NEUROIMMUNOMODULATION: ROLE OF OPIOIDS AND ENDOGENOUS OPIOID-LIKE PEPTIDES IN MURINE IMMUNE SYSTEM

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

ACTH	Adrenocorticotropic hormone
BSA	Bovine serum albumin
ConA	Concanavilin A
CRF	Corticotropin-releasing factor
DS	Dextran sulfate
HBSS	Hank's balanced salt solution
IL-2	Interleukin-2
β-LPH	β-lipotropin
LPS	Lipopolysaccharide
MSH	Melanocyte-stimulating hormone
PBS	Phosphate buffer saline
PEC	Peritoneal exudate cell
PFC	Plaque-forming cell
PHA	Phytohemagglutinin
TCA	Trichloroacetic acid

CHAPTER I

INTRODUCTION

Mind and Body

The relationship between the body and the mind has been one of the basic concerns of philosophers throughout history. The seventeenth century French philosopher Descartes (15) thought that the body and the mind are two and separate, not one and interconnected. Yet, for centuries, anecdotal evidence has accumulated suggesting that the mind can influence vulnerability to diseases. Almost 2000 years ago, the Greek physician Galen noted that depressed women were more likely to get cancer. In addition placebos, which are made up of inactive substances in the place of drugs, have often been prescribed by doctors because these have often proven to be effective in the treatment of patients (29).

The whole organism is now believed to be an open system in dynamic interaction with its environment. Two systems have been developed for the organism to adapt to its environment. These are the nervous (neuroendocrine) and the immune systems. The nervous (neuroendocrine) system is affected by cognitive stimuli that can be physical, emotional, or social. The immune system on the other hand is affected by non-cognitive stimuli such as viruses, bacteria, or tumors. Both types of stimuli are presented to the body through the environment (5, 13).

An interaction between the two systems has been shown (7, 8, 18, 60). Bullock (7), using horseradish peroxidase, found a direct innervation of the thymus from the brain stem and the spinal cord. The innervation of the thymus, spleen, lymph nodes and bone marrow have been known for some time (8, 60). Many, however, assumed that the nerves mainly regulated blood flow. This was questioned by Felton and his colleagues (18) who showed that lymphoid tissues have nerve fibers which not only surround blood vessels, but are also found among lymphocytes, through which they twist and turn. They have also showed that noradrenaline-secreting nerves are found in those areas of lymphoid tissue that have T cells, but not those having B cells. Thus the connections are not random.

Another mode of interaction between the nervous (neuroendocrine) and immune systems is chemically, the mediators of stress being good examples. Stress can be defined as any physiological, psychological, or behavioral response, within the organism, elicited by provocative agents (16).

Stress has been shown to enhance the induction and development of experimental tumors in rats and mice. Stress has also been shown to augment immune functions and retard tumor growth (46). Stress chronicity, timing and intensity appear to play a role in determining the nature of its effect on the immune system (54). The importance of timing can be shown by the work of Amkraut et al. (1). These researchers have examined the effect of timing on the development of Moloney sarcoma virus (MSV)-induced tumors, using brief electric shocks as a stressor. The electric shocks were administered for 4 hrs (10 periods per hr, for a period of 10 sec) on each of 3 consecutive days, prior to or following

virus inoculation. Reduction in the size of tumors was found in mice shocked prior to inoculation with MSV. On the other hand, an enhancement in tumor size was noticed when the shocks were administered after virus inoculation.

It gradually became clear that many immunological parameters were affected by the nature and intensity of exposure to stressors. Acute exposure to a stressor, for example, suppressed the humoral immune response, while repeated exposure resulted in an apparent adaptation of the animals to the stressor, and in some cases, an enhanced response (52). The exposure of mice to sound stress for up to 20 days suppressed the activation of splenic B lymphocytes using lipapolysaccharide (LPS) as a mitogen, but more extended exposure resulted in an enhanced response (36). Decreased T cell responses to mitogenic stimulation occurred for two weeks following peer separation in monkeys raised together from infancy. Mitogen responses returned back to baseline within several weeks of reunion (43).

Stress classically has been shown to act through the hypothalamic-pituitary-adrenal (HPA) axis. The stressor excites the hypothalamus, which acts as a bridge between the brain and the endocrine system. The hypothalamus then releases corticotropin hormone-releasing factor (CRF). The CRF acts on the pituitary gland to elicit increased production and release of adrenocorticotropic hormone (ACTH) into the general circulation. Upon reaching the adrenal cortex, ACTH triggers the secretion of corticoids, mainly glucocorticoids, such as cortisol or corticosterone (47, 57).

