complex than that associated with acupuncture analgesia This is likely to result from a greater variability in the intensity, frerquency, duration, location, and ot er parameters of TNS. Despite this diversity in experimental paradigm, some general consistencies are apparent in the literature. While only 4 of 13 studies of TNS analgesia have reported naloxone antagonism, all 4 studies utilized low-frequency TNS. On the other hand none of 6 studies utilizing high-frequen TNS found a naloxone antagonism (see table 3-B for references). The effects of TNS on endorphin levels have been less well studied. As seen in table 3-B, 3 of the 6 reported studies have found an increase in endorphin levels, while the remainder have found no effects. Such results should be interpreted with the caveats discussed above in mind. Overall, these results are strikingly consistent with reports in the animal literature (e.g., Han *et al.* 1984) and suggest the posseibility that certain types of sensory stimulation either inactivate opiate systems or activate opiate hyperalgesia systems as discussed above. Nevertheless, these results are consistent with an emerging picture that low-freguency, high-intensity acupuncture-like TNS (cf. Sjolund and Eriksson 1979) invokes endogenous opioid mechanisms. Such studies, taken together, probaably provide the most convincing evidence available that endogenous opioids can function to modulate pain transmission in man.

In conclusion, acupuncture and transcutaneous nerve stimulation appear to be forms of counterirritation that activate both opiate and nonopiate systems. The variable clinical outcomes observed following these treatments probably result from differential recruitment of segmental, extrasegmental, opiate, and nonopiate pain inhibitory systems, all of which are now known to be activated by these types of stimulation in animals.

Stress analgesia in man

A manipulation related to, but not identical with, counterirritation analgesia is a phenomenon most generally referred to as "stress analgesia." This issue

was discussed above in relation to animal studies, and similar caveats should be taken under consideration in human studies. These studies have utilized environmental manipulations tht are either severe physical or psychological stressors. The stressors catalogued in this section include surgery, labor, and childbirth, application of overtly painful stimuli such as cold pressor am or ischemic pain, chronic pain anticipation of pain, and chronic stressfu states such as life threatening disease.

TABLE 4

EVIDENCE FOR THE INVOLVEMENT OF OPIOID PEPTIDES IN STRESS ANALGESIA IN MAN

NALOXONE	ENDORPHIN LEVELS			
	B-ENDORPHIN		ENKEPHALIN	
	PLASMA	CSF	PLASMA	CSF
(6) <	(8) >	(7) >	(11) =	(12) <
(9) <	(3) >			(10) <
(15) <	(11) >			
(5) <	(1) >			
(14) <	(2) >			
(13) <				
(4) <				

REFERENCES FOR TABLE 4

1. Atkinson *et al.* 1983
2. Cohen *et al.* 1982
3. Delke *et al.* 1985
4. Frid and Sin er 1979
5. Frid *et al.* 1981
6. Jungkinz *et al.* 1983
7. *Katz et al.* 1982
8. Pancheri *et al. 1985*
9. Pertovaara *et al.* 1982a
10. Puig *et al.* 1982
11. Smith *et al.* 1985
12. Szczudlik and Lypka 1983
13. Willer and Albe-Fessard 1980
14. Willer *et al.* 1981
15. Willer *et al.* 1982a

TABLE 4 - The effect of various opiate manipulations on analgesia and endorphin levels resulting from stress. Symbols: "<" = attenuates analgesia or endorphin level; "=" = no effect on analgesia or endorphin level; ">" = increases endorphin levels. Numbers in the table refer to numbered citations listed alphabetically below the table. Abbreviation: CSF = cerebrospinal fluid.

Examination of table 4 reveals a strikingly consistent outcome for studies of this sort. It can be seen that in eight of eight studies in which a “stressful” manipulation increased nociceptive thresholds, naloxone at least partially reversed this increase. Another study (Janal *et al.* 1984 utilized vigorous exercise, at least a possible stressor, to increase pain threshold and also showed naloxone reversibility. In addition, and not surprisingly, since beta-endorphin is coreleased with ACTH, stress resulted in an increase in plasma beta-endorphin levels in five of five studies. Although such a result is not convincing alone, it is certainly consistent with the notion that endogenous opioids may underlie changes in pain threshold produced by stress. In addition, such results are consistent with the results of counterir-ritation studies discussed above with respect to the observation that naloxone

reversibility is more likely to occur with hi intensity peripheral stimulation (see table 3) In conclusion, considering the diversirty of procedures used in counterirration studies and stress studies, a generally convincing picture of opioid involvement in pain modulation in man emerges. Many important questions about the nature of this involvement remain unanswered. The most important of these are the particular endogenous opiate involved and its site of action. Answers to such questions are unlikely to come from human studies, since invasive procedures are probably necessary to acquire such information. The consistency of the animal and human studies, however, indicates the likelihood that such questions may be studied with animal models and verified in man.

Placebo analgesia in man

A placebo manipulation may well be considered a form of stress, at least under some circumstances. Naloxone has also been used to examine whether endogenous opiates are involved in placebo analgesia. Levine and coworkers (1978) reported that naloxone antagonized placebo analgesia in postsurgical patients. Although this conclusion as been questioned on technical rounds (Goldstein and Grevert 1978; Korczyn 1978; Skrabanek 1978), little conflicting data have been published. Gracely *et al.* (1983) have reported that naloxone results in an increase in pain levels experienced by post surgical patients that is independent of placebo effects.On the other hand, Levine and Gordon (1984). in a study at rectified the technical problems of their earlier one Levine *et al.* 1978), found convincing evidence for at least a partial reversal of placebo analgesia by naloxone. A difficulty with all of these studies is that surgical stress itself is a powerful activator of endogenous opioid systems (see above and may influence the experimental results. A carefully controlled study utilizing experimentally induced ischemic pain (Grevert *et al.* 1983) indicates that a artial antagonism of placebo analgesia can occur in the absence of any effects of naloxone on baseline pain responsivity. At this point, it seems most likely that endogenous opioids mediate at least some components of placebo analgesia. It should be kept in mind, however, that placebos, like other analgesic manipulations discussed above, are multidimensional manipulations likely to ativate multiple pain inhibitory and possibly pain facilitatory systems. Thus, hypnosis, a manipulation that has at least a superficial resemblance to placebo procedures, consistently has failed to be antagonized by naloxone (Barber and Mayer 1977; Goldstein and Hilgard 1975; Mayer *et al.* 1976; Spiegel and Albert 1983). The possibility that opiates are involved in some aspect of placebo analgesia appears articularly reasonable considering the fact that footshock analgesia can be classically conditioned in rats. Placebo analgesia can easily be conceived of as a classical conditioning paradigm wherein the placebo manipulation (e.g., injections, pills) serves as the conditioned stimulus and prior medication or treatment serves as the unconditioned stimulus. The observation by Grevert *et al.* (1983) that placebo effects tend to extinguish with repeated trials supports such a conceptualization.

Although explanations of this sort are clearly speculative, they are indicative of the wealth of concepts from experimental pain research now available for clinical evaluation. Our increasing knowledge of pain modulatory systems has the potential not only of providing explanations of current therapies but of suggesting new approaches for the control of pain. The preponderance of current pain therapies involve either the surgical destruction of neural tissue or the use of addictive drug. Such procedures offer great difficulties for the prolonged treatment of chronic pain. If multiple pain inhibitory systems

could be activated pharmacologically or otherwise in an alternating sequence, the problems of tissue destruction and addiction could be circumvented.

OTHER PEPTIDES

In addition to the endogenous opioids. other peptides have been reported to be involved in endogenous analgesia-producing mechanisms in various ways. Thus, neurotensin and substance P produce analgesia upon central administration by themselves; CCK, on the other hand, has been demonstrated to modulate analgesia produced by other manipulations.

Substance P

In addition to the numerous papers that have proposed substance P (SP) as a transmitter of primary afferent neurons in the spinal cord and medulla, it has been demonstrated that SP possesses analgesic properties, as well. Systemic and intracerebroventricular (ICV) admmistration of SP produce analgesia in mice (Stewart *et al.* 1976; Starr *et al.* 1978; Hall and Stewart 1983a; Meszaros *et al* 1981; Oehme *et al.* 1980) and in rats following both systemic (Mohrland and Gebhart 1979) and ICV (Malick and Goldstein 1978; Meszaros et *al.* 1981) injections. It should be noted that this analgesic effect when compared with that produced by opiates is very small (Frederickson *et al.* 1978; Frederickson and Gesellchen 1980) and has failed to be observed at all, particularly following systemic administration, by others Goldstein and Malick 1979; Growcott and Shaw 1979; Hayes and yers 1979; Inoki *et al.* 1977).

The analgesic action of SP is antagonized by naloxone Frederickson *et al* 1978; Frederickson and Gesellchen 1980; Malick and Goldstein 1978; Stewart *et al* 1976). Since SP does not bind to opiate receptors (Teremus 1975; Szreniawsky *et al.* 1980) SP a arently releases opioid peptides. Both the periaqueductal central gray (PAG) and the spinal cord are likely substrates for this SP effect because (1) SP has been demonstrated to produce analgesia both after administration into the PAG (Mohrland and Gebhart 1979 and following intrathecal administration (Doi and Juma 1981, 1982b , (2) SP causes a release of opioid peptides in both the PAG (del Rio *et al* 1983) and the spinal cord (Tang *et al.* 1983), and 3) tolerance to and opiate cross-tolerance with the analgesic action of SP has been demonstrated following intraventricular injections (Naranjo *et al.* 1984).

The apparent paradox that intrahecal injection of SP may induce both analgesia and "hyperalgesia" (Akerman *et al.* 1982; Lembeck *et al.* 1981; Matsumra *et al.* 1985; Yashpal and Henry 1983; Yasphal *et al.* 1982) [but see Piercey *et al.* (1981) for absence of any change in nociceptive threshold] has been partially resolved by Yashpal and coworkers. They showed that the SP-induced analgesia occurs after 10-15 min, when "hyperalgesia" disappears. Although these findings are consistent with results of others, they are in conflict with those obtained by Han *et al.* (1984). The latter administered SP antibodies into the PAG and intrahecally prior to inducing electroacupuncture analgesia. Whereas the SP antibodies reduced electroacupuncture analgesia following intracerebral administration, confirming the importance of the analgesic effects of SP at this site in this behavior, they potentiated electroacupuncture analgesia upon intrahecal administration. In view of the fact that the hyperalgesic effects of SP have been shown to be generally short-lived [up to 10 minutes (Yasphal and Henry 1983; Yasphal *et al.* 1982)]

while the analgesic effect has lasted in some studies for over 30 minutes (e.g. Doi and Juma 1981, 1982b; Yashpal and Henry, 1983), the predominant hyperalgesic action of spinal SP on electroacupuncture is surprising.

Although the analgesic effect of SP has been reliably observed and its mechanism understood, the very existence of SP-induced analgesia remains enigmatic. First, it seems to negate a role for SP as a transmitter of pain, as the analgesic effects clearly abolish its presumably algesic ones. Second, since it has been proposed that the presumed hyperalgesia results from spinal convulsive-like excitation (Bossut *et al.* 1986), the physiological relevance of the analgesic action is to be doubted.

Neurotensin

NT has been administered to mouse and rat via three different routes: intracerebroventricularly (ICV), intracistemally (IC), and intrathecally (IT). As NT affected the pain threshold in a different way depending on the route of administration, the literature is reviewed accordingly.

IC administration. The analgesic effect of NT has been observed in mice (Clineschmidt and McGuffin 1977) and rats Clineschmidt *et al.* 1979). Whereas NT analgesia in mice was dose-related and could be observed on the hot plate test, writhing test Clineschmidt and McGuffin 1977; Davis *et al.* 1983; Osbahr *et al.* 1981), and tail immersion test (Nemeroff *et al.* 1979, it could be demonstrated only on the hot plate test and not on the tail-flick test in rats (Clineschmidt *et al.* 1979). Naloxone (1-5 mg/kg failed to antagonize NT-induced analgesia in both species (Clineschmidt and McGuffin 1977; Clineschmidt *et al.* 1979. Osbahr *et al* 1981). however the observation that repeated pretreatment with morphine reduced the analgesic effect of NT (Luttmger *et al.* 1983) could be viewed as cross-tolerance, thus suggesting some involvement of opiate mechanisms.

NT analgesia is reduced by thyrotropin releasing hormone (Osbahr *et al.* 1981) and its structural analogs Hemandez *et al.* 1984). The mechanism of this antagonism is, as yet, not fully understood.

IT administration. The behavioral effects of a drug injected IC may be caused at both spinal and supraspinal sites of action. One way to test whether spinal sites are involved in NT-induced analgesia is administering the drug IT.

The results of IT administration of NT are somewhat confusing. In rats NT in doses up to 90 micrograms [3,000 times the dose producmg hot plate anal esia in this species following IC administration Clineschmidt *et al.* 1979)], failed to produce hot plate analgesia when administered IT (Clineschmidt *et al.* 1982; Martin and Naruse 1982 . These results seem to indicate that the site of action for NT-induced analgesia is supraspinal.

However, Yaksh *et al.* (1982) reported that doses of 1-100 micrograms of neurotensin injected IT produce analgesia on the hot plate, and even some analgesia on the tail flick test. Moreover, this grou reported that this analgesia was fully reversible by naloxone (10 or 15 mg/kg). Consistent with this report are the finding that IT-administered NT produced naloxone (2 mg/kg) reversible analgesia in the mouse Hylden and Wilcox 1983). However, this analgesia was not observed on the tail flick reflex but rather

ICV administration When NT is administered ICV to mice, it readily produces analgesia on the writhing test, and this analgesia is not reversible by naloxone (5 mg/kg) (Nicolaides *et al.* 1985). However, results obtained in the rat are more equivocal. Thus, while 20 micrograms of NT ICV in the latter species induced analgesia in one stud (Martin *et al.* 1981), 5 micrograms did not in others (Kalivas *et al.* 1982; Parolaro *et al.* 1983). Although these observations seem consistent with the view that these negative findings result from an insufficient dose of NT, hot plate analgesia from the cisterna magna was reported following 25 ng of NT (Clineschmidt and McGuffin 1977). It seems, therefore, hardly likely that the anatomical substrate for NT analgesia is located in the immediate vicinity of the lateral ventricles; structures in the brainstem in the immediate proximity of the cisterna seem better candidates. This conclusion was not confirmed by a mapping study, in which both periventricular structures (medial preoptic area, amygdala), mesencephalic (rostral periventricular gray), and rhombencephalic (medial pars of the reticular formation were equally effective injection sites for inducing hot plate analgesia with NT in rats (Kalivas *et al.* 1982).

Conclusion NT undoubtedly has analgesic properties, but neither its mechanism nor its site of action are understood. Thus, it is possible that NT induces analgesia upon IT administration by releasing endogenous opioids. This would explain some of the results using this paradigm (Hylden and Wilcox 1983; Yaksh *et al.* 1982) but not the lack of analgesia obtained by others (Clineschmidt *et al.* 1982; Martin and Naruse 1982).

The increase in latency to response reported from discrete injection of NT into supraspinal areas (Kalivas *et al.* 1982) may well reflect true analgesia in some cases, but not all. Thus, although it is not known whether NT excites neurons in amygdala or PAG upon iontophoretic application, in view of its effects in spinal cord and cortex this is a likely possibility. If NT causes general neuronal excitation, it could well cause analgesia in the PAG, as do electrical stimulation (Mayer *et al.* 1971) and injection of glutamate Behbehani and Fields 1978; Urca *et al.* 1980). Also, electrical stimulation Abbott and Melzack 1978) and morphine (Rodgers 1977, 1978; VanRee 1977 in forebrain produce analgesia. Further work is needed in order to decide these issues.

CCK

The earliest experiments with CCK in systemic doses of over 50 micrograms/kg in the mouse (Zetler 1980, 1983) and intracerebral or intrathecal doses of 10-40 ng in the rat (Juma and Zetler 1981) demonstrated the analgesic potential of this peptide. The observation that this anal esia was reversible by naloxone made these authors suggest that it was mediated by endogenous opioids. However, at these and higher doses it failed, unlike morphine, to depress ascending A-delta and C fiber activation (Doi and Jurna 1982a), suggesting a different mode or site of action.

More recent experiments indicate that lower doses of CCK, although playing a role in opiate analgesia, have effects opposite to the one imtially described. Thus, 3 micrograms/kg of systemic and 3.6 ng of intrathecal CCK antagonize the analgesia produced by morphine and opiate mediated foot shock analgesia (Faris *et al.* 1983). Similar effects were observed by others (Itoh *et al.* 1985; Han *et al.* 1985). The antagonistic effect of CCK fo lows an inverted "U" dose response curve with doses lower and higher than the aforementioned producing less effect.

Subsequent work with proglumide, a CCK antagonist, showed that this compound, after both systemic and intrathecal administration, significantly otentiated analgesia induced by morphine, endogenous opioids, and various behavioral manipulations that release endogenous opioids in rats (Watkins *et al.* 1984b, 1985; Tang *et al.* 1984) Anothe CCK antagonist, benzotript, has similar effects. Also, morphine analgesia in rats immunized a ainst CCK is potentiated (Faris 1985). Proglumide (Suberg *et al.* 198 or CCK antibodies (Suberg *et al.* 1985) potentiate the morphine inducea , naloxone reversible suppression of nociceptive spinal cord dorsal horn neurons indicating that the interaction between CCK and morphine occurs at the same site.

The antagonist action of CCK on opiate action and roglumide's potentiation seems not to be limited to analgesia. Itoh and Katsuura (1981) found that CCK significantly reduced the duration of catalepsy following intraventricularly injected beta-endorphin in rats, whereas proglumide potentiated it Itoh *et al.* 1985; Katsuura and Itoh 1985). Similarly, proglumide was found to potentiate morphine's effect on open field motility in the rat (Ben-Horin *et al.* 1984).

The interaction between CCK and opiate action has some very interesting clinical implications. Thus, it has been demonstrated in rats that proglumide prevents or reduces tolerance induced by repeated morphine administration (Watkins *et al.* 1984b; Tang *et al.* 1984). Together with the recent demonstration that proglumide potentiates morphine analgesia in humans as well (Price et al. 1985),. these findings promise new perspectives both for the use of opiates in treating pain and for treating opiate addicts.

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Mechanism of Development of Tolerance and Dependence to Opioids in Neuroblastoma x Glioma Hybrid Cells and Mice

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Neuroblastoma x glioma (NG108-15) hybrid cells are enriched with opioid receptors (Klee and Nirenberg 1974; Chang and Cuatrecasas 1979). In these cells opiates and opioid peptides regulate the synthesis of cyclic AMP at two different levels. A rapid receptor-mediated inhibition of adenylate Cyclase is followed by a delayed and long-lived compensatory increase in its activity. It has been suggested that the latter effect may be responsible for narcotic dependence and tolerance (Sharma et al. 1975a,b 1977; Traber et al. 1975; Lampert et al. 1976). Ascorbic acid suppresses the etorphine-induced compensatory increase in the levels of cAMP with little or no effect on the short-term response of NG108-15 hybrid cells to the effector agents (Sharma and Khanna 1982). These studies suggest that ascorbic acid may play a role in the Prevention of the development of tolerance in therapeutic applications of narcotics as analgesics. The mechanism by which ascorbic acid prevents the development of tolerance to opiates is still obscure.

Effect of Ascorbate on Etorphine-Induced Alterations in Cyclic AMP Levels

Results reported earlier from this laboratory show that ascorbic acid suppresses the etorphine-induced compensatory increase in the levels of cAMP in NG108-15 hybrid cells (Sharma and Khanna 1982) It was of interest to study the effect of variation of ascorbate concentration on etorphine-induced

compensatory increase in basal and PGE_1-stimulated cAMP levels. Results in tl show that in the absence of ascorbate, etorphine led to a compensatory increase of 80 percent in the basal and 65 percent in the PGE_1-stimulated cAMP levels. A progressive decrease in the compensatory response is seen as the concentration of ascorbate is raised to 10mM. Initially, at lower concentrations of ascorbate the decrease is more pronounced for PGE_1-stimulated cAMP levels.

Preexposure of NG108-15 hybrid cells toascorbate did not abolish stimulatory or inhibitory effects of drugs on cAMP levels (Sharma and Khanna 1982). indicating that receptors for opiates and PGE_1 were functionally coupled to adenylate cyclase. The effect of preincubation of NG108-15 hybrid cells with ascorbate on etorphine-induced compensatory increase in the levels of cAMP was studied. In this experiment cells were preexposed to ascorbate for 4 hours. After washing, the second incubation was with and without etorphine for an additional 4 hours. The monolayer cultures were washed free of ascorbate and used to determine basal and PGE_1-stimulated cAMP levels as in t1. Results in t2 show that etorphine-induced basal and PGE_1-stimulated compensatory increase in cAMP levels observed is of the same order of magnitude in the cells preincubated with or without ascorbate. The data from tl and t2 clearly show that simultaneous presence of ascorbate and etorphine is necessary to suppress the etorphine-induced compensatory increase in basal and PGE_1-stimulated cAMP levels. These studies have been carried out in a cultured cell line of neuronal origin. Therefore, we studied the effect of ascorbic acid on development of tolerance and dependence in vivo in mice.

The in vivo effects of ascorbate on morphine-induced analgesia, physical dependence, and tolerance were studied. In mice ascorbate, when administered along with morphine, suppresses the development of tolerance and physical dependence on the drug, without significantly affecting its analgesic properties. Mice receiving morphine along with ascorbate became less tolerant to and dependent on the drug. The duration of morphine-induced analgesia, however, is progressively reduced with an increase in the amount of ascorbate. However, ascorbate does not alter the intensity of morphine-induced analgesia (Khanna and Sharma 1983). Ascorbate at lg/kg body weight did not alter the pH of blood and did not have an effect on the levels of lipid-peroxides in blood and brain. These results suggest that action of ascorbate is not mediated by its oxidation and lipid peroxide formation.

TABLE 1

Suppression of opiate-induced compensatory increase in c-AMP levels by ascorbate

NG108-15 hybrid cells (1.2 x 10^6, passage 20) were plated in (Medium A) OMEM (Flow Labs U.K.) supplemented with 0.1 mM-hypoxanthine, 1µM-aminopterin, 16 µM-thymidine, and 10 percent of fetal bovine serum (Flow Labs) in 60 mm Falcon petri-dishes. When 60-80 percent confluent, the culture plates were divided in five groups: (i) control with no effector agents; (ii) etorphine (1.6 µM); (iii) etorphine 1.6 µM and sodium ascorbate (1 mM); (iv) etorphine 1.6 µM and sodium ascorbate (5 mM); (v) etorphine 1.6 µM and sodium ascorbate (10 mM). The cells were incubated for 4 hours at 37°C, washed with medium B [DMEM with 25 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)]. Incubations were carried out in 3 ml of medium B with 0.5 mM Ro20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidine-2-one]. Amounts of intracellular levels of c-AMP were determined over a period of 15 minutes with and without PGE_1 (10 µM) by the method of Gilman (1970). Protein in each petri dish was estimated by the method of Lowry et al. (1951). Values are the average of three individual experiments (each experiment had four plates in a group).

Additions during cell growth for 4 h	cAMP (Pmole/mg protein) Basal	PGE_1
	328	3800
Etorphine	584	6263
Etorphine + ascorbate 1 mM	535	3954
Etorphine + ascorbate 5 mM	308	4400
Etorphine + ascorbate 10mM	192	3542

TABLE 2

Compensatory increase in cAMP levels after preincubation of cells with ascorbate

NG108-15 hybrid cells ($1.4x10^6$, passage 20) were plated in medium A in 60 mm petri dishes. When 60-80 percent confluent the culture plates were divided in two groups (eight plates in each group): (i) control with no effector agents; (ii) sodium-ascorbate (5mM). The cells were incubated for 4 hours at 37°C, washed with 4 x 3 ml medium A, and each set of culture plates was divided into two groups: (ia) control with no effector agents; (ib) etorphine 1.6 µM; (iia) control with no effector agents; (iib) etorphine 1.6 µM. Incubations were carried out in 4 ml medium A for 4 hours at 37°C and washed with medium B. 3ml medium B with 0.5 mM Ro20-1724 was added, and the plates were incubated in presence of naloxone with and without PGE_1 (10 µM) for 15 min. Intracellular cAMP levels were determined as in tl. Each value is the average of three individual experiments (each experiment had four plates in a group).

Additions during cell growth		cAMP (pmole/mg protein	
Preincubation 1 (4 h)	Preincubation 2 (4 h)	Basal	PGE_1
-	-	433±49	5821±254
-	Etorphine	782±36	8063±422
Ascorbate	-	371±19	5216±397
Ascorbate	Etorphine	679±42	7976±510

Mechanism of Ascorbate Action

Ascorbic acid may exert its effect at the level either of binding of opiates to the receptor or of coupling of the receptor to other components of the adenylate cyclase system, i.e., inhibitory guanine nucleotide regulatory protein (Ni) and catalytic unit of adenylate cyclase.

Effect of Ascorbate on (^{3}H) Etorphine and (^{3}H) DADLE Binding

Effect of ascorbate on binding of (15,16-^{3}H) etorphine to intact NG108-15 hybrid cells was studied as a function of ascorbate concentration. Ascorbate at 1 and 5 mM concentration inhibited the specific binding of etorphine by 28 percent and 56 percent (t3). Similar effect of sodium ascorbate on D-Ala^2-(tyrosyl-3,5-^{3} H) enkephalin (5-D-leucine) binding was observed. Specific binding was inhibited by 24 percent, 56 percent, and 68 percent, respectively, in the presence of 1 mM, 5 mM, and 10 mM ascorbate (t4). These studies are compatible with those of Dunlap et al. (1979).

In order to ascertain that ascorbate does not destroy opiate receptors, NG108-15 hybrid cells in monolayer were preincubated with 1 and 5 mM ascorbate, washed and assayed for (^{3}H) etorphine binding. There was no significant change in specific (^{3}H) etorphine binding in the cells preincubated with ascorbate as compared to that in controls (t5). These results show that ascorbic acid does not destroy opiate receptors.

Effect of Ascot-bate on GTPase Activity

Klee et al. (1984) proposed that opiate inhibition of adenylate cyclase in NG108-15 hybrid cells is the direct result of stimulation of a low Km GTPase component associated with Ni protein of the adenylate cyclase complex. Adenylate cyclase shuttles between two states, an active one with bound GTP and an inactive one with bound GDP. Stimulatory receptors facilitate exchange of GTP for bound GDP, whereas inhibitory receptors (including opiate receptors) stimulate low Km GTPase activity, which converts GTP bound to the Ni complex to GDP (Ross and Gilman 1980; Katada and Ui 1979, 1982a,b; Koski et al. 1982).

We have studied the short- and long-term effects of sodium ascorbate and etorphine on low Km GTPase activity and thus Ni in NG108-15 hybrid cells. Etorphine stimulates low Km GTPase activity of NG108-15 hybrid cells by 54 percent. This acute stimulatory

effect of etorphine is reversed by opiate antagonist naloxone (t6).

TABLE 3

Effect of sodium ascorbate on binding of (^{3}H) etorphine to monolayer cultures of NG1O8-15 hybrid cells

NG108-15 hybrid cells ($2x10^4$, passage 20) were plated in medium P in 24 well Falcon culture plates. When 80 percent confluent, the wells were washed with medium
Incubations were carried out in 2 ml of medium B containing 2 nM (15,16-^{3}H) etorphine at 37°C in a water bath. Specific binding was determined by subtracting the nonspecific binding measured in the presence of 2.5 µM naloxone from total binding at each concentration of sodium ascorbate. Each value is the average of four individual determinations and is expressed as Mean ± S.D.

Addition	(^{3}H) etorphine binding CPM/mg protein
None	750 ± 101
Sodium ascorbate 0.1 mM	770 ± 58
Sodium ascorbate 0.5 mM	697 ± 47
Sodium ascorbate 1.0 mM	537 ± 56
Sodium ascorbate 5.0 mM	327 ± 20

TABLE 4

Effect of sodium ascorbate on binding of (^{3}H) DADLE to monolayer cultures of NG108-15 hybrid cells

Conditions of the experiment were the same as in t3. Specific binding of 5nM D-Ala^2-(tyrosyl-3,5-^{3}H) enkephalin (5-D-leucine) was measured.

Addition	(^{3}H) DADLE binding CPM/mg protein
None	295 ± 14
Sodium ascorbate 1 mM	225 ± 18
Sodium ascorbate 5 mM	131 ± 11
Sodium ascorbate 10 mM	92 ± 5

TABLE 5

Binding of (^{3}H) etorphine to NG108-15 hybrid cells, preincubated with ascorbate

NG108-15 hybrid cells were plated in 60 mm Falcon petri dishes as described in tl. When 60-80 percent confluent, the monolayer of cells was washed with 5 x 3 ml of medium B, and plates were divided in three sets: (i) control with no effector agents; (ii) sodium ascorbate (1 mM); (iii) sodium ascorbate 5mM. Incubations were carried out in 3 mlof medium B, for 60 min at 37°C. washed with 5 x 3 ml of medium B, and incubated in 3 ml of medium B containing 2 nM (15,16-H) etorphine at 37°C in a water bath. Specific binding was determined as described in t3.

Preincubation 60 min	(^{3}H) etorphine binding CPM/mg protein
None	1085 ± 134
Sodium ascorbate 1 mM	1243 ± 78
Sodium ascorbate 5 mM	1153 ± 133

TABLE 6

Effect of etorphine and sodium ascorbate on GTPase activity in membranes prepared from NG108-15 hybrid cells

Membranes were prepared and used for GTPase assay as described by Koski and Klee (1981). All experimental conditions were the same except that membranes were collected by centrifugation at 48,000 g for 50 min. Concentration of effector agents were naloxone, 2.5 mM; etorphine, 1 μM; sodium ascorbate, 5 mM; PGE_1, 10 μM.

Each value is the difference of GTPase activity obtained in the absence and presence of 50 μM GTP determined in triplicates and is expressed as Mean ± S.D.

Additions	GTPase activity (+ PGE_1) pmole of Pi formed/min/mg protein
-	35.3 ± 1.3
Naloxone	35.5 ± 3.8
Etorphine	54.6 ± 0.89
Etorphine + naloxone	33.5 ± 1.11

These results are consistent with the earlier observations of Koski and Klee (1981) on opiate stimulation of low Km GTPase activity. Recently we have shown that NG108-15 hybrid cells grown with etorphine or ascorbic acid for 4 hours had decreased low Km GTPase activity. Inhibition of the low Km GTPase by ascorbic acid may be the mechanism by which ascorbic acid inhibits the etorphine-induced compensatory increase in cAMP formation (manuscript under preparation).

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Development of Spinal Substrate for Nociception in Man

Veena Bijlani, Ph.D.; Tilat A. Rizvi, M.Sc.; and S. Wadhwa, MS.

The dorsal horn of the spinal cord is the first site of interaction between primary afferents and intrinsic neurons. There is considerable evidence in literature about the existence of a variety of descending fibers taking part in the spinal neuronal circuitry. The small diameter nociceptive fibers have been shown to establish synaptic contacts with the neurons of lamina I, II, and III(Kerr 1975; Bishop 1980; Christensen and Perl 1970; Willis and Coggeshall 1978). Spino-thalamic tract neurons are dispersed in lamina I, IV, V, and VI (Trevino *et al.* 1978; Kerr 1975; Carsten and Trevino 1978). The small neurons of lamina II and III do not appear to project for long distances (Trevino and Carsten 1975). Kerr (1975) has suggested these to be inhibitory neurons. The precise under-standing of function of neuronal circuitry requires that the afferent and efferent synaptic connections and the transmitters of its constituent be identified.

Studies were done on the dorsal gray of human fetal spinal cord ranging-in age from 8 to 37 weeks of intrauterine life with a view to identify neuronal circuits that modify/modulate the afferent inputs.

MATERIAL AND METHODS

Present study was conducted on 30 human embryos and fetuses ranging in age from 8 to 37 weeks of intra-uterine life obtained by hysterotomy and still birth autopsies. The fetal age was calculated on the basis of last menstrual period, crown rump length, biparietal diameter, and external features of the fetuses, while that of premature infant was assessed on the basis of crown heel and biparietal length. Lower cervical segments of spinal cord were processed for various techniques.

The neurons and afferent fibers were identified in the

Golgi preparation (100 μ thick celloidin sections), and circuitry was reconstructed using camera lucida. One Golgi impregnated neuron was processed for electron microscopy using the method of Fairen *et al.* (1977).

1 μrn semitnin sections were cut to identify different zones for ultrastructural studies, and electron microscopy was done to study synaptogenesis.

Immunohistochemical localization of substance P, enkephalin, and serotonin at different age periods was carried out with the help of avidin biotin peroxidase complex technique using monoclonal antibody (Sera Lab). GABA was localized using polyclonal antibody (Sera Lab) with PAP technique of Sternberger (1974).

RESULTS

Morphologically, on the basis of axonal projections and dendritic arborization, different types of neurons could be identified in different zones of dorsal horn. Interneurons (encased of Lima and Coimbra 1983) and projection neurons in marginal zone (Bijlani et al. 1986), islet, stalked, bipolar and multipolar neurons in substantia gelatinosa, and multipolar and antenna neurons in deeper zone were typed in Golgi preparation at all age periods (unpublished observation).

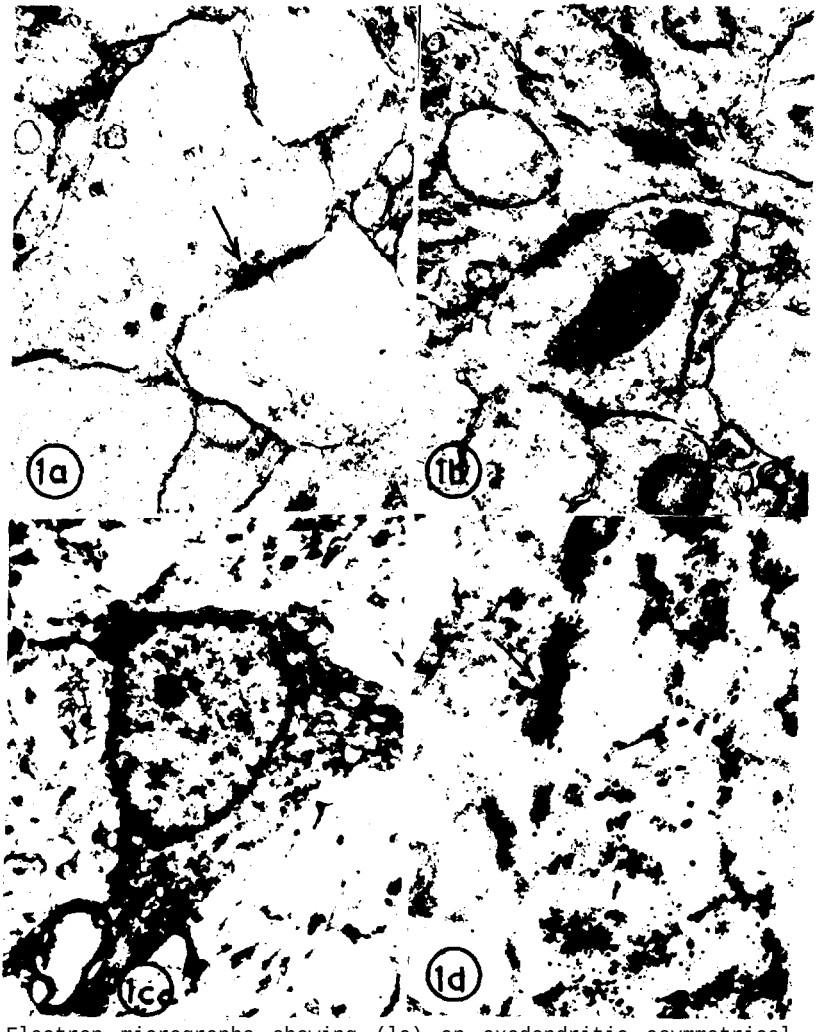
One interneuron at 13 weeks (encased) after Golgi impregnation was processed for electron microscopy. Different types of axodendritic and axosomatic synapses with clear spherical, dense core and pleomorphic vesicles were found on the dendrite and soma of this neuron (figs. 1c, 1d).