Some effects on the immune system of increased corticosterone concentration in the blood plasma are (a) decreased circulating

lymphocytes (lymphocytopenia), (b) thymus involution, and (c) loss of tissue mass of the spleen and peripheral lymph nodes (45, 57). Also, some mineralocorticoids are released affecting salt and water metabolism (25).

In addition to ACTH, stress causes the release of β-endorphin from the pituitary gland into the blood stream (23). Also during stress, enkephalins and catecholamines are released from the adrenal medulla into the blood stream (24). Recent studies have suggested that these substances also interact with the components of the immune system and modify the immune response (37).

Opioids and Endogenous Opioid-Like Peptides

β-endorphin and the enkephalins belong to a group of compounds, found in mammalian tissues, having an activity similar to opium in terms of relief from pain. Opium has been known, for more than 2000 years, to be an analgesic. The principal alkaloids produced from opium and having an analgesic action are morphine (10% of opium), codeine (0.5%), and thebaine (0.2%).

There is much confusion concerning the terminology of drugs having an activity similar to opium (or morphine). Words like "opiates," "opioids," "endogenous opioid peptides," and "endorphins" have often been used interchangeably without a definite delineation between them. In this thesis, the following terms "opioids," "endogenous opioid-like peptides," and "opioid antagonists" will be employed to have the following meanings.

The term "opioids" is used to designate all exogenous substances that bind specifically to any of several types of opioid receptors and produce some agonistic activity. This group includes, but is not restricted to, compounds derived from opium. Some substances, on the other hand, such as naloxone, bind to the receptors for opioids, but exert little or no agonistic activity. They are designated here as "opioid antagonists" because they will prevent the binding and subsequent action of opioids. The term "endogenous opioid-like peptides" specifies all compounds produced in mammalian tissues that bind specifically to one or more of the opioid receptor types. This group is made up of the endorphins, the enkephalins, and the dynorphins.

The presence of highly specific opioid receptors in the mammalian nervous system was first demonstrated by binding studies using morphine, and other compounds produced from opium (4). It was not clear, however, why receptors for compounds produced from opium were present in mammalian tissues. It was, therefore, logical to hypothesize, and look for, endogenous ligands for these receptors.

The actual presence of these endogenous compounds was revealed when researchers succeeded in isolating them from mammalian tissues. Teschemacher et al. (56) demonstrated the presence of a 31 amino acid peptide in the bovine pituitary gland with opioid-like activity. This peptide was ultimately termed β-endorphin (49). Hughes, Kosterlitz and coworkers (27), using pig brain, were able to isolate two pentapeptides with opioid-like activity. They termed them methionine (met-) and leucine (leu-) enkephalin.

β-endorphin is the most potent naturally occurring analgesic agent. It is derived from the parent compound pre-pro-opiomelanocortin

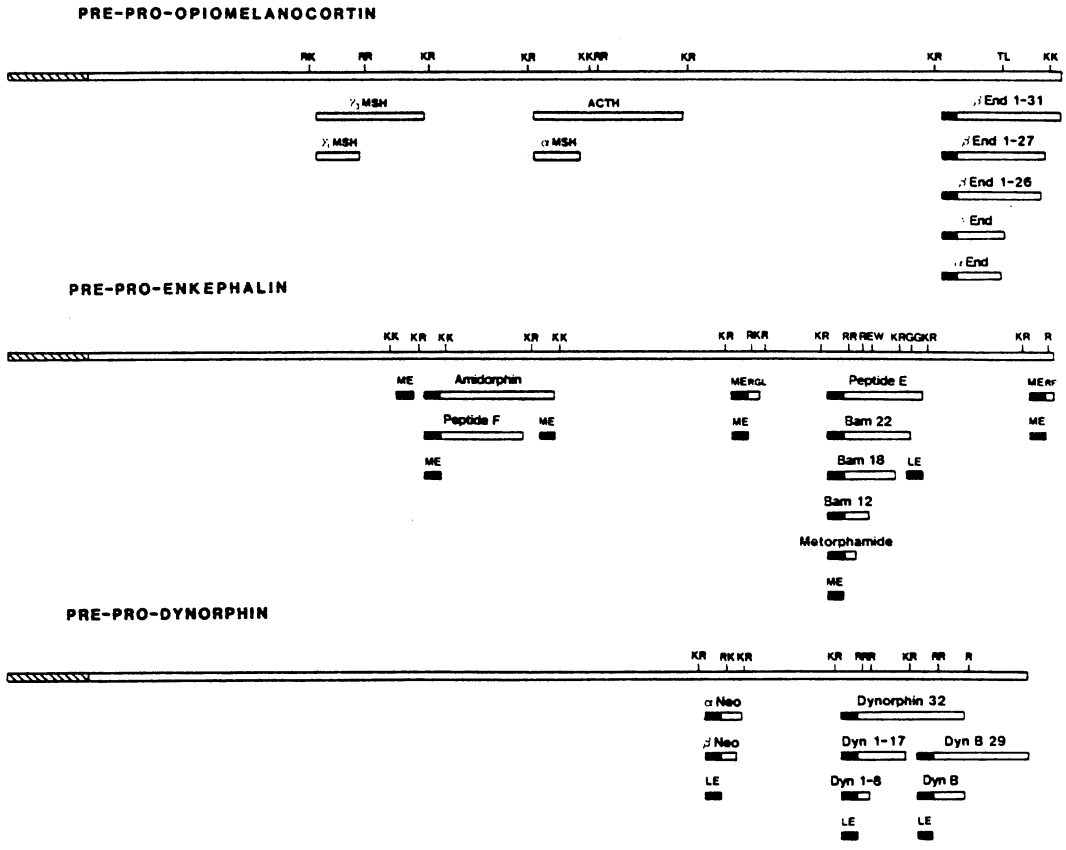
(Figure 1). This latter contains ACTH and MSH in its mid portion and β-LPH in its C terminus. β-LPH is the direct precursor of β-endorphin which constitutes the C terminal 31 amino acids of pre-pro-opiomelanocortin. Two other forms of endorphin termed α- and γ- also exist (4, 17, 22).

β-endorphin is present in highest concentration in the pituitary gland possibly due to the high content of its precursor, β-LPH, there. It can also be found in the brain, especially in the hypothalamus; and is also found, though in small quantities, in the pancreas, the antrum, the eye, and the placenta of some species. It is also present in the reproductive organs of the male rat (10, 22, 51).

α-endorphin is also derived from pre-pro-opiomelanocortin (Figure 1). It is present in small quantities in the brain. It is a peptide made up of 16 amino acids, which are identical to the first 16 amino acids of β-endorphin (4).

Each of the enkephalins, met- and leu-enkephalin, is made up of five amino acids, differing only at their C terminal amino acid. The amino acid sequence of met-enkephalin is Tyr-Gly-Gly-Phe-Met, while that of leu-enkephalin is Tyr-Gly-Gly-Phe-Leu (11). The enkephalins are not derived from pre-pro-opiomelanocortin, although met-enkephalin has the same amino acid sequence as the N terminal five amino acids of β-endorphin. Met-enkephalin, in fact, is the end product in the processing of the precursor peptide pre-pro-enkephalin, where six copies exist. Leu-enkephalin is the end product in the processing of pre-pro-dynorphin as well as pre-pro-enkephalin. Three copies of leu-enkephalin are derived from the first compound, and one copy is derived from the latter (Figure 1) (17, 58).

Figure 1. Molecular origin of endogenous opioid-like peptides. The opioid active core sequences are represented by solid bars (▬) and the putative signal regions of the precursors by thatched bars (▨). The following abbreviations have been used: MSH, melanocyte stimulating hormone; ACTH, adrenocorticotrophic hormone; End, endorphin; ME, met-enkephalin; LE, leucine-enkephalin; Neo, neoendorphin; Dyn, dynorphin; R, arginine; K, lysine; T, threonine; L, leucine; E, Glutamic acid; W, tryptophan; G, glycine; and F, phenylalanine. The structures are for bovine pre-pro-opiomelanocortin, bovine pre-pro-enkephalin and porcine pre-pro dynorphin (17).