Mild substance P immunoreactivity was localized at the age of 8 weeks in the tangential plexus of fibers in superficial dorsal horn and in laterally curving fibers. Immunostained ventrally curving fibers could also be seen in the region of substantia gelatinosa (figs. 2a, 2d). Intensity of immunoreactivity increased with the age (fig. 3).

Serotonin inmunoreactivity was also found to be of the same pattern as that of substance P at 12 weeks in the superficial dorsal horn. Eoth medially and laterally curving fibers picked up intense reaction. Intermediate horn was found to have network of immunostained fibers (fig. 2b). Immunoreactivity again intensifies with the increasing age.

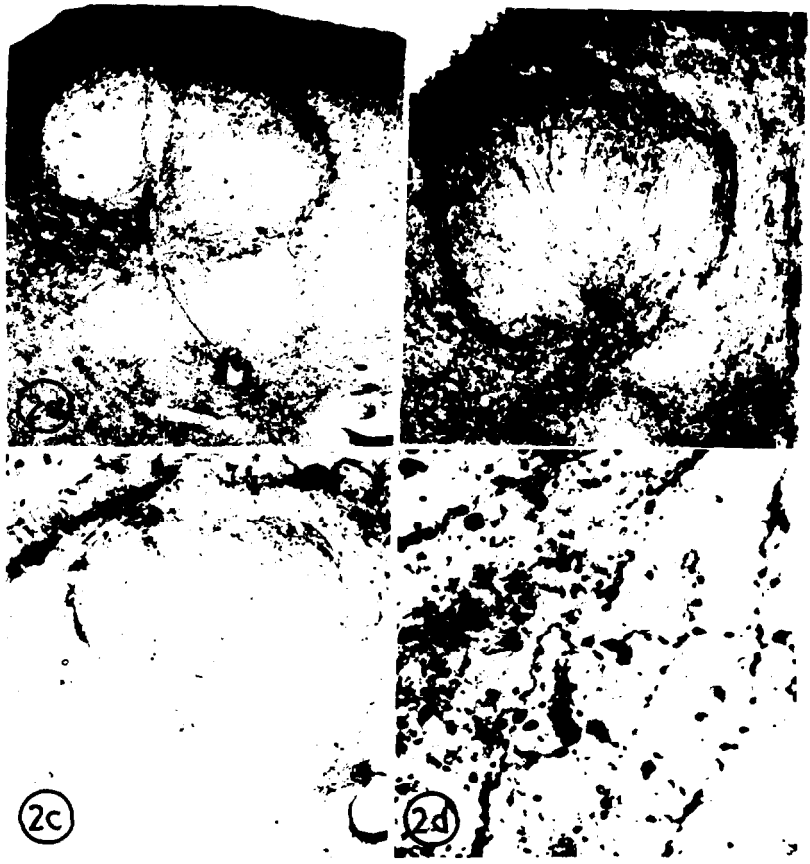
Enkephalin was localized at the age of 12 weeks of gestation in the laterally curving fibers and in intermediate gray. At 14 weeks onward mild immunoreactive positive cells were found in marginal zone and substantia gelatinosa. At the same age superficial dorsal gray: i.e., the region capping the dorsal horn,

FIGURE 1



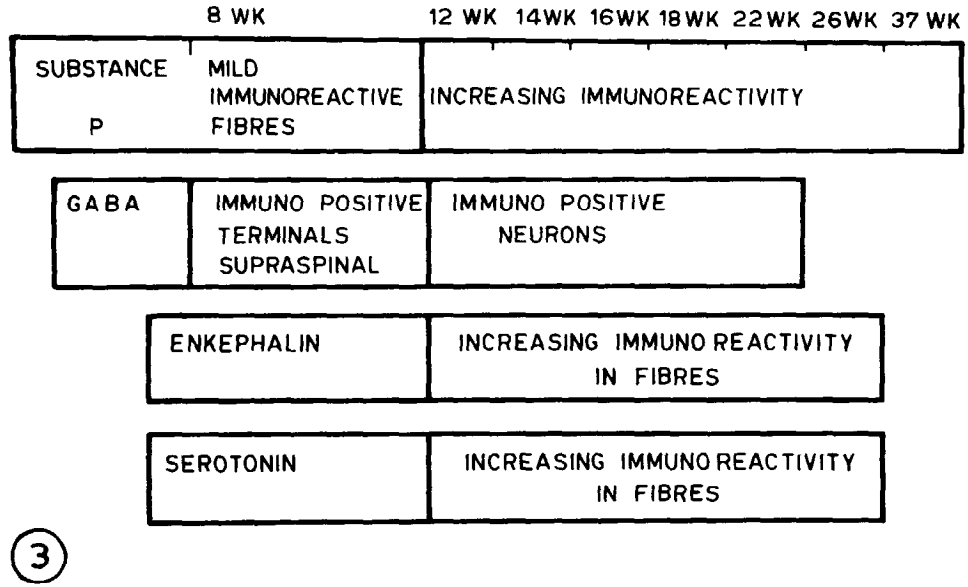
Electron micrographs showing (la) an axodendritic asymmetrical synapse (arrow) with clear spherical and dense core vesicles at 18 weeks of intrauterine life in marginal zone. X 35640.
(1b) serial types of synapses from deeper zone at 25 weeks of age. X 35640.
(1c) Golgi impregnated neuron from marginal zone after gold toning at 13 weeks of age. X 6385.
(1d) asymnetric synapse (arrow) on the soma of gold toned neuron(c) with clear spherical vesicles. X 46827.

FIGURE 2



Photomicrographs showing immunoreactivity in the dorsal horn of spinal cord at the age of 16 weeks of gestation.
2a - Substance P X 75.
2b - Serotonin X 75.
2c - Enkephalin X 75.
2d - Substance P immunoreactive fibres under high power X 750.

FIGURE 3



Comprehensive chart illustrates the appearance of various neurotransmitters in the dorsal gray at different age periods.

and laterally curving fibers were also immunopositive (fig. 2c).

GABA was first localized in terminals on dorsal side of superficial dorsal gray at 8 weeks, while at 10 and 12 weeks immunostained cells were seen in the marginal zone, substantia gelatinosa, and intermediate gray (unpublished observation).

At ultrastructural level dense core vesicles were seen in axonal terminals at 8 weeks. From 12 weeks onward axosomatic synapses with plemorphic vesicles were identified (Rizvi *et al.* 1986). These vesicles could contain either GABA or serotonin or even enkephalin. Asymmetric synapses were seen at 18 weeks (fig. la), while axoaxonic synapse was visible at 20 weeks (Rizvi *et al.* 1986). Serial types of synapses could be visualized at 25 weeks (fig. 1b).

DISCUSSION

Gobel (1978a) stated that all neurons in the marginal zone are projection neurons. Our studies are not in agreement with Gobel, as we have identified both projection (Waldeyer 1888) and interneurons at 13 weeks in that marginal zone on the basis of cell size, location, and dendritic morphology (Bijlani et *al.* 1986). We could also identify "encased neuron", specifically described by Lima and Coimbra (1983) in rat spinal cord as having a prominent dorsally directed dendrite situated dorsolaterally. Encased neuron picked up from Golgi section reembedded in araldite, was sectioned for electron microscopy (fig. 1c) and was found to have axosomatic (fig.1d) as well as axodendritic synapses. This neuron appears to be contacted by descending supraspinal fibers and could thus be involved in modulation mechanisms (Lima and Coimbra 1983). We also found this neuron situated in close proximity of GABAergic terminals at 8 weeks. No GABA positive cells were seen in dorsal horn at this age period (unpublished observations). The punctate appearance of GABA positive terminals in the region of Lissauer tract suggests the presence of GABA in the bulbcspinal fibers.

Cajal (1909) and Gobel (1978b) have reported central and limiting cells, islet, arboreal, and lamina II-III border cells in substantia gelatinosa, respectively. In our Golgi studies at 13 to 14 weeks we could find primitive stalk cell with axon projecting to lamina I. According to Gobel (1978b), these neurons are equivalent to the limiting cells of Cajal (1909) and may be excitatory interneurons, but according to Bennett *et al.* (1982) they contain enkephalin, an inhibitory neurotransmitter.

We could also find islet cell with axon in its vicinity in the same lamina (Bijlani *et al.* 1985). According to Gobel (1978b), these neurons may be inhibitory interneuron of Golgi type II class and central cells of Cajal (1909).

In deeper zone we could find antenna neurons with dendrites directed toward substantia gelatinosa. According to Willis *et al.* (1979), some spinothalamic tract cells in monkey do send dorsally directed dendrites through lamina II to lamina I so it may correspond to spinothalamic tract cell.

Substance P is putative neurotransmitter for dorsal sensory neurons (Otsuka and Konishi 1977; Nicoll *et al.* 1980). Recently Bossut *et al.* (1986) stated that substance P does not appear to be a primary afferent transmitter for pain in the spinal cord. Present investigations have suggested that substance P appears very early in ontogeny, as it was visible at 8 weeks embryonic spinal cord in our study (Mehra and Bijlani 1986). The neuropeptide has earlier been reported in the dorsal horn of human spinal cord at 12 weeks (Charnay *et al.* 1983) with immunofluorescent technique. Our immunohistochemical studies corroborate with Charnay *et al.* (1983) but with earlier ontogenic appearance (figs. 2a,2d).

The role of descending fibers in the modulation of sensory pathways at spinal level is well illustrated (Fields and Basbaum 1978; Mayer and Price 1976). Activation of serotoninergic pathways has been described to inhibit nociceptive responses of spinothalamic neurons (Messing and Lytle 1977). In dorsal horn serotoninergic axons have been described by Dahlstrom and Fuxe (1965) that originate from brain stem cells containing 5 HT. In our study serotonin positive immunoreactivity was localized at the age of 12 weeks using monoclonal antibodies. Serotonin positive neurons could not be identified in the spinal cord, thus supporting supraspinal origin of these fibers. Serotoninergic terminals gave an appearance of honey comb network around unstained neurons in substantia gelatinosa, supporting close proximity of gelatinosa neurons to supraspinal serotoninergic terminals.

At ultrastructural level visualization of axosomatic and axodendritic synapses with pleomorphic vesicles may correspond to serotoninergic terminals (Rizvi *et al.* 1986). Ruda and Gobel (1980) have described in detail the mode of action of descending serotoninergic fibers on islet, stalked, and projection neurons and

have stressed the modulatory role in nociception. Our observations indicate the existence of a morphological substrate corroborating Ruda and Gobel's (1980) findings.

Neuromodulatory role of enkephalin in the pain sensory system was supported by physiological (Basbaum and Fields 1978; Mountcastle 1980) and anatomical (Hokfelt *et al.* 1977; Glazer and Basbaum 1981) findings. Enkephalin has been localized immuno-histochemically in the spinal cord of man (DeLanerolle and LaMotte 1983) and monkey (Aronin *et al.* 1981) and in human fetuses and infants (Charnay *et al.* 1984) by immunofluorescent technique. Enkephalin immunoreactive fibers in these studies were predominant in the marginal layer and substantia gelatinosa (Elde *et al.* 1976; Sar *et al.* 1978), while enkephalin containing cell bodies were located in the substantia gelatinosa (Ditirro and Ho 1980). Opiate receptors have been described to be located in the superficial dorsal horn on primary afferent fibers (Atweh and Kuhar 1977), thereby indicating the formation of axoaxonic synapses (Jessel *et al.* 1979) and causing presynaptic inhibition by enkephalinergic neurons of substantia gelatinosa (Mudge *et al.* 1979).

In contrast, physiological studies have shown that enkephalin acts at postsynaptic sites in dorsal horn (Henry *et al.* 1980). At electron microscopic levels in rat dorsal horn enkephalin has been localized in axon terminals (Pelletier and Leclere 1979) and found to form synapses with aendrites and soma (Hunt *et al..* (1980). For the first time, enkephalin positive neurons were found in lamina I, II, III, and V at ultra-structural level (Aronin *et al.* 1981). According to these authors, enkephalin binds mostly to opioid receptors contained on dendrites and soma within the spinal cord, while direct synapses by enkephalinergic axons with primary afferent fibers form a secondary and not so common site of interaction. Therefore, earlier concept that opiate receptors are on primary afferent terminals and form axoaxonic synapses for presynaptic inhibition appears to be contradicted.

In present investigation on developing dorsal gray, enkephalin immunoreactivity was localized in the dorsal gray at the age of 12 weeks in fibers entering to intermediate dorsal horn through lateral funiculus. From 14 weeks, immunoreactive fibers were found in superficial dorsal gray, i.e., marginal zone and substantia gelatinosa (fig. 2c). Laterally curving fibers also were found to show immunoreactivity. Our immunohistochemical findings corroborate with the immunofluorescent finding of Charnay *et al.* (1984).

At 17 weeks, in addition to immunoreactive fibers, some cells positive for enkephalin could also be visualized, suggesting the presence of enkephalin in substantia gelatinosa neurons. The intensity of reaction in fibers and cells increased from 12 weeks to 25 weeks (the specimen used for immunohistochemical localization).

Curtis and Johnston (1974) and Levy (1977) have described GABA as a major neurotransmitter involved in mediation of presynaptic and postsynaptic inhibition and presynaptic felicitation. Wolf (1981) has described relationship between GABA positive neurons and development of excitatory synapses in developing visual cortex and has specified that in prenatal phase GABA has extra synaptic function. Light *et al.* (1979) and Kumazawa and Perl (1978) have stressed the roles of GABAergic synapses in the integration of afferent information.

In the present investigations on human fetal material, GABA was localized using polyclonal antibodies. At 8 weeks of intrauterine life, immunopositive terminals were identified in dorsal most-regions of the spinal cord. These appear to be in the region of Lissauer tract and could be the terminals of bulbospinal pathways. At 12 weeks of intrauterine life, GABA positive cells were found in the marginal zone, substantia gelatinosa, as well as in the deeper zones. These findings support the results of Barber *et al.* (1982) in the rat spinal cord. These authors, however, identified GAD positive cell bodies.

At ultrastructural level presence of various types of synapses with morphologically different types of vesicles, namely, clear spherical (fig. 1b), dense core (fig. 1a), and pleomorphic vesicles (Rizvi *et al.* 1986), suggest the presence of different transmitter, e.g., substance P, serotonin, GABA, and enkephalin.

CONCLUSIONS

In conclusion, present investigations suggest that among various neurotransmitters that have been described in the literature to play a role of mechanism of nociception, substance P of dorsal root origin appears at 8 weeks and at the same age GABA in the bulbospinal fibers is present. Serotonin and enkephalin neurotransmitters are present at 12 weeks in the fibers while enkephalinergic cell bodies are visualized at 14 weeks onward. The presence of these transmitters indicates the possibility of the existence of various components of morphological

substrate for nociception in spinal cord at very early stages of human ontogeny. All the same, possibility of neurotrophic role of these various transmitters cannot be ruled out.

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Differential Effects of Opioid Peptides Administered Intracerebrally in Loci of Self-Stimulation Reward of Lateral Hypothalamus and Ventral Tegmental Area-Substantia Nigra

Jitendra Singh, Ph.D., and T. Desiraju, Ph.D.

INTRODUCTION

In recent years the knowledge on the wide distributions of the opioid neurons, fibre terminals, and their receptors has been growing (Atweh and Kuhar 1983; Akil *et al.* 1984; Snyder 1984, 1986). The opioid peptide neuronal and receptor mechanisms are considered to participate in a range of homeostatic integrations in the different subsystems of the brain, including the stress response and coping, pain control, analgesia, neuro-endocrine, cardiovascular, and other visceral regulations, and the mechanisms of reward, mood, and psyche. The present state of knowledge generally indicates that the beta-endorphin neurons and the epsilon, mu, and delta receptor mechanisms might be predominantly involved in the visceral and neuro-endocrine regulations, the enkephalins and the delta receptors involved primarily in the psychic and other limbic neuronal mechanisms, the dynorphins and the kappa receptors primarily involved in the analgesic and sedative neuronal mechanisms, and with several overlapping roles also in the functioning of these neurons and receptors in different subsystems.

The enkephalins were shown to have the reward properties in intra-ventricular self-administration experiments (Stein and Belluzzi 1979), the leu-enkephalin was more self-administered than the met-enkephalin or the morphine. The self-administration of morphine and of D-ala^2-mets-enkephalinamide (and its blockade by naloxone) into the self-stimulation sites of lateral hypothalamus was also reported (Olds 1979; Olds and Williams 1980), but these results were argued to be artifacts by Bozarth (1983), who stated that the morphine self-administration property was not present in lateral hypothalamus, but present only in the ventral tegmental area (Bozarth and Wise 1981).

Shaw et al. (1984) reported that while the intra-ventricularly injected morphine caused a reduction of spontaneous motor activity, the electrical self-stimulation (SS) of medial prefrontal cortex (MPC) was facilitated. Similar results were also observed by them with the intra-ventricular injection of the enkephalin analogue BW 180 (tyr-D-ala-gly-phe-D-leu), but these effects were indirect according to them as direct injections of morphine or naloxone into MPC caused no change

in the SS of MPC. Naloxone (intraperitoneal) has also been shown to cause a small but significant reduction in the SS of lateral hypothalamus (Ichitani et al. 1985). Broekkamp et al. (1976) reported that the SS of LH was inhibited by injecting morphine into central grey. In contrast, microinjections of morphine or of D-ala^2-met^5-enkephalinamide administered into ventral tegmental area facilitated the SS of lateral hypothalamus and the effect of morphine was antagonized by systemic naloxone (Phillips et al. 1983). They also reported rewarding effects in the conditioned place preference paradigm and that such a facilitatory effect was attenuated by haloperidol or by lesions of dopamine pathways (Phillips et al. 1983).