PRE-PRO-OPIOMELANOCORTIN
RK
RR
KR
KR
KKRR
KR
KR
TL
KK
γ3 MSH
ACTH
β End 1-31
γ1 MSH
α MSH
γ1 End 1-27
γ1 End 1-26
γ End
α End
PRE-PRO-ENKEPHALIN
KK
KR
KK
KR
KK
KR
RKR
KR
RR
REW
KRGGKR
KR
R
ME
Amidorphin
MERGL
Peptide E
MERF
Peptide F
ME
ME
Bam 22
ME
ME
Bam 18
LE
Bam 12
Metorphamide
ME
PRE-PRO-DYNORPHIN
KR
RKKR
KR
RRR
KR
RR
R
α Neo
Dynorphin 32
β Neo
Dyn 1-17
Dyn B 29
LE
Dyn 1-8
Dyn B
LE
LE

The enkephalins are found in the brain, the gut, and the adrenal medulla, where it has been localized to chromaffin cells containing catecholamine by immunocytochemistry (22, 24).

Opioid Receptors

The concept that there are several types of opioid receptors originated from the observation that, in man, nalorphine had a dual action, antagonizing the analgesic effect of morphine and also acting as an analgesic in its own right. It was concluded that the analgesic effect of nalorphine was mediated by a receptor which is different from that for morphine. That concept was reaffirmed by finding different pharmacological profiles among several opioids (39).

The existence of multiple opioid receptors is now well established. The receptors that are generally accepted are the μ, κ, δ, and to a lesser degree, ∂ types (Table I). The μ-type favors morphine-like drugs, such as morphine and pethidine, and the opioid antagonist naloxone. The κ-type favors cyclazocine-like drugs (i.e., ketocyclazocine and ethylketocyclazocine) as well as the dynorphins. The δ-type favors the enkephalins, including the synthetic compound (D-ala^2, D-leu^5) enkephalin. The ∂-type favors N-allylnormetazocine (SKF 10047). β-endorphin appears to be equally active at both μ and δ receptors (3, 9, 39, 48, 63).

Opioid compounds bind stereospecifically, and the pharmacological activity resides in the (-) isomer, while the (+) isomer is devoid of

TABLE I

BINDING OF OPIOIDS TO MULTIPLE OPIOID RECEPTORS

Opioid Receptor Type	Ligand
μ	morphine, pethidine, naloxone, and β-endorphin
κ	ketocyclazocine, ethylketocyclazocine and the dynorphins
δ	met-, leu-enkephalin and β-endorphin
∂	N-allylnormetazocine

this activity.[1] In the morphinan series, for example, levorphanol, the (-) isomer has all the analgesic activity where as dextrorphan has none.

Little information about the membrane components responsible for binding has yet accumulated, because of resistance to solubilization and purification in an active form. Simon et al. (50) claimed that a single high molecular weight binding component of 370,000-380,000 was observed following extraction with Brij 36-T, a non-ionic detergent, but they did not publish its sepharose elution profiles. The chemical nature of opioid receptors are still under investigation, and some work suggests that they contain protein and lipid components.

Physiological Effects of Endogenous Opioid-Like Peptides

Non-Immune Systems

Endogenous opioid-like peptides have been known primarily for their behavioral effects. Their central administration into the brain causes analgesia. Also, some endogenous opioid-like peptides cause dream-like euphoria, and reality escape, similar to morphine which has been known for centuries to have mood altering properties. Moreover, a role for these peptides in mental diseases has been suggested, when it was shown that an intra-cerebroventricular administration of β-endorphin in rats induced catalepsy (30). Catalepsy is a condition characterized by waxy rigidity of the muscles.

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[1]The (+) isomer is dextrorotatory because it rotates a plane of polarized light in a clockwise direction, which a (-) isomer is levoratatory because it rotates the plane of polarized light in the opposite or counter-clockwise direction.

Endogenous opioid-like peptides are also involved in the physiological regulation of cardiovascular functions at various levels. They increase the vagal tone and decrease the central sympathetic activity resulting in bradycardia and hypotension. They are also mobilized if the baroreceptor-mediated and other hypotensive mechanisms are insufficient to keep the blood pressure under a certain limit (4, 55).

Besides their addiction liability, suppression of normal breathing, which can be observed sometimes at an analgesic dose level, is probably the main contraindication of their clinical application. This respiratory depressant action of opioids can be reversed by opioid antagonists, such as naloxone, which actually explains their efficiency in the treatment of acute opioid poisoning (4, 11).