The above studies indicate the need for investigations of the role of the opioid peptides administered into the different regions of SS to understand the intrinsic and interlinked components in the mechanisms of the brain reward neuronal systems. The present study was designed to investigate the effects of enkephalins and dynorphins administered directly into the loci of self-stimulation of ventral tegmental area-substantia nigra (SN-VTA) and of lateral hypothalamus (MFB-LH) on the self-stimulation operant rates of the same loci. Such studies have not appeared in the literature so far.

METHODS

Wistar rats weighing 260-320 g were implanted with bipolar SS electrodes made up of 28 g stainless steel wire, and bipolar cannula-cum-wire electrode made up of the same type of wire joining to 22 g cannula needle. The cannula-wire electrode (both insulated except for tips) served either as bipolar SS electrode or for microinjections of chemicals through the cannula into the SS site. In each rat one bipolar wire electrode and one bipolar cannula electrode were implanted, one in MFB-LH and the second in ipsilateral SN-VTA . In different rats the placement of the two types of electrodes was interchanged between the regions. After a few days of post-operative recovery, the self-stimulation rates of each of these sites were shaped and stabilized reproduceably in a few sessions. The stimuli used were sine waves of 50 Hz, 0.25 sec train duration for each pedal press, and current intensities ranging between 75 μA and 200 μA as required to get the maximum possible SS without apparent side effects. The duration of a control or a test session was 15 min. At least three test sessions (with drug) and three control sessions (with vehicle) were made for each dose, and all repeated in at least three rats. 33 rats were used in the present study. All intra-cerebral injections (drug or vehicle solutions) were made through the cannula slowly in about 60 seconds in a constant volume of I μl. At the end of the experiment, the placements of the electrodes were noted from frozen sections cut at 60 μ stained with cresyl violet, and marked on the charts of atlas of Paxinos and Watson (1982). Data were processed by using the F test (Anova) to test the homogeneity of variance and by Student's 't' test to know the levels of significance of the effects.

The chemicals used were: leu^5-enkephalin; met^5-enkephalin; leu^5-enkephalinamide; dynorphin-A[1-13] (all these of Sigma, USA); D-ala^2-D-leu^5-enkephalin (BW 180C of Wellcome Research Laboratories,

UK); naloxone-HCI (Endo Laboratories, USA).

RESULTS

The leu^5-enkephalin (leu-enk) was administered (500 ng) into SN-VTA and one hour and two hours later, the SS operants were recorded. It was observed that the SS of the site was reduced by 48% at 1 hr and by 70% at 2 hrs from the level of the control value obtained under the vehicle injection (Fig. 1), the SS of the other site (MFB-LH) was not changed. In the reverse experiment, administration of the leu-enk in MFB-LH has not changed the SS of the same site but has caused a facilitation of the SS of the SN-VTA (Fig. 1). A similar pattern of response was obtained with the leu^5-enkephalinamide also (Fig. 2).

The D-ala^2-D-leu^5-enkephalin (ala-leu-enk or BW 180c) caused, contrary to the above opioids, a facilitatory effect on the SS of SN-VTA when it was admiristered either in the SN-VTA or in the MFB-LH (Fig. 3). This opioid also has not influenced the SS of the MFB-LH. Naloxone hydrochloride (30 mg/kg ip) has brought down the facilitated effect of SN-VTA back to about the normal level (Fig. 3).

The met^5-enkephalin (met-enk) administered in doses of 500 ng or 2 μg either into SN-VTA or into the MFB-LH has not been found to influence the SS of either region (Fig. 4).

Dynorphin-A[1-13](dyn) administered in doses of 500 ng or 1 μg into SN-VTA has caused facilitation of its SS. Administration of the same into the MFB-LH has not influenced either its SS or that of the SN-VTA (Fig. 5).

In summary, the leu-enk or its amide have caused reduction in the SS of SN-VTA when administered into the same site. When these opioids were administered into the MFB-LH its own SS was not influenced, but caused indirect facilitatory effects on the SS of SN-VTA. Met-enk caused no changes of SS in both these regions. Contrary to leu-enk, dynorphin-A[1-13] caused facilitatory effect in the SN-VTA, and also no effect through MFB-LH. Ala-leu-enk caused both types of facilitatory effects on the SS of SN-VTA, when it was administered into it and also into the MFB-LH, without changing the SS of the MFB-LH.

DISCUSSION

There seems to be no study made previously to find the effects of the above cited opioid peptides on the SS of SN-VTA and of MFB-LH, after administering them into the same SS sites.

It was previously reported (Shaw et al. 1984) that only the intraventricular administrations of BW 180 (ala-leu-enk) up to 40 μg doses, or morphine up to 20 μg, caused facilitation of the SS of the medial prefrontal cortex (MPC), but no effects were observed when morphine or naloxone was administered into the MPC. As the ventricular drug could act at many sites in the brain, the authors interpreted the effects as indirect ones on the MPC. These authors have also reported that

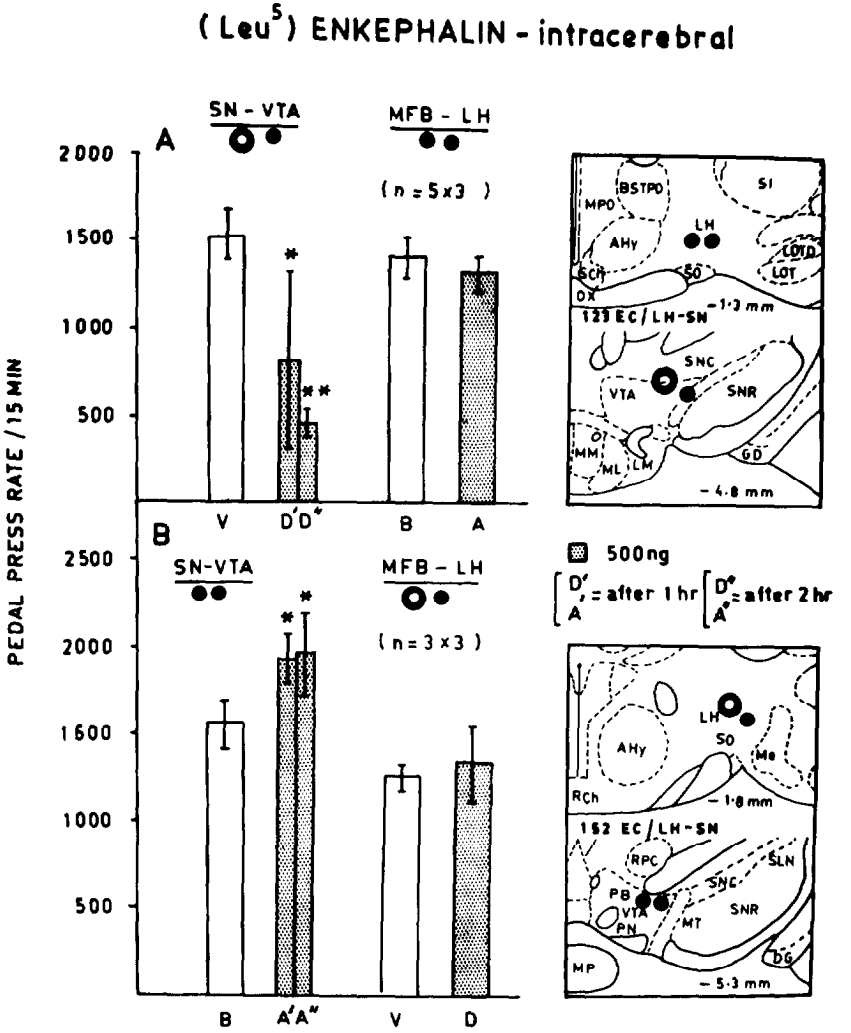

FIGURE 1

Effects of slow microinjection (1 µl in 60 sec) of leuenkephalin either into SN-VTA (A) or into MFB-LH (B) on the electrical self-stimulation of the injected site and of the other site. Cannula site is open circle, filled circle is wire. Typical placement sites of histology indicated in the atlas drawings of hypothalamus and midbrain. A', one hour or two hours (A") after the drug is administered in other site; B, before the drug is administered in the other site; D', one hour or two hours (D") after the drug is administered in the same site; V, after the vehicle (saline) is administered in the same site. 'n' indicates the data of number of rats and the number of sessions conducted on each of them. * = $P < 0.005$; ** = $P < 0.001$.

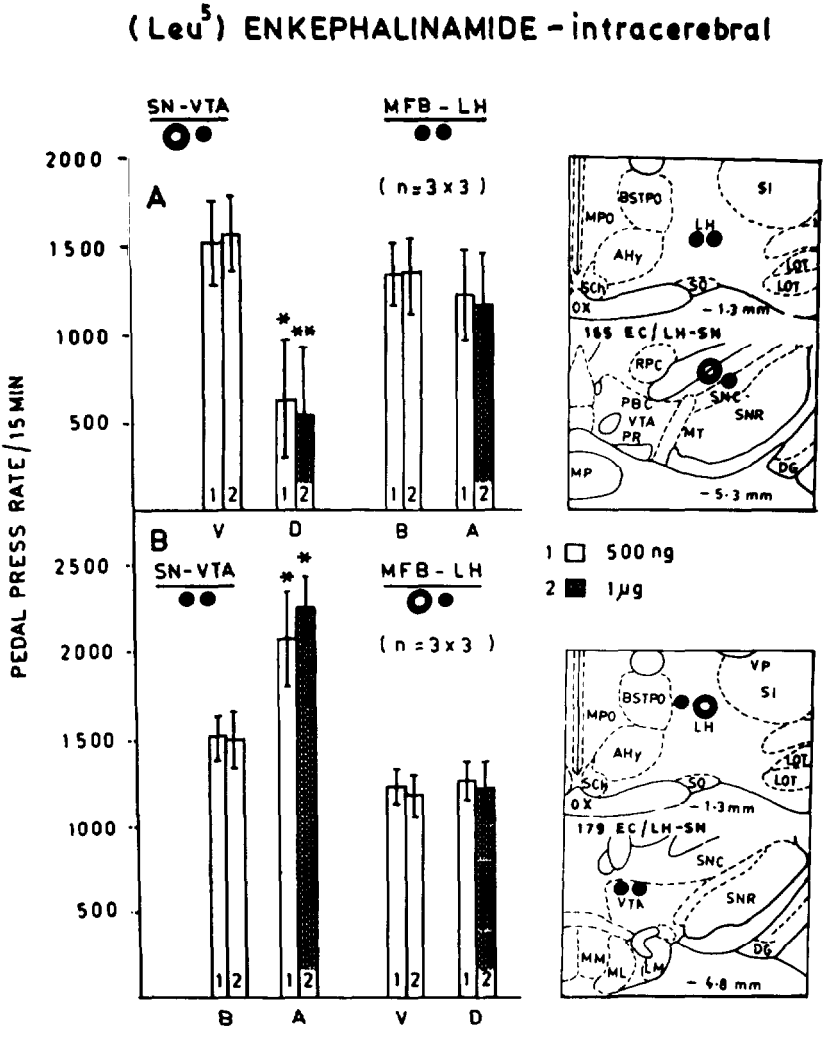

FIGURE 2

Effects of leu-enkephalinamide in two doses represented by the two sets of bars (1,2). Self-stimulation sessions were conducted one hour after the drug (D) or the vehicle (V) administration. After testing the injected site, the other site was tested. Other symbols as in Figure 1.

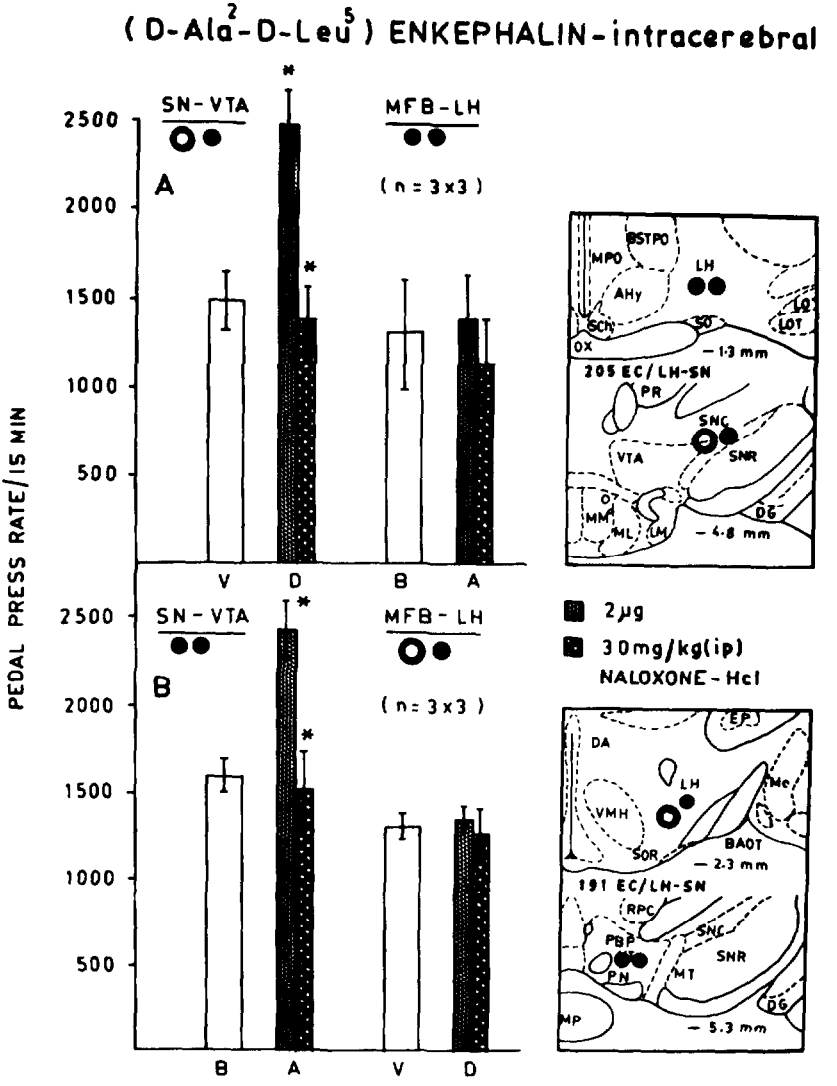

FIGURE 3

Effects of D-ala^2-D-leu^5-enkephalin, and of intraperitoneal (ip) naloxone hydrochloride administered during the enkephalin effect. Naloxone effect studied from 15 min after its administration. Symbols as in Figure 2.

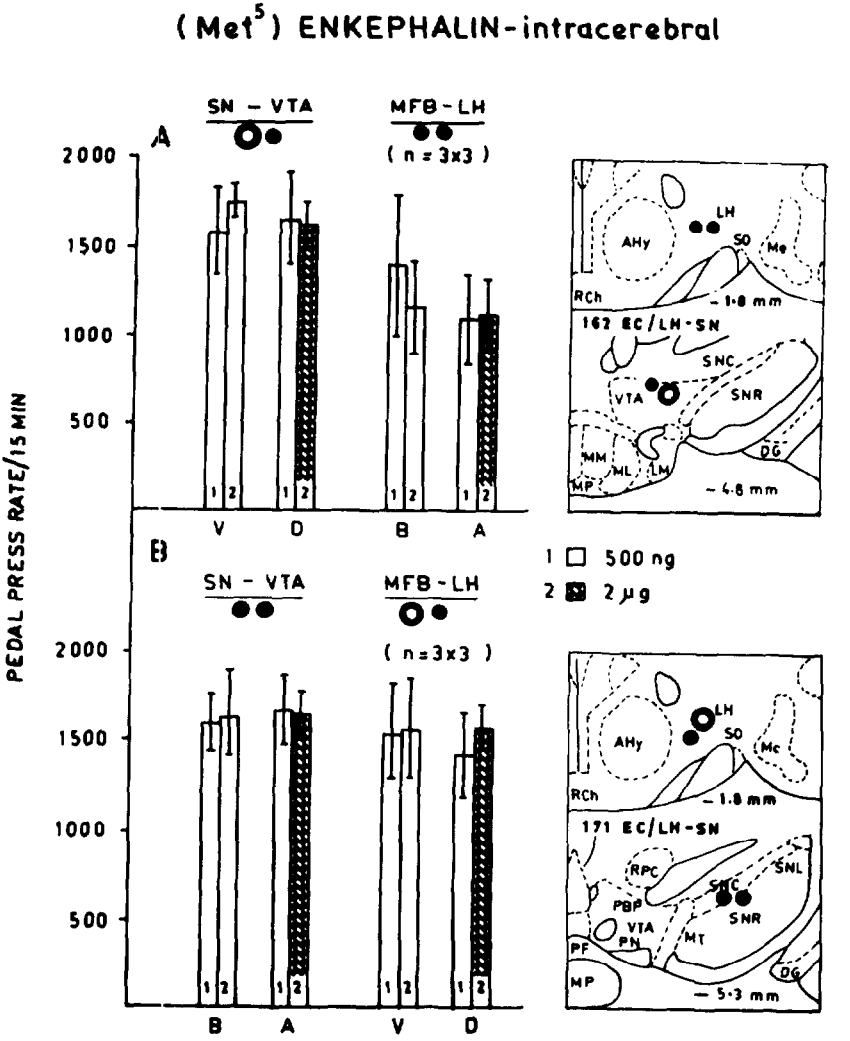

FIGURE 4

Effects of met-enkephalin in two doses represented by the two sets of bars (1,2). Symbols as in Figure 2.

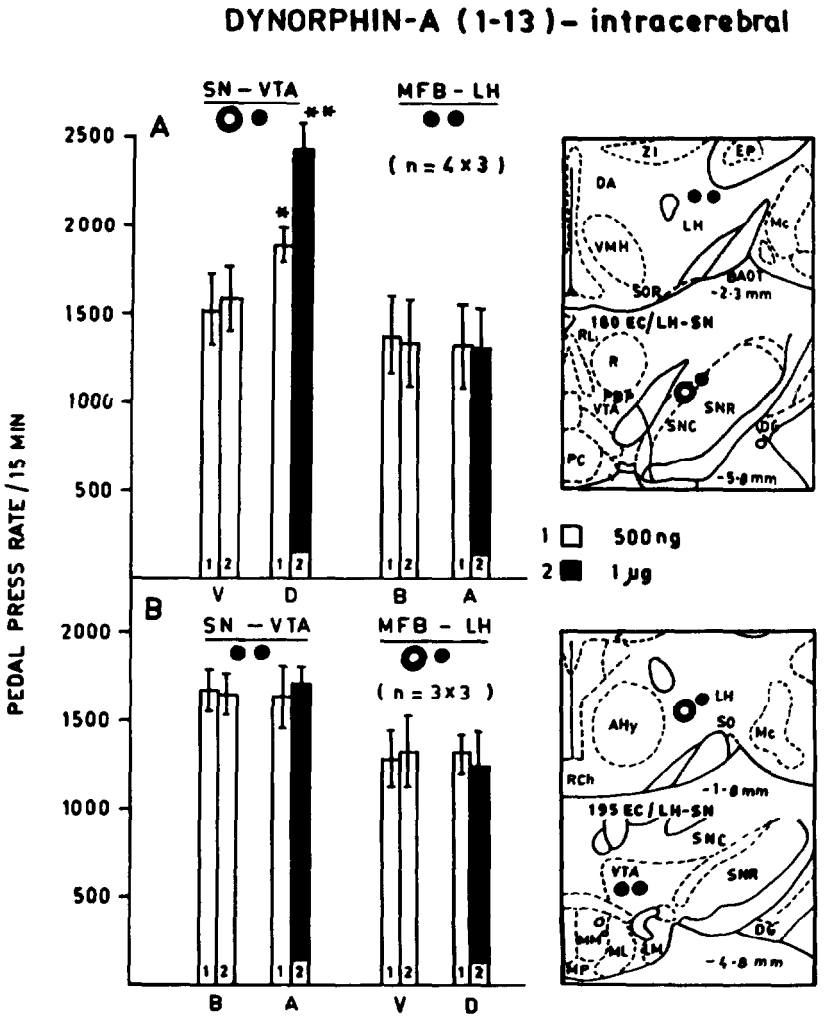

FIGURE 5

Effects of dynorphin-A[1-13] in two doses represented by the two sets of bars (1,2). Symbols as in Figure 2.

the intraventricular BW 180 caused a reduction of the spontaneous motility, particularly in the dose range of 20-40μg. In our local study on SN-VTA and MFB-LH, only a dose of 2 μg of the ala-leu-enk has been used and the effects on their self-stimulation have been very significant. Further, we have also observed that the facilitatory effect was relieved by administration of naloxone hydrochloride intraperitoneally. Naloxone has not significantly affected the normal SS of SN-VTA or of MFB-LH in our study, in contrast to the report of Ichitani et al. (1985) who observed a small but significant reduction of the SS of lateral hypothalamus under naloxone (5mg/kg ip). The effects of naloxone hydrochloride on the SS could depend on the regional differences and on the conditions of reinforcement, the FR-30 schedules being most affected and the CR schedules least affected. Schaefer and Michael (1985) observed the SS (FR-30) of the periventricular grey to decrease in a dose-dependent manner, by about 70% at a naloxorie dose of 30 mg/kg given sub-cutaneously. At this dose, they have also observed slight reduction (about 15%) in the ambulatory activity.

Phillips et al. (1983) reviewed the observations to show that morphine or ala-met-enk administration into SN-VTA caused facilitation of the SS of lateral hypothalamus, whereas our results with the ala-leu-enk administration into the SN-VTA have not caused any change in the SS of MFB-LH. Probably the difference in the opioids used and the receptors upon which they act could be the reason for the opposite patterns of the results. Our present study with the leu-enk, met-enk, ala-leu-enk, and dyn has also disclosed such differences in the patterns of the effects on SS of the SN-VTA and the MFB-LH. In the studies of Phillips et al. (1983) the effect of morphine or of ala-met-enk on the _SS of the injected site (SN-VTA) was not studied, whereas we have found that with the ala-leu-enk the SS of the injected site (SN-VTA) was facilitated. We have also observed the converse, i.e., administration of the ala-leu-enk into the MFB-LH has not changed the SS of the injected site and yet led to a change of the SS of the SN-VTA. This could imply that at the MFB-LH the ala-leu-enk could have acted on an additional interlinked substrate besides on the SS substrate (intrinsic) of MFB-LH and that the additional substrate could have been linked to the SN-VTA intrinsic substrate of SS. In other words, the opioid effects probably differentiated in the MFB-LH the intrinsic SS substrate from a separate substrate present there and interlinked to the SN-VTA mechanism of SS.

The mode of synaptic transmission actions of opioids and the details about differerces in the proportions of the opioid receptor types present on the neurons of the MFB-LH and the SN-VTA are not adequately available to enable to infer the possible mechanisms underlying the effects seen in the present study. A review of the previous studies (North and Williams 1983) indicates that morphine would enhance K^+ conductance and cause inhibition of noradrenergic neuronal firing. These effects could widely differ according to the types of the neurons and the sites in the brain. There was also an opposite type of observation that morphine when administered iontophoretically led probably indirectly to an increase in the firing of dopaminergic neurons at SN-VTA and that iontophoressed naloxone had not reversed this effect,

whereas the non-DA neurons in the vicinity were suppressed, which could be reversed by naloxone (Gysling and Wang 1983). It has been reported that the leu-enk has primarily the delta receptor activity (Akil et al. 1984). Dynorphin-A [1-13] is known to be very potent at both mu and kappa receptors, and its fibre terminals are present only in SN and not in LH, which has the cell bodies (Akil et al. 1984). Ala-leu-enk also labels preferentially the delta sites. Hence, the opioid peptides used in the present study would be primarily acting upon the delta, mu, and kappa receptors of the non-opioid and the opioid neurons and axon terminals in the MFB-LH and SN-VTA. However, the differences in the proportions of these receptor types present in the two regions, and the organization of the types of neurons and terminals in the linkages of circuits in which these receptors are located in the two regions, are unknown. The mu receptors are probably more predominant in the MFB-LH (Akil et al. 1984), whereas the delta and kappa may be relatively more in the SN-VTA substrates of SS. It is hypothesized from the present results that the leu-enk in the MFB-LH could be inhibiting a projection mechanism inhibitory to the SS system of SN-VTA, thereby releasing the facilitation of the SN-VTA. The opposite type of effects of the leu-enk and the ala-leu-enk on the SS of SN-VTA, when these are administered in the SN-VTA, is probably due to their differences in actions on the receptor types present in the SN-VTA. The dynorphin-A actions suggest that the kappa receptors seem to be involved in the SS mechanism of SN-VTA and not in that of MFB-LH.

The differential effects of the above opioids on the SS of the SN-VTA and the MFB-LH emphasize the differences in the neural mechanisms of the SS of the two regions. Our previous study (Singh and Desiraju 1986) of the SS of the two regions with local administrations of haloperidol and apomorphine also disclosed that the two regions have separate synaptic types of intrinsic mechanisms of SS, besides having an inter-linkage mechanism. As the opioids have shown either inhibitory or facilitatory effects on the SN-VTA, and little such effects on the MFB-LH, the possibility arises whether this striking difference could be due to the dopaminergic neurons and receptors present in the SN-VTA. This difference in effects could also as well be due to the opiate receptors present on non-dopaminergic neurons and terminals located in the SN-VTA.

SUMMARY

Leu-enkephalin, leu-enkephalinamide, ala-leu-enkephalin, met-enkephalin and dynorphin-A[1-13] were administered in microinjection into one of the self-stimulation sites of SN-VTA or MFB-LH and the electrical self-stimulation (SS) of the injected site and of the second site was recorded. The study revealed that the leu-enkephalin and the leu-enkephalinamide inhibited the SS of SN-VTA and produced no effect on the SS of MFB-LH, when administered into these sites. The MFB-LH injection, however, facilitated the SS of SN-VTA. The effect of ala-leu-enkephalin injection in MFB-LH was similar to the above, but the effect of the injection in SN-VTA was different in that it caused

the facilitation of its SS and not the depression as seen with leu-enkephalin. Met-enkephalin injections in the two regions caused no direct or indirect changes of the SS of the regions. Dynorphin injection in SN-VTA facilitated its SS, like the injection of ala-leu-enk, but dynorphin injections in MFB-LH produced no effects. The results essentially demonstrate the differences in the effects of the different opioids in the reward system of the SN-VTA, and it is discussed that these differences are probably due to the preferences in the types of the receptors upon which these opioids act in the SN-VTA neuronal organisation. The results also demonstrate the major difference in the organisation of the reward substrate of the MFB-LH from that of the SN-VTA, as the effects of the opioids in the MFB-LH are markedly differert or none compared to the effects in the SN-VTA.

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National Institute on Drug Abuse, 1988

Peptides and Thermoregulation

Rakesh Shukla, Ph.D., and Bhola N. Dhawan, M.D.

Many neurotransmitters are involved in the central regulation of body temperature under normal conditions, in fever, and during exercise and heat and cold stress. Emphasis on amines and prostaglandins has declined to some extent, and a major focus in recent years has been on the peptides that might participate in central nervous system (CNS) mediation of temperature control. Interest in -the neurobiology of peptides has developed coincident with their identification and chemical characterization in mammalian brain. Several of these peptides were originally isolated from pituitary tissue or the alimentary canal, but they also occur in various regions of the CNS. Although the central roles of most of the peptides are still not adequately understood, many neuropeptides are known to have profound effects on regulation of body temperature, and the available data on the neuropeptides will be briefly reviewed.

1. OPIOIDS

The endogenous opioids are unique in that their ability to alter thermoregulatory mechanisms is shared by nonpeptide opioids. While evidence is accumulating that opioid ligands and opioid receptors play a functional role in thermoregulation, an exhaustive study in different species has not yet been undertaken for opioid peptides. Much less information is available on the opioid peptides than on morphine.

A. Morphine

Most studies have been conducted with morphine, although some investigations have also been carried out with other opioids. Several reviews have appeared on the effects of morphine on body temperature, the most comprehensive being those by Clark (1979). Clark and Lipton (1983, 1985), and a recent review by Adler et al. (1988). The effects of morphine are species-dependent. A hypothermic response is predominant in the dog, rabbit, and pigeon; a hyperthermic response in cat, cattle, goat, and horse; and a dual response (low--dose hyperthermia and high-dose hypothermia) in rat, mouse, and primates (Clark and Lipton 1985). A biphasic response,

hypothermia followed by hyperthermia, in rat and mouse is also reported (Rosow et al. 1980). All these responses to morphine are blocked and antagonized by naloxone. There is also some interaction with calcium channel antagonists (Pillai and Ross 1986b). Morphine responses are dependent on ambient temperature: a hypothermic response is predominant at ambient temperature below thermoneutrality; biphasic responses appear as the ambient temperature increases, and ultimately hyperthermia predominates at the highest ambient temperatures (Adler et al. 1988). Recent studies have demonstrated that morphine responses are also dependent on the route of administration (Adler et al. 1984; Geller et al. 1986), and route is a critical determinant not only of quantitative effects but of qualitative effects as well. The exact mechanisms involved in the body temperature changes produced by morphine are still not known with certainty. The effects may result primarily from the actions on oxygen consumption, however. Morphine-induced hypothermia is associated with decrease in oxygen consumption and metabolic heat production in a variety of species (Lotti et al. 1966; Lin et al. 1980). Recent studies by Adler and his colleagues (1986, 1988) suggest that body temperature responses to morphine are due to the change in thermal set point in hypothalamus. In dog, an increased heat loss after morphine administration has been demonstrated (see Clark and Lipton 1985 for a review and references). Some interaction of morphine with dopaminergic and cholinergic systems in thermoregulation has been shown by Cox et al. (1975) and Adler et al. (1986).

B. Naloxone

Naloxone has been used by several investigators to establish evidence for involvement of opioid receptors. Naloxone, per se, however, does not have an effect on thermoregulation in rat (Pillai and Ross 1986a) or man (Fuenmayor and Cubeddu 1986) though in morphine-dependent rats naloxone and related compounds reduced rectal temperature by a central action since quarternary derivatives were inactive (Katovich et al. 1986). The drug interactions are not always well defined. Thus naloxone only partially blocked ethanol hypothermia, which was completely blocked by kappa antagonist MR 2266 (Pillai and Ross 1986a), whereas it completely antagonized microwave blockade of amphetamine hyperthermia (Lai et al. 1986). The blphasic temperature changes with phencyclidlne are antagonized by naloxone, by the ε and κ antagonist SKF 10,007 as well as by the pure k antagonist MR2266 (Hiramatsu et al. 1986).

C. Endogenous Opioids

Identification of endogenous peptides and their precursor molecules in the brain encouraged intense research efforts to determine both the in vivo and the in vitro effects of these substances and the possible functional roles played by the μ, κ, and δ opiold receptors. Recent data utilizing more selective ligands in rat suggest that μ opioid receptors play a role in hyperthermic response. Morphine, a prototypic μ ligand, and DAGO, a μ selective receptor agonist, produce hyperthermla in several species

of animals (Adler et al. 1988) and prompt rats to seek warmer temperatures. On the other hand, κ receptors seem to be involved in hypothermia, as U50488H, a κ receptor agonist, produces dose-related hypothermia. A role for δ receptors in thermoregulation cannot be ruled out completely.

a. ß-Endorphin. If nonpeptide opioids act at receptors that are stimulated by oploid peptides, the thermoregulatory effects of endogenous opioids and of morphine and related agonists ought to be alike. When ß-endorphin is injected centrally to a variety of species, it induces patterns of temperature change that closely resemble those associated with central or peripheral administration of morphine (Clark and Lipton 1985). Relatively low doses of morphine and ß-endorphin raise the level at which temperature is regulated in the cat, rat, mouse, and rabbit (Rezvani et al. 1982), primate (Murphy and Lipton 1983), and fish (Kavaliers 1982). Evidence from studies on rabbits indicates that this change in regulation may be due to impaired input from peripheral warmth sensors (Rezvaini and Heath 1984). Such an effect would be of greater significance in a warmer environment and could account for the greater increase in body temperature at usual ambient temperatures than in a cold environment after injection of a given dose of ß-endorphin to cats, rabbits, etc. In contrast to the above species, which have been used most often for the study of opioid-induced temperature changes, ß-endorphin lowers the temperature of chickens and hamsters (Clark and Lipton 1983).

In those species in which small doses of ß-endorphin induce hyperthermia. naloxone and similar antagonists ought to lower temperature if' ß-endorphin contributes to maintenance or elevation of body temperature. Both naloxone and naltrenone prevented a rise in the temperature of rats secondary to restraint or handling, or lowered temperature when stress was minimized by habituation of the animals to the experimental procedures (Clark and Lipton 1983; Pae et al. 1985). Except for such reports, evidence is not available to indicate that reasonable doses of opioid antagonists appreciably affect temperature. So it is unlikely that ß-endorphin, which has been readily antagonized in most studies, plays a major role in normal thermoregulation, even in hot or cold environments (Clark et al. 1983). Likewise, naloxone has not altered responses to pyrogen in the cat, rat (Clark and Lipton 1983), guinea pig (Kandasamy and Williams 1983), and sheep (Duranton and Bueno 1984). So a role for ß-endorphin in fever is unlikely. This view is also supported by the fact that antipyretics failed to antagonize the hyperthermic action of opioid peptides in the cat, mouse (Clark and Lipton 1983), guinea pig (Kaidasamy and Williams 1983), and rabbit (Kandasamy and Williams 1983; Rezvani and Heath 1984). It is likely that the endorphin effects are mediated via a mechanism independent of the arachidonic acid cascade.

b. Enkephalins. Very little information is available on the potential thermoregulatory actions of enkephalins. Met-enkephalin is a relatively weak agonist in altering body temperature (Clark and Lipton 1985), probably because of its rapid inactivation (Miller 1981). To obtain measurable temperature changes, larger doses (0.1 mg to 10 mg centrally) of the enkephalins (as compared to ß-endorphin) have been required. The usual response at room temperature has been hyperthermia (Clark and Lipton 1985), although there have been exceptions (Sakurada et al. 1983). Shukla et al. (1982) have demonstrated that Met-enkephalin induced hyperthermia varied inversely with ambient temperature over a temperature range of 10° to 33°C In the desert rat Mastomys natalensis.

A major difference between Met-enkephalin and ß-endorphin is that the hyperthermic response to the former is less readily antagonized by naloxone (Clark and Lipton 1983). Hence the lack of appreciable effect of naloxone on temperature does not rule out the mediation of thermoregulatlon by enkephalins. Assessment of this possibility has been hampered by the lack of a selective enkephalln antagonist. A number of relatively stable and more potent enkephalin analogs have been tested (see 1985 review by Clark and Lipton). Unfortunately, these have generally been more readily antagonized by naloxone, and act more like ß-endorphin than like the parent enkephallns, probably because the structural modifications that increase stability tend to shift sensitivity away from naloxone-insensitive types of receptors. Recently Shukla et al. (1988) suggested an enkephalinergic and serotonergic interaction in the thermoregulation of the desert rat.

c. Other Opioid Peptides. Dermorphin produces a naloxone-reversible hypothermia which is unaffected by antagonists of other putative neurotransmitters such as serotonin, catecholamines, GABA, acetylchollne, etc. (Tatara et al. 1986).

Currently available data do not permit a definitive statement regarding the role of opioid peptides in thermoregulatlon. It is, however, abundantly clear from data reviewed above that multiple oploid receptors are involved. It appears likely that several types of transmitters/modulators are involved in regulation of body temperature in health and disease and that their relative importance varies in various situations. It is necessary to study the effect of specific agonists and antagonists at various opioid receptors in several species, not only at different ambient temperatures but also during pyrexia induced by different agents to assess their roles. It is also necessary to study their interaction with other putative neurotransmitters and to analyze in depth their effect on heat production and heat loss mechanisms. There is evidence to indicate that some of the peptides may be acting at sites such as the septum (see below). Such sites have not been investigated for the effect of oploid peptides.

II. ANTIPYRETIC PEPTIDES

A. ACTH/MSH

ACTH and MSH, which were first isolated from pituitary tissue, are widely distributed within the brain (Krieger and Martin 1981). They were first shown to reduce temperature when given centrally to rabbits (Lipton et al. 1984). Subsequently, ACTH and MSH were found to be antipyretic when administered centrally or peripherally in doses that had no effect on normal temperature (Murphy et al. 1983). The parent molecule ACTH (1-39) was actually the first peptide with documented antipyretic activity. Most of the recent research, however, has been focused on MSH. This peptide is about 25,000 times more potent than acetaminophen as an antipyretic (Murphy et al. 1983). MSH reduces fever whether administered i.c.v., i.v., or into the stomach of rabbits (Murphy and Lipton 1982). Its central site of action may be the septum, as injections of MSH into the septum are also antipyretic. ICV administration of ACTH reduces endotoxin-induced fever in squirrel monkeys, and MSH is likewise antipyretic when given i.v. to these primates (Lipton and Shih 1985). Corticotropin-releasing factor (CRF), which releases ACTH/MSH from the pituitary, also reduces fever in a dose-related fashion when given i.c.v. to rabbits.

The precise mechanism of action of MSH in reducing fever has not been determined. However, it is unlikely that antipyretic doses directly inhibit central heat production and conservation pathways, since MSH has no effect on the temperature of a febrile rabbit exposed to cold (Richards and Lipton 1984). The doses required to induce the antipyretic and hypothermic effects of peptides differ. These effects appear to represent distinct actions within the CNS. Studies on hyperthermic responses to arachidonates suggest that central release of MSH could mediate the antipyretic effect of acetaminophen

B. Arginine Vasopressin (AVP)

The role in thermoregulation of arginine vasopressin, the antidiuretic hormone in man, has been studied in the past. Most of the supportive data have been obtained recently, however. Studies of temperature-induced changes in plasma vasopressin levels or diuresis have produced conflicting data. Concentration of AVP did not change in the pig during exposure to warm, neutral, or cold environments (Forsling et al. 1976). Cooling of the rostral hypothalamus in cold and thermoneutral environments, or heating in thermoneutral and warm environments, likewise did not affect vasopressin levels. However, increasing core temperature to 42°C caused a rise in plasma vasopressln that could be completely suppressed by reducing preoptic/anterior hypothalamic temperature. Brisk water diuresis was observed with preoptic/anterior

hypothalamic cooling in rhesus macaques, presumably due to inhibition of vasopressin release (Hayward and Baker 1968). These findings suggest a relation between vasopressin and central temperature control mechanisms. They give no indication, however, that this relationship is important under normal circumstances. The evidence for arginine vasopressin as an endogenous antipyretic is strong but not yet conclusive. It includes direct suppression of fever by the application of AVP in a sensitive, dose-dependent manner by push pull perfusion into the region of the septum in animals given intravenous pyrogens, and the appearance of AVP in the perfusate after perfusion of the septal region with an artificial interstitial fluid during fever. The vasopressin concentration is inversely related to the magnitude of fever.

Hemorrhage, a well-known stimulus for the release of vasopressin into the circulation, was found to be without effect on normal body temperature but to suppress the fever elicited from intravenous pyrogen. The application of an antibody to AVP in the septal region led to a markedly greater rise in body temperature than would normally occur from a fixed dose of endotoxin given intravenously (Cooper 1987). AVP itself is without effect on normal body temperature in the sheep when perfused through the ventral septal area. Zeisberger et al. (1980) and Merker et al. (1983) have demonstrated that the pregnant guinea pig close to term and several hours post partum is refractory to endotoxin. In the nonpregnant guinea pig during fever, AVP reactivity was increased in fibers projecting from the hypothalamus to the septum and amygdala (Zeisberger et al. 1983).

Arginine vasopressin suppressed pyrexia caused by intraventricular injection of PGE when the AVP was perfused through the rostral diencephallc loci at which PGs caused fever (Ruwe et al. 1985). Evidence of the endogenous antipyretic action of AVP has been found in rats (Kovacs and De Wied 1983). Banet and Wleland (1985) found that AVP infused into the lateral septum of the rat suppressed the heat production caused by hypothalamic cooling but did not affect vasomotor tone in the skin. The reduction in thermoregulatory increase in heat production that led to a fall of body temperature during cold exposure might explain the antipyretic action of AVP. Wilkinson and Kasting (1986) psesented evidence to indicate that vasopressin reduces the set point for body temperature in febrile rats.

Only one study (Lee et al. 1985) has evaluated the role of AVP in a subhuman primate, macaque monkeys, where intraventricular AVP had no effect on the normal body temperature or fever, except at a dose of 65 ng, where the mean fall of body temperature of 0.5°C appeared to be due to a larger response in one animal. AVP has also been shown to cause hyperthermia in rabbit (Lipton and Glyn-Ballinger 1980) but is without effect in cats and causes hypothermia or hyperthermia when microinjected into the preoptic area in the rat (Naylor et al. 1986).

Arginine vasopressin might be a mediator of fever within the anterior hypothalamus/preoptic anterior hypothalamus and an antipyretlc within the ventral septal area. Such a dual function would be consistent with a negative feedback system to modulate the magnitude of' fever, a useful function if fever is a beneficial accompanimert of disease. A role for AVP as a fever mediator as well as an endogenous antipyretic is, however, far from conclusive.

III. OTHER PEPTIDES

A. Thyrotropin-Releasing Hormone (TRH)

TRH is the most extensively studied peptide, and it usually induces a brief increase in temperature (Clark 1979). Cats respond with hypothermia (Metcalf 1974; Myers et al. 1977), however, as do rats (Lin et al. 1980) and nonhibernating ground squirrels (Stanton et al. 1981). TRH-induced temperature changes are independent of its action on the thyroid. Effects of TRH on body temperature were determined over a range of ambient temperatures, but the pattern of body temperature change in the majority of reports is consistent with increase in the level of regulation. TRH has frequently been observed to have a general excitatory action that would tend to raise temperature by increasing heat production. It can enhance locomotor and other behavioral activities alone and can reverse behavioral depression, hypnosis, and anesthesia induced by other agents (Clark and Lipton 1983).

The hyperthermic effect of TRH administered into the preoptic/anterior hypothalamus of rats was unaffected by pretreatment with aspirin, but was reduced by adrenergic antagonists (Chi and Lin 1983). However, based on resistance to inhibition by adrenergic antagonists, other investigators have concluded that the noradrenerglc system is not essential for this response in mice given TRH by intraperitoneal injection (Pawlowski and Kwiatek 1983). In another study, TRH given intraperitoneally to mice caused hyperthermia that was associated with increased plasma noreplnephrlne and eplnephrine concentration (Boschi et al. 1983). Simultaneous injection of these catecholamlnes increased hyperthermia. Adrenal demedullation suppressed both the increase in plasma catecholamines and TRH-induced hyperthermia, but not the hyperthermic response to TRH plus catecholamines. Hypophysectomy also reduced ¨RH hyperthermia, in contrast to earlier reports, but not the increase of plasma catecholamines or the effect of combinations with exogenous catecholamines. These authors concluded that both the adrenal and pituitary have essential roles in TRH-induced hyperthermia. When injected into the hypothalamus of pigeons, TRH induced hyperthermia, shivering, and vasodilation that was probably compensatory (Lathi et al. 1983). Evidence that TRH might be involved in the maintenance of body temperature has been presented by Prasad et al. (1978, 1980). In this study, a

dose-related decrease in temperature occurred when rabbit TRH antiserum was injected into the lateral ventricle of rats. and this response was prevented if the antiserum was neutralized with TRH.

B. Neurotensin (NT)

Neurotensin was initially isolated from bovine hypothalamus (Carraway and Leeman 1973). The primary temperature response to central administration of NT in a variety of species is hypothermia, which is enhanced in cold environments. Neurotensin has been reported to have less than one-thousandth the potency of bombesin in rats exposed to cold (Brown et al. 1977), whereas under similar conditions in mice both of these peptides were equipotent (Mason et al. 1980). Injections of NT into 223 brain sites in rat (Kalivas et al. 1982) indicated that regions responsive to its hypothermic action are distinct from sites at which it inhibits nociception. Administration of NT into the periaqueductal gray had no clear effect on normal body temperature (Kalivas et al. 1982). The N-terminal fragments of NT were inactive, but a C-terminal fragment (8-13) did lower temperature (Widdowson et al. 1983). Neurotensin also lowered the temperature of guinea pigs and hamsters only in a cold environment and did not change the temperature of fish, frogs, lizards, pigeons, ground squirrels, woodchucks, and rabbits (Prange et al. 1979).

The research on the mechanism of action of NT has been limited to studies on interactions with other substances. Intracisternally administered NT potentiated the hypothermic response of ethanol (Luttinger et al. 1981) and of dopamine agonists (Jolicoeur et al. 1983), but inconsistent effects were obtained with pentobarbital-induced hypothermia (Nemeroff et al. 1977). The hypothermic response to NT in rat was antagonized by i.c.v. administration of prostaglandin E2 and by calcium chelator EGTA (Lee et al. 1983) but not by TRH. somatostatin, or naloxone. The hypothermic response was also antagonized by amine and cholinergic antagonists (Morley et al. 1982) and was enhanced by thyroidectomy (Nemeroff et al. 1980). Since central administration of EGTA and PGE2 alone causes hyperthermia, a nonspecific antagonism may be responsible for preventing the hypothermic effect of NT at thermoneutral environment. Mason et al. (1982) suggested that release of central prostaglandlns may oppose the hypothermic response of NT in cold environment. Lee and Myers (1983) concluded that NT renders the animals poikilothermic, because NT caused hypothermia in rats in thermoneutral and cold environments but not in hot (34°-4O°C) environment.

C. Bombesin

Bornbesin was originally isolated from frog skin (Anastasi et al. 1972, 19751. The central administration of bombesin caused hypothermia in rat and mouse at normal laboratory temperatures. The magnitude of the fall in the temperature has generally been dose

related. However, systemically given bombesin did not affect the temperature of rats in the cold (Brown et al. 1977; Brown and Vale 1980). It was subsequently found that injections of bombesin into the PO/AH of rats increased locomotor activity and enhanced both heat reinforcement and heat escape behavior (Hawkins and Avery 1983). It was concluded that the effects on behavior are secondary to the change in activity and do not demonstrate that bombesin lowers the regulated temperature. Avery and Calisher (1982) showed that in a thermoneutral environment i.c.v. bombesin caused dose-related decrease in body temperature and food intake of food-deprived rats.

D. Somatostatin

Nemeroff et al. (1979) found that intracisternal administration of somatostatin in mice caused hyperthermia at normal laboratory temperature. Brown et al. (1981) did not find any change in body temperature of rat after intracisternal administration of somatostatin in cold environment. Lateral ventricular administration of somatostatin also did not alter the temperature of rabbits (Lipton and Glyn 1980). In only one study somatostatin caused hyperthermia at high ambient temperature and hypothermia at low ambient temperature (Chandra et al. 1981). The pattern of associated effector changes was unusual in that the hyperthermic response at ambient temperature was associated with vasoconstriction and an increase in metabolic rate, whereas the hypothermic response at lower ambient temperature was associated with vasodilation and decreased metabolism. Morley et al. (1982) elicited a small decrease in temperature of rats with i.c.v. injection of somatostatin (10 µg). which was even less if given with TRH. Somatostatin did not appreciably affect changes in temperature induced by bombesin or neurotensin. Intracisternal administration of somatostatln 10 min after pentobarbital did not alter the hypothermic response to the barbiturate (Breese et al. 1975). However, a slight enhancement by i.p. somatostatin of pentobarbital-induced hypothermia has been reported in the mouse (Prange et al. 1975).

E. Angiotensin II

The major response to central administration of angiotensin in most of the species has been a fall in temperature; the monkey, when experiments were conducted in a hot environment, was an exception (Clark 1979). Later, Lin and coworkers (1980) reported that central injection of angiotensin to rats did not alter body temperature when the ambient tenperature was 30°C but caused comparable dose-related hypothermia at lower ambient temperatures. The hypothermic response was inhibited by pretreatment with propranolol or by depletion of whole brain catecholamines with 6-hydroxydopamine. Depletion of brain serotonin by 5,6-dihydroxytyptamine did not inhibit the response to angiotensin, nor did prior administration of atropine or phentolamine. They

concluded that the hypothermic action of angiotensin II is due to its effects on the mechanisms that promote reduced metabolism and increased heat loss. The heat loss effecters vary in different species.

IV. KININS

Small hyperthermic responses, which were associated with increased muscle tone, ear vessel vasodilation, and piloerection were inhibited by pretreatment with indomethacin and acetaminophen. These responses were recorded after lateral ventricular administration of bradykinin to rabbit (Almeida et al. 1978). However, i.c.v. administration of bradykinln to rat (Francesconi and Mager 1981) and to mouse (Nemeroff et al. 1979) did not alter body temperature. Similarly, substance P caused a small rise in temperature of rabbit when given in the third ventricle. This hyperthermic response was reduced by pretreatment with indomethacin, phenobarbital, or phentolamine but not by acetazolamide, atropine, capsaicin, or propranolol (Krupin et al. 1982). In the rat, substance P was consistently ineffective in altering temperature whether given i.c.v., i.v., or intracisternally at any ambient temperature.

V. CONCLUDING REMARKS

Attention has been focused on the role of neuropeptldes in thermoregulation, because many of them are found within neuroanatomical sites important to temperature control and exert profound effects on body temperature. As with several other classes of putative neurotransmitters, the effect seen is dependent on species, ambient temperature, degree of restraint imposed on the subject, route of drug administration, and several other factors. For neuropeptides also the effect observed is dependent on several factors. A limiting factor has been the unavailability of selective pharmacological antagonists in many classes. Specific antisera to many peptides have, however, become available in recent years. The physiological relevance of a peptide to temperature control could be studied by inactivation of the endogenous peptide through local treatment with specific antibodies. There is evidence that certain peptldes such as bombesin, which disrupt thermoregulation, have no physiological significance in temperature control (Clark and Lipton 1983). For many of the peptides, the data available are not yet adequate to determine their roles as mediators in central thermoregulatlon. As noted above, however, convincing data exist to support an antipyretic role for certain peptides, such as ACTH/MSH.

To understand the physiological role of central peptldes in thermoregulation. several methodological factors should be considered in future studies. The effect of peptides should be tested in animals exposed to cold, hot, and thermoneutral environments. This will make it possible to assess the functional nature of the peptide effect. Past research clearly reveals that

species differences do exist for the temperature responses obtained by the central administration of peptides. These may reflect either real differences in the utilization of specific peptides in temperature control or artifactual differences due to physical factors such as variation in spread of peptide to active sites after intracerebral injections. Many systems of the brain are involved in thermoregulation, and multiple contributions of a given neuropeptide to thermoregulatory control may lead to conflicting results when the peptide is injected into different brain regions. It is already known that endogenous peptide levels are affected by stress, and it has been shown that opiate-induced temperature responses are reversed by restraint stress. Stress may be reduced by avoiding restraint; all all studies, of course, appropriate use of control animals is essential to minimize misinterpretation of data, since stress can naver be eliminated from the experiments with conscious animals. The route of administration also plays an important role. Thus it has been shown that intracerebroventricular administration of most agonists, including μ, κ, and ε agonists, produced hyperthermia. possibly because mostly in receptors are reached by that route (Geller et al. 1986).

It is also important to recall that the thermoregulatory apparatus utilizes other major body systems (respiratory, cardiovascular, and others). Thus, for example, a centrally acting peptide that primarily affects the distribution of peripheral blood flow could secondarily alter body temperature and be thought erroneously to act directly on thermoregulatory controls. Direct actions on thermoeffectcr pathways can generally be discovered in experiments carried out in disparate thermal environments. These considerations underline one obvious conclusion: only through triangulation of information from experiments using different methods can the importance of a neuropeptide to thermoregulation be established. Techniques that are relatively new in this field, such as assay of peptides in brain samples taken from febrile, hypothermic, and hyperthermic animals, and immunoneutralization coupled with pyrogen administration or direct thermal challenge, can provide convincing evidence about the role of specific peptides in physiological control of body temperature. There are technical difficulties and other problems with these approaches, but there is little doubt about the advantages they provide.

A second major question in research on neuropeptides is whether they may eventually have some clinical application. In the case of thermoregulation, as with several other functions, it is too early to predict. There is considerable hope, however, that applications will be found once the roles of central neuropeptides are understood. We speculate that the mechanism of action of temperature-altering agents such as pyrogens may be explainable in terms of alterations in central peptides. Naturally occurring changes in these peptides with aging, metabolic diseases, etc., may contribute to the increased incidence of dysthermia in certain populations. Further, the discovery of the temperature-altering

effects of peptides may lead to new therapeutic compounds for antipyresis. for induction of surgical hypothermia, and for treatment of dysthermias such as heatstroke and accidental hypothermia.

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Endogenous Opioids and Immune Responses: An Experimental study

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ABSTRACT

Possible involvementofendogenousopioids inhumoral immune responses has been explored in the experimental animals. Opioid agonists like morphine and leu-enkephalin significantly enhanced antigen-induced histamine release from the peritoneal mast cells of sensitised rats in vitro; this was effectively antagonised by naloxone. Naloxone itself inhibited antigen-induced histamine release. Animals were effectively protected against anaphylactic shock by naloxone which also antagonised morphine-induced increase in anaphylactic mortality. Naloxone reduced haemagglutination titre to sheep red blood cells and IgE antibody titre as measured by passive cutaneous anaphylaxis. Thus, endogenous opioids appear to be involved in the mediation of humoral immune responses. They seem to act at various steps in the immune mechanism viz (i) antibody production and (ii) release of mediators of hypersensitivity reactions.

INTRODUCTION

In the cities we live in, we are surrounded all over by a polluted atmosphere containing billions of heterogenous substances which are foreign to our system. Although most of us can withstand an onslaught by these agents, a considerable number of individuals respond violently and develop various types of immunological disorders. It is, however, not known why only some and not all persons respond violently to these offending substances. It has been reported that many psychosocial situations and stressful conditions can modify host resistance to a variety of illnesses including allergic and infectious disorders and influence the immune processes of an individual (Solomon and Amkraut 1981). Hypothalamus is known to be a centre for correlation and co-ordination of the autonomic and endocrinal activities with the emotions of an individual. Stein et al. (1976) observed that in sensitised animals lesion of the anterior hypothalamus produced significant protection against anaphylaxis, lowered the titre of the circulating antibodies, and also depressed the delayed type of

hypersensitivity reactions. Hypothalamus is rich in neurohormones and neurotransmitters, which may affect immune functions. Opioid peptides are one such group of hypothalamic neuropeptides. A number of reports indicate that administration of opioids or opioid antagonists can modulate the immune competence (Lancet 1984). Hence, it was considered worthwhile to explore the possible role of endogenous opioids in immune responses.

MATERIALS AND METHODS

The experiments were carried out mainly on Wistar rats (100-150 gms) and Swiss mice (20-30 gms) of either sex. They were obtained from the local source and fed on Hind Lever Laboratory animal feed with water available ad lib. Animals were sensitised using egg albumin (EA) or sheep red blood cells (SRBC) as antigens. Freund's complete adjuvant and B. pertussis were used as adjuvants. The results were statistically analysed by Chi^2 test or t test as appropriate.

Acute Systemic Anaphylaxis In Mice

Four groups of mice (15-25 in each group) were sensitised by injecting subcutaneously 0.2ml EA(25mg/ml) along with 0.5ml of triple antigen (containing 20,000x 10°B. pertussis organisms) and 0.2ml of Freund's complete adjuvant. On the 12th day following sensitisation, control group received 0.2 ml of normal saline; one group was given naloxone 10mg/kgi.p., second group receivedmorphine in a dose of 10mg/kg i.p and third group was administered naloxone (10mg/kg) along with morphine (10mg/kg). Thirty minutes after the drug pretreatments all the groups were challenged with 0.2 ml EA i.v., and mortality was recorded after two hours.

Histamine Release From The Peritoneal Mast Cells Of Sensitised Rats In Vitro

Histamine release from the peritoneal mast cells was studied by the method of Chakravarty et al. (1973). Rats were sensitised by injecting subcutaneously 0.5 ml (25 mg/ml) along with 0.5 ml triple antigen (containing 20,000 x 10°B. pertussis organisms) and 0.5 ml Freund's complete adjuvant. Rats were sacrificed by exsanguination on the 14th day; peritoneal cell suspensions were prepared and divided into 10-12 samples. Cells were usually pooled from two sensitised rats. Samples were treated with various drugs under investigation (opioid agonists and antagonist) and incubated at 37°C under gentle shaking for 10 minutes. The antigen (0.5 mg/ml EA) was added to the cell suspension and the incubation, thereafter, continued for another ten minutes. The reaction was terminated by placing the samples in ice cold water. Adequate controls using agonists or antagonist alone were included. Histamine content of different samples was estimated by biological assay using isolated guinea pig ileum. Control histamine release as well as spontaneous release values were detected from the results.

Haemagglutination Titre To Sheep Red Blood Cells (SRBC)

Twogroups of 6 mice were immunized by injecting 1 x 10^9SRBC i.p. One group was given naloxone 5 mg/kg twice daily from day 1 to 5 and the control group received normal saline in the same schedule. Animalswere bled on the 8th day and sera separated. Two fold dilutins (0.025ml) of the seraweremade inthemicrotitre plates with normal saline. To each well 0.025 ml of 1% (V/V) SRBC was added. The plates were incubatedat 37°C for one hour and then observed for haemagglutination.

Passive Cutaneous Anaphylaxis

This was studied in two groups of 6 rats sensitised with 0.5 ml EA (25mg/ml) with 0.5 ml triple antigen (containing 20,000 x 10^6B. pertussis organisms) and 0.5 ml of Freund's complete adjuvant subcutaneously. The test group received naloxone 5 mg/kg twice daily from day 1 to 5 while the control group received normal saline. Animals were bled on the 6th day and sera collected. Serial dilutions of the sera were made,and O.1ml of different dilutions was injected into the shaven dorsal skin of normal rats. 72 hours later i.v. challenge was given with EA (0.5ml) and Evan's blue (1%,0.2ml) and blueing reaction at test site was observed after one hour.

RESULTS

Effect On Acute Systemic Anaphylaxis

Pretreatment with naloxone (1Omg/kg) significantly protected animals against anaphylactic shock; mortality was reduced from 56% in the control group to 32% in the naloxone pretreated group ($p<0.01$). Morphine pretreatment (10mg/kg) enhanced anaphylactic mortality which was effectively antagonised by naloxone ($p<O.001$; Table 1).

Effect On Antigen Induced Histamine Release

Opioid agonists like morphine (100 µM) and leu-enkephalin (10 µM) significantly enhanced antigen induced histamine release from the peritoneal mast cells of sensitised rats in vitro; this enhancement was effectively antagonised by naloxone (Table 2(i) and 2(ii)). Naloxone (1 µM) pretreatment itself significantly inhibited antigen induced histamine release ($p<0.01$, Table 3).

Effect On Haemagglutination Titre To SRBC

In the control group in all the 6 i.e., 100% animals the detectable level of haemagglutination titre was between 1/128 and 1/2048. Pretreatment with naloxone reduced the detectable level of haemagglutination titre to 1/2 - 1/16 in all the 6(100%) animals (Table 4).

TABLE 1. Effect of Naloxone and Morphine on Acute Systemic Anaphylactic Mortality in Mice

Group	No. of Animals	Senitization (Day 0)	Drug Pre-treatment (Day 12)	Chal-lenge	% Mortality	p
1	25	EA+DPT+FA	Normal Saline	EA	56.00	
2	25	EA+DPT+FA	Naloxone 10mg/kg I.P.	EA	32.00	<0.01
3	23	EA+DPT+FA	Morphine 10mg/kg I.P.	EA	78.26	<0.01
4	15	EA+DPT+FA	Morphine 10mg/kg I.P. Naloxone 10mg/kg I. P.	EA	33.33	<0.001

EA- Egg albumin 0.2ml(25mg/ml)
DPT- Triple antigen 0.5 ml (containing 20,000,X 10^6B. pertussis organisms)
FA- Freund's complete adjuvant 0.2 ml

TABLE 2(i) Effect of Morphine and its Modification by Naloxone on the Antigen Induced Histamine Release from Peritoneal Mast Cells of Sensitized Rats in Vitro

Expt. No.	% Histamine Release Egg Albumin (EA) (0.5mg/ml) A	EA+Morphine (100 µM) B	EA+Morphine+Naloxone (1 µM) C
1.	16.0	31.6	4.4
2.	10.5	27.7	12.4
3.	17.4	32.4	7.3
4.	31.7	39.4	21.7

The differences between columns A and B ($p<0.01$) and B and C ($p<0.01$ are significant by t test for paired comparison.

TABLE 2(ii) Effect of Leu-enkephalin and its Modification by Naloxone on the Antigen Induced Histamine Release from Peritoneal Mast Cells of Sensitized Rats in Vitro

Expt. No.	% Histamine Release		
	Egg Albumin(EA) (0.5mg/ml) A	EA+Leu-enkephalin (10 μM) B	EA+Leu-enkephalin+ Naloxone (1 μM) C
1.	17.4	34.1	5.5
2.	10.5	24.0	12.0
3.	16.0	34.0	4.0
4.	31.7	49.6	6.7

The differences between columns A and B ($p<0.001$) and B and C ($p<0.05$) are significant by t test for paired comparison.

TABLE 3. Effect of Naloxone on the Antigen Induced Histamine Release from the Peritoneal Mast Cells of Sensitized Rats in Vitro

Expt. No.	% Histamine Release	
	Egg Albumin (EA) (0.5 mg/ml) A	EA+Naloxone (1 μM) B
1.	11.9	4.3
2.	33.3	3.7
3.	16.0	5.0
4.	10.5	0.5
5.	17.4	6.7
6.	31.7	10.6

The difference between columns A and B is significant ($p<0.01$) by t test for paired comparison.

Table 4. Effect of Naloxone on Haemagglutination Titre to Sheep Red Blood Cells (SRBC) in Mice

Group	Pretreatment	No. of Animals	Antibody Not Detectable	Detectable Level of Haemagglutination titre 1/2-1/64	1/128-1/2048
1.	SRBC ($1x10^9$ I.P.) + Normal Saline (from day 1 to 5)	6	0	0	6 (100%)
2.	SRBC + Naloxone (5mg/kg BD- from day 1 to 5	6	0	6 (100%)	0

Animals wre bled on 8th day after the sensitization with SRBC.

Effect on Passive Cutaneous Anaphylaxis

The detectable level of IgE antibody in the control animals was between 1/1 - 1/10 in 33.33% and up to 1/100 in 66.67% of the animals. In the naloxone treated group, on the other hand, in 50% of the animals the antibodies could not be detected, while in 16.67% cases it ranged between 1/1 - 1/10 and up to 1/100 in 33.33% cases (Table 5).

Discussion

Hypothalamus and other areas of the brain have several neurotransmitters including endogenous opioids. Recently it has been reported that hypothalamus is activated by the immune system which by a feed back mechanism may modulate the immune responses (Besedovsky and Sorkin 1977; Stein et al. 1976). Opioid agonists and antagonists have been shown to modulate the immune competence (Lancet 1984). An agent may affect the immune system by acting atone or more than one step in the immune mechanism. Our experimental studies were confined to finding out the roleofendogenous opioids in humoral immune responses in albino rats and mice using egg albumin and sheep red blood cells as antigens and Freund's complete adjuvant and B.pertussisas adjuvants.

In the initial experiments, role of opioids in acute systemic anaphylaxis was studied. Naloxone, an opioid antagonist when given in a dose of 10mg/kg i.p., significantly protected animals against anaphylactic shock. This corroborates the findings of Amir (1982) who showed that administration of naloxone and naltrexone provided a

TABLE 5. Effect of Naloxone on Passive Cutaneous Anaphylaxis in Rats

Group	Pretreatment	No. of Animals	Antibody NOt Detectable	Detectable Level of IgE Antibody 1/1-1/10	1/100
1.	EA+DPT+FA + Normal Saline (from day 1 to 5)	6	0	2 (33.33%)	6 (66.67%)
2.	EA+DPT+FA + Naloxone (mg/kg BD- from day 1 to 5)	6	3 (50%)	1 (16.67%)	2 (33.33%)

EA- Egg albumin 0.5 ml (25mg/ml)
DPT- Triple antigen 0.5 ml (containing 20,000 x 10^6B. pertussis organisms)
FA- Freud's complete adjuvant 0.5 ml

significant protection against anaphylactic shock. Morphine, an opioid agonist, however, enhanced anaphylactic mortality which was effectively antagonised by naloxone (Table 1). The present findings that apparent blockade of opioid receptors protects against anaphylactic shock suggest a role for endogenous opioids in the pathophysiology of systemic anaphylaxis, a condition associated with antigen antibody interaction.

To further elucidate the involvement of endogenous opioids in immune mechanisms, experiments were done to study the effect of opioids on histamine release, the latter being an important mediator of antigen-antibody reaction. Opioid agonists like morphine (a μ receptor agonist) and leu-enkephalin (a delta receptor agonist) produced a significantincreaseintheantigen induced histamine release from the peritoneal mast cells of sensitised rats in vitro. This increase was effectivelyantagonised bynaloxone (Table 2(i) and 2(ii)) suggesting thereby that the potentiating effect of opioid agonists on antigen induced histamine release is mediated by opioid receptors and both μ and delta receptors appear to be involved. Involvement of μ and delta opioid receptors has also been reported for IgE mediated serotonin release from rat mast cells (Yamasaki et al. 1982). Naloxone 'perse' also significantly inhibited antigen induced histamine release from peritoneal mast cells of sensitised rats in vitro (Table 3) indicating a role of endogenous opioids in the mediation of antigen induced histamine release. The protective effect of naloxone against anaphylactic shock observed in the present study (Table 1) could be because of its inhibitory effect on the mediator release.

Experiments were also done to find out the role of endogenous opioids on antibody production. Naloxone administration effectively reduced haemagglutination titre to SRBC in mice (Table 4), suggesting an effect on IgG and/IgM antibodies. Naloxone pretreatment also suppressed IgE antibody production as measured by passive cutaneous anaphylaxis in the present study. In the naloxone treated group antibody production was completely inhibited in 50% of the animals (Table 5). Thus, it is observed that apparent blockade of opioid receptors by naloxone suppressed formation of anti SRBC and IgE antibodies,i.e.,endogenous opioids, appear to be involved in the synthesis of these immunoglobulins.

In conclusion, the present findings suggest that endogenous opioids appear to be involved in the mediation of humoral immune responses. They seem to act at various steps in the immune mechanism viz(i) antibody production and (ii) release of mediators of hypersensitivity reactions.

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An Update of Selected Topics in the Biology and Chemistry of Opioid Peptides

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The discovery of enkephalins by Kosterlitz and colleagues (Hughes et al. 1975) was immediately followed by an era of synthesis of a large number of opioid peptides. At present, a great deal of attention is being paid to conformational analysis, receptor biochemistry, mechanism(s) of action, addiction mechanisms, and physiological actions of opioids. The chemistry and biology of opioids have been extensively reviewed (Udenfriend and Meienhofer 1984; Rapaka 1986; Rapaka and Hawks 1986; Rapaka et al. 1986; Olson et al. 1987; Dhawan and Rapaka 1988). This article is intended to focus on selected topics only and is not a thorough review. More detailed information is available from the above reviews. In this article, the importance of using pure peptides and the need for extensive characterization of these peptides is emphasized. In addition, some of the recent advances in synthetic methodology, rationales for some of the newly synthesized μ-, δ-, and κ-specific ligands, conformational properties of opioids, the role of lipids in conformational induction of llgands, and a few selected biological aspects (such as immunomodulation by opioids) are briefly discussed.

Numerous opioid peptides have recently been synthesized and evaluated for various biological activities. In order to obtain dependable data, a pure compound is essential and purity of the compound cannot be overemphasized. Much more rigorous analytical testing should be undertaken for commercial samples and for those compounds that are synthesized by solid phase peptide synthesis. As there is no way of establishing absolute purity, as many criteria as possible should be employed to establish the purity of the product. If the peptide is synthesized in-house, the material is likely to be checked for purity at each stage and the end product is therefore likely to be pure. Due to the ready availability of new products from several commercial suppliers, and the facilities for custom synthesis, a large number of studies now utilize commercial samples. Recently. Brown et al. (1986) reported on the problems associated with commercial peptides. Samples of the tachykinin-related peptides were analyzed from several commercial sources. Some of the problems encountered were

these: (1) peptides had incorrect sequences; peptides might have an incorrect residue, or material supplied might be a mixture of the peptides; (2:) peptide content varied as much as three- to sixfold; (3) for radiolabeled compounds, radioactivity might not be associated with the authentic substance. These findings indicate that results obtained with these compounds will make the data worthless and adequate quality control checks must be made for these compounds prior to their use. Amino acid analysis, sequencing data, TLC data in several systems, HPLC analysis in at least two solvent systems, elemental analysis, and any other pertinent physicochemical data should be obtained prior to their use. In addition, optical purity and radiochemical purity (in the case of using labeled products) of the peptides is also critical. A measure of optical rotation alone will not guarantee an optically pure product. Enzyme digestions (Rapaka et al. 1976, 1977) to establish the optical purity, along with utilization of synthetic methods that yield optically pure products, might be helpful. Radiochemical purity of the labeled product should be determined, and since some of the labeled products are unstable, chemical purity should also be determined prior to use.

Batch-to-batch variation should always be monitored. This problem may be much more important with solid phase peptide synthesis than with synthetic material obtained from conventional methods. All batches should be compared with a reference standard batch. Recently, Taylor et al. (1986) reported that anomalous binding of DPDPE resulted due to batch variability, and the results from the impure batch indicated a two receptor model instead of a single receptor model. HPLC purification of the material yielded chromatographically pure compound and gave the predicted results. Hence, even a minor component can potentially lead to erroneous conclusions.

The chemical complexity of peptides makes the analysis of peptides very difficult and demands utilization of innovative, extremely specific, and sensitive methods. In spite of the recent advances (Desiderio et al. 1986; Fridland and Desiderio 1987; Sternson 1987) in analytical techniques for the analysis of peptides, adequate methodology for the analysis of extremely small amounts of opioid peptides, their precursors and metabolites, is not currently available. FAB-MS and LC analysis is increasingly being utilized successfully in the area of peptide analysis (Canova-Davis et al. 1988).

The developmert of solid phase peptide synthesis (SPPS) was instrumental in providing a large number of peptides for structure-activity studies. Following the development of solid phase peptide synthesis by Merrifield (1963), many other methods have been explored to simplify and to automate the synthesis. None of them have really been widely accepted. Recently, Houghten and colleagues have reported a modified solid phase method that is simple (Houghten 1985; Houghten et al. 1986a,b. 1988). This method permits simultaneous synthesis of up to 100 or more peptides

of up to 15 residues in length. The synthesis is fast, and these analogs can be synthesized within a month. No special equipment is needed, and the procedure is not labor-intensive. This method is referred to as the simultaneous multiple peptide synthesis (SMPS), and it involves placing the solid phase resin in a porous polypropylene packet. Several such packets (up to 100 or so) containing the protected amino acid resin can be deprotected together in a Nalgene bottle. Coupling steps are performed in different containers. After the coupling step, the resin packets are returned to the common reaction bottle and the synthesis is continued. At the end of the desired synthesis, the peptide can be cleaved utilizing an apparatus that permits simultaneous cleavage of several samples (up to 24). After the peptides are liberated from the resin, they are purified by routine methods. Thus SMPS permits the synthesis of a large number of peptides with reduced time and cost, and it appears to be suitable for the synthesis and evaluation of a large number of analogs for structure-activity studies.

Another interesting approach to obtain complex and difficult-to-synthesize peptides for structure-activity studies is through the expression of synthetic genes (for a review, see Taylor, this volume). Standard methods of peptide synthesis, either conventional or solid phase methods, are too cumbersome for the preparation of large numbers of peptides, their deletion analogs, and analogs incorporating natural and unnatural amino acid residues. The approach of utilizing synthetic genes and recombinant DNA technology is an attractive alternative to conventional peptide synthesis. This involves synthesis of the desired oligonucleotide sequence and preparation of mutant gene coding for the desired analogs followed by expression. Mutagenesis of the synthetic gene code of the natural structure is the critical step in the generation of the analogs. Mutagenesis may be induced in either a random or a more controlled fashion. Chemicals such as hydrazine, nitrous acid, and sodium bisulfite have been used, as well as physical methods, such as UV irradiation. Other methods include purposeful introduction of one or more restriction enzyme recognition sites to achieve chosen mutations and oligonucleotide directed mutagenesis. Oligotide directed mutagenesis appears to be the most commonly used, and the phosphorothiate DNA method for mutagenesis is one of the most efficient methods to achieve an oligonucleotide directed mutagenesis. Methodologies for the expression of the synthetic gene and purification of the product have been reviewed extensively (see Marston 1986).

Numerous μ-, δ-, and κ- selective opioid analogs have been synthesized, and several reviews have already appeared (Morley 1980; Hansen and Morgan 1984; Morley and Dutta 1986; Udenfriend and Meienhofer 1984). Here, some of the recently synthesized analogs are discussed briefly. One of the usual approaches was "conformational restriction" by cyclization. Synthetic analogs, H-Tyr-Cyclo [-D-A2bu-Gly-Phe-Leu-] (DiMaio and Schiller 1980; DiMaio et al. 1982), homologs containing amino acid residues of

varying chain lengths in position 2 (Schiller et al. 1985; Schiller 1986), and related retro-inverso homologs (Berman et alN 1983) were μ-selective compounds. Later, a cyclic tetrapeptide

H-Tyr-D-Orn-Phe-Asp-NH_2

was designed by Schiller et al. (1985). and pharmacological characterization revealed that it was one of the most μ-selective compounds.

Another approach to the development of P-receptor specific peptides was utilized by Hruby and colleagues (1988). In this approach, a synthetic analog related to the somatostatin fragment, D-Phe-Cys-Phs-D-Trp-Lys-Thr-Cys-Thr(ol), which interacts weakly with opioid receptors as well, was utilized as a template to the development of opioid ligands of high receptor affinity. Following a detailed structure-activity study, a number of μ-receptor selective analogs, such as

H-D--Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP),

were developed. Some of these analogs exhibited decreased affinity for somatostatin binding and a high μ/δ specificity. This template was further extended to analogs containing tetrahydroisoquinoline moieties. This represents a successful manipulation for modulation of one major physiological activity into another activity. Conformational studies on these analogs might shed light on conformation-receptor selectivity relationships.

Recently, novel μ-selective analogs with antagonist activity were reported by Judd et al. (1987). The analogs were obtained by modifications of the Tyrl residues of D-Ala^2-Met^5-enkephalinamide, and the modified tyrosine residues were m-Tyr, ß-methyl-m-Tyr, α,, ß-dimethyl-Tyr and N-phenethyl-m-Tyr. Biological evaluation of these analogs revealed that (1) shifting of phenolic-OH from the ortho to meta position enhanced relative antagonist activity, (2) addition of ß-CH_3 to m-Tyr enhanced P-selectivity. (3) analogs with P-selectivity had a common energy access ble 2-3 B 11' turn, (4) α, ß-dimethyl-m-OH, Tyr^1-analog had a low affinity and an N-phenylethyl analog was a pure antagonist. A rationale for design of peptides based on structural comparison to an opiate compound [3-(m-OH phenyl)-piperidine] was presented.

One of the earliest successful syntheses of receptor-selective ligands was that of a s-receptor specific compound, D-Pen^2, D-Pen^5-enkephalin. Two other examples of δ-selective analogs are linear herapeptides, DTLET and DSLET. and these analogs have been extensively used as δ--receptor probes. Recently, Roques and colleagues reported the synthesis of analogs of

H-Tyr-Ser-Gly-Phe-Leu-Thr-OH and H-Tyr-Thr-Gly-Phe-Leu-Thr-OH (Delay-Goyet et al. 1988). The three analogs reported are [D-Ser2(0-t-butyl), Leu^5] enkephalinyl-Thr^6, DSTBULET; [D-Ser^2(O-t-butyl), Leu^5] enkephalinyl-Thr^6 (o-t-butl), BUBU; and [D-Thr^2(0-t-butyl), Leu^5]-enkephalinyl-Thr^6 DTTBULET. DSTBULET displays a higher affinity for rat brain membrane preparation than DSLET and [D-Pen^2, Pen^5] enkephalin. DSTBULET has a high specific binding, and it is resistant to degradation by peptidases present in the crude rat brain membrane preparation (a $t_{1/2}$ of 1 hr, compared to $t_{1/2}$ of 10 min for Met-enkephalin). BUBU is an even more selective δ-receptor ligand. However, DTTBULET is an inactive ligand. These ligands are apparently influenced by the large conformational changes induced by the bulky t-butyl groups. It was suggested that these analogs are better probes, as the other two δ-selective analogs, DPDPE and DPLPE, although highly δ-receptor specific, have variable affinity depending on the preparation, toxic effects are exhibited at higher concentrations, and more important, have comparatively lower affinity for the receptor site.

Although a large number of μ- and δ-selective peptides have already been reported, very few κ-receptor selective peptide ligands have been reported to date. Recently, Schiller et al. (1988) reported conformationally restricted dynorphin analogs such as [D-Orn^2 Asp^5]-dynorphin A-(1-8). However, these peptides are no longer κ-receptor selective, but μ-receptor selective. This led the authors to hypothesize that these cyclizations resulted in overall folded conformations, which are incompatible with the conformational requirements of the κ-receptor.

Conformational aspects of the peptides are being actively investigated to help design potent and receptor specific compounds, and to understand conformation-receptor selectivity relationships. ^{1}H NMR studies were performed on a number of δ-selective penicillamine-containing enkephalin analogs by Mosberg and colleagues (Mosberg 1987; Mosberg et al. 1987). Discrepancies of conformation-dependent NMR parameters between DPDPE and DPLPE (which have similar δ-selectivities) were observed. It appears that the Gly residue is flexible and as such plays a crucial role, altering its local conformation to allow similar spatial relationships between key groups in different analogs leading to δ-affinity. From NMR data, conformational features of DPDPE and DCDPE appear very similar. However, DPDPE is much more δ-selective and less potent as compared with DCDPE. This suggests that gem-dimethyl groups of Pen^2 in DPDPE lead to adverse reaction. The adverse steric reaction may be much greater at μ-sites than at δ-receptor sites. Studies on the ring expanded analogs of DPDPE and DPLDPE revealed that increasing the ring size led to a reduction in δ-receptor selectivity and that expanded conformations favored μ-receptor binding, whereas compact conformations are favorable for δ-receptor binding. Analogs containing bulky t-Leu residue(s) revealed that bulky substituents at residue 2 decrease affinity to δ-receptors and even more so to μ-receptors.

In a related study by Belleney et al. (1987), conformational properties of DTLET, (H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH) and D-Pen^{2}, L-Pen^{5}-enkephalin (DPLPE) were determined by ^{1}H NMR spectroscopy. The results, surprisingly, indicated similarities in the overall structure for the two-folded backbone conformations for the constrained DPLPE and the flexible peptide DTLET. Relative orientations of the Tyr and Phe residues were found to be similar. Interestingly, the Tyr-Phe distance was 10 A° for both the peptides, which reflects a quasilinear backbone orientation. This extended conformation may be associated with δ-receptor selectivity. From these studies it was hypothesized that incorporation of sterically bulky residues at positions 2 and/or 6 in DSLET and DTLET would reinforce structural analogy to DPDPE-like peptides, thus leading to inhibition of μreceptor binding. However, this should still permit δ-receptor affinity due to the inherent flexibility of the linear hexapeptide. The above hypothesized analogs have been recently synthesized and were shown to have high affinity as well as high selectivity for the s-receptor (Delay-Goyet et al. 1988). In spite of a large number of studies, conformation-receptor selectivity relationships are not adequately understood.

The role of the lipid phase of the membrane for the facilitation of ligand-receptor interaction has been addressed earlier (Schwyzer et al. 1982; Gysin and Schwyzer 1983; Erne et al. 1985; Sargent and Schwyzer 1986; Schwyzer 1986). Erne et al. (1985) demonstrated that interaction of dynorphln A-(1-13) with the lipid membrane results in the formation of an α-helix at the N-terminal (from 1-9, and helix formation beyond the 9th residue is inhibited by Pro 10). The N-terminal message portion is perpendicularly inserted into the hydrophilic membrane. Recently conformational features of dynorphin A-(1-13) and interaction of dynorphin A-(1-13) with lipids were investigated by Renugopalakrishnan and colleagues (Rapaka et al. 1987; Huang et al. 1988; Renugopalakrishnan et al. 1988a,b; Renugopalakrishnan and Rapaka 1988) utilizing NMR. CD, FT-IR and Raman spectroscopy. These studies indicated that in aqueous solution dynorphin has an open structure, and the segment Arg_{6}-Arg_{7}-Ile-Argg- manifests considerable side chain mobility on the NMR time scale and N-terminal segment may be disordered. In the presence of lipid, a steep increase in molar ellipticity value starting at λ;240^{nm} indicated a large conformational change. This is consistent with the observations of Erne et al. (1985). Recently, Schwyzer (1986) estimated the conformation, orientation, and accumulation of dynorphin A-(1-13) on the surface of lipid membrane. Schwyzer used α-helical conformation of dynorphln as the most stable conformation interacting with the hydrophilic phase. He calculated the Gibbs free energy, ΔG for the transfer of increasing number, m, of n-terminal residues from "random" coil conformation in water to α-helical conformation in the hydrophobic phase to predict the preferred conformation of dynorphin and its orientation and accumulation on the surface of neutral lipid membrane. A model of dynorphin was derived in which the hydrophobic segments of the

lipid were in contact with the a-helical segment and the "random" coil segment was protruding into the aqueous phase. This ingenious study presents a plausible explanation of the events leading to the observed interaction of dynorphln A-(1-13).

The role of neuropeptldes in general, and of oplold peptides in particular, is being increasingly studied in the regulation of the immune system, as the neuropeptides appear to be agents of communication between the immune system and the brain (for reviews, see Morley et al. 1987; Plotnikoff et al. 1986a). ACTH suppresses γ-interferon production (Johnson et al. 1984). VIP inhibits the egress of small lymphocytes (Moore 1984). substance P is chemotactic and cross-reacts with the chemotactic receptor of rabbit neutrophlls (Marasco et al. 1981; Ruff et al. 1985; Payan et al. 1983), and somatostatln inhibits the proliferation of T-lymphocytes (Payan et al. 1984). Thus, the studies to date indicate that the neuropeptides may play an important role in immunomodulation and that such modulation may involve the central nervous system, the endocrine system, and the autonomic nervous system (Plotnikoff et al. 1985; Morley et al. 1987; Wybran 1985; Donohoe et al. 1985; Blalock and Smith 1985).

Two interesting and independent observations, one by Wybran et al. (1979) demonstrating the presence of morphine-like and Met^5-enkephalin-like receptors on blood lymphocytes, and the other by Hazum et al. (1979) demonstrating the presence of ß-endorphln receptors on lymphoblastold cells, generated a great deal of interest on the role of oplold peptldes in immunoregulation. Human blood T cells have receptors for morphine and enkephalls, whereas B cells have receptors for ß-endorphin. and T and B cell involvement may be a critical factor in generating an immune response. For researchers in the drug abuse area, study of the role of morphine and other opiates might yield clues to the higher Incidence of bacterial and viral infections in opiate addicts. It has been shown that morphine decreases the abillty to eradicate infections (Tubaro et al. 1983; for a minireview, see Yahya and Watson 1987). Morphine injections into lateral ventricles suppress natural klller cell activity (Shavit et al. 1986), and heroin addicts have decreased T-lymphocyte activity (Singh et al. 1980), thus suggesting that prolonged abuse of opiates impairs the immune system. On the other hand, enkephallns have been shown to prolong the survival time of tumor-bearing mice, enhance lymphocyte blastogenesis in mice, Increase size of thymus in mice. and, in humans, to show significant increase in active T cell rosettes and increase natural killer (NK) cell activity (Plotnikoff et al. 1985). Similarly, ß-endorphin enhances NK activity and active T cell rosettes. The enhancement of NK cell activity is important for defense against bacterial and viral infections. Here it is interesting to note that the NK cell stimulating abllity appears to be associated with the N-terminal sequence of ß-endorphin (Kay et al. 1983). In addition, both Met^5-enkephalln and ß-endorphin enhance interferon production (Mandler et al. 1986).

Morphine depresses T cell E-rosetting (Donohoe et al. 1985), and it was shown by Tubaro et al. (1983) that polymorphonuclear cell activity for ohagocytosis and superoxide radical production by polymorphonuclear cells are depressed. In contrast, ß-endorphin and dynorphin enhance superoxide radical formation (Sharp et al. 1985). It is very interesting that the opiates and opioid peptides act similarly at the analgesic receptors, where they appear to mediate diffe-ent immunologic responses. It is worthwhile that a large number of opiates and oploid peptide analogs be tested for their actions on the immune system and to develop SAR relationships that will lead to the design of therapeutically useful peptide drugs. This is particularly interesting in terms of initial encouraging results obtained with methionine enkephalin in the treatment of AIDS patients (Plotnikoff et al. 1986b). It appears that there is an exciting possibility of obtaining new leads for management of this condition from centrally acting peptides.

Awareness of the important role opioid peptides may be playing in regulation/modulation of physiological activities/behavior has been increasing. There also appears to be a close interaction between the enkephalirs and other naturally occurring peptides and other putative neurctransmitters like dopamine (Radhakishun et al. 1988) and 5-HT (Foster and Roberts 1988). It should be rewarding to study the interaction with other neurotransmitters, particularly the biogenic amines, in greater detail. Among the central autonomic activities possibly regulated by the opioid peptides, the effects on cardiovascular regulation (Said 1987) and thermoregulation (Shukla and Dhawan, this volume) appear to be particularly significant. The role in reaction to stress has been extensively studied, the initial studies having been undertaken with morphine. The recent work has been reviewed by Olson et al. (1987).

There is evidence that some of the central effects of clonidine and central antihypertensives like centhaquin may be mediated via the opioid receptors (Srimal et al. 1988). Phencyclidine may be exerting some of its effects Via μ- or κ-receptors, particularly its effects on body temperature (Hiramatsu et al. 1986).

In the past few years, considerable data have been generated on the role of opioid peptides in the control of eating, which is stimulated by opioid agonists (Majeed et al. 1986) in several species (Kavaliers and Hirst 1986; Yirmiya et al. 1986), as well as that of fluid intake, perhaps through a homeostatic control of water balance (Spencer et al. 1986). It is interesting to note that their effects may depend on the taste. Thus agonists were reported to enhance eating of palatable foods (Jackson and Cooper 1986). This behavior could be suppressed by naltrexone (Kirkham et al. 1986), which also lowered pleasure rating for sweet food and selected odors (Fantino et al. 1986). The possible role of opioids in pathogenesis of several functional disorders of gut has been reviewed by Dhawan et al. (1988).

Recent evidence indicates an important role of opioid systems in the regulation of sexual behavior in both sexes. Initial reports suggest an inhibitory effect of opioid peptides on male sexual behavior via the medial preoptic area (Band et al. 1986), but if the male sex drive is low they may mediate rewards of sex (Miller and Baum 1986). They also mediate sexual behavior in the female, particularly the proceptive behavior (Wiesner et al. 1986). and interaction with 5 HT in such regulatory effects has been suggested (Allen et al. 1986).

There are several neurological disorders in whose etiology opioid peptides may be involved (Dhawan et al. 1988). More definitive data need to be generated in these disorders, but a major limiting factor is the nonavailability of suitable animal models for conditions such as Alzheimer's disease. There is an urgent need also to develop suitable models for neurodegenerative disorders, nerve injury, and other neurological conditions (Taylor et al. 1987). It will be useful to develop sensitive in vitro bioassay systems involving critical biochemical parameters having predictive capabilities.

With the increased availability of data in species other than the rat and mouse, it has become abundantly clear that significant species differences do exist in the effects of opioid agonists, and these help in better understanding of their possible physiological roles. Several investigators have in recent years included multiple species in their studies (Davis et al. 1986; Fanselow and Sigmundi 1986) to assess the central roles of peptides. Recently, studies have been extended, not only to other species of laboratory animals and nonhuman primates, but also to insects, fish, and some unnatural ones like the bat, deer mice, and ground squirrel (Shukla et al. 1988; see Olson et al. 1987 for additional references).

It is difficult to explain some of the results obtained with the opioids based on the currently available data, but more comparative studies are indicated. It is also necessary to extend studies to different age groups of animals, since age-related changes in modification of stress responses (Iny et al. 1986) as well as analgesia (Hoskins et al. 1986) have been reported. It is therefore important to carry out carefully controlled studies before drawing firm conclusions regarding various regulatory roles being assigned to opioid peptides.

A great deal of research work has been carried out during the past decade on opioids, and yet the mechanisms of their various biological actions are far from clear. It is hoped that a greater understanding of their role will finally result in the development of therapeutically useful drugs and help in the alleviation of pain and human misery.

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