Contrary to their respiratory and cardiovascular action, the intestinal effects of endogenous opioid-like peptides are primarily mediated by peripheral opioid receptors situated in the gastrointestinal tract. These endogenous opioid-like peptides inhibit spontaneous peristalsis mostly after meals. They also decrease pancreatic and intestinal fluid secretions (55).

Endogenous opioid-like peptides act also as thermoregulators. They induce hyperthermia by elevating the thermoregulatory set point of the organism's thermostat in the anterior hypothalamus resulting in inhibition of heat-escaping behavior (4).

Immune System

During stress, β-endorphin was found to be released from the pituitary gland (23). The variations in its secretion from the pituitary

gland, and in its circulating plasma level were not correlated with changes in the cerebral concentration of the peptide. That meant that its effect might be directed somewhere else.

The effect of opioids and endogenous opioid-like peptides on the immune system was first suggested by the pioneering work of Wybran in 1979 (62). Wybran et al. suggested the presence of met-enkephalin-like and morphine-like receptors on human peripheral blood T lymphocytes. They performed active rosette assays between T lymphocytes and sheep red blood cells and reported that met-enkephalin increased the percentage of active T rosettes while morphine decreased it. Plotnikoff and coworkers (35) have reported an increase in active T cell rosettes after addition of met-enkephalin but not leu-enkephalin. Enkephalins have also been reported to decrease the spleen weights of mice at both 10 and 30 mg/kg doses (42).

McCain and coworkers (33), using human T lymphocytes, reported that β-endorphin decreases blastogenesis at a concentration of 10^{-7} M, but could not show it at 10^{-9} M. PHA was used as the mitogen. The addition of naloxone did not have a reversing effect, which suggests that the action of β-endorphin on PHA-mediated blastogenesis is via a non-opioid receptor mechanism. In contrast, Gilman (20) has reported an enhancement of rat lymphocyte proliferative responses using PHA and ConA as mitogens, and β-endorphin as a modulator of the response. When a mixture of lipopolysaccharide and dextran sulfate (LPS/DS) was used no significant effect was shown. These results suggest that the effect is mediated through T lymphocytes and that B lymphocytes are not involved. Using naloxone did not reduce the effect. In contrast to β-endorphin, α-endorphin and [D-ala^2-met^5] enkephalin did not modify proliferative

responses to mitogen. Plotnikoff and coworkers (41) have reported that leu-enkephalin increases lymphocyte blastogenesis using PHA as a mitogen. This effect was noticed in dilutions of 1:100 and 1:250 of PHA but not in 1:500. Also an increase in lymphocyte blastogenesis was noticed for met-enkephalin at concentrations ranging between 10^{-4} - 10^{-12} M using dilutions of 1:100 of PHA. At lower concentrations of PHA (dilution 1:500), only high doses of ($2x10^{-4}$ M) enkephalins were significant.

In contrast, Wybran (61) could not show any significant effect of enkephalins using human peripheral blood lymphocytes. No effect on mitogen-induced proliferation was observed except in the case of leu-enkephalin at a very narrow concentration range (10^{-5} M).

Mathews et al. (32) have published data showing that β-endorphin (10^{-14} M) and met-enkephalin (10^{-9}) could significantly increase the spontaneous cytotoxicity induced by natural killer (NK) cells against ^{51}Cr labeled K562 target cells. The increased NK activity was dose dependent. Naloxone inhibited the augmentation of NK activity produced by β-endorphin and met-enkephalin. Thus, in this assay, β-endorphin activity appears to be mediated via classical opioid receptors. By using soft agarose single cell analysis assays, they showed that β-endorphin could increase the number of effector to target cell conjugates and the number of active NK cells. They could not show any modification of NK activity with α-endorphin (10^{-12} - 10^{-6} M), leu-enkephalin (10^{-12} - 10^{-6} M), or morphine (10^{-12} - 10^{-6} M).

Wybran et al. (61) have also attempted to determine whether morphine and several enkephalins could modify the human NK activity of peripheral blood mononuclear cells against ^{51}Cr radiolabeled K562 cells. The

results can be summarized as follows. Morphine, leu-enkephalin and met-enkephalin increase NK activity by approximately 30 percent and usually at a concentration ranging between 10^{-8} and 10^{-5} M. However, D-ala^2-met^5 enkephalin increased NK activity less than the previously mentioned compounds (around 20%) and in a smaller range of concentration. The increase in NK activity elicited by each of these compounds was reversed when each compound at 1 μM was incubated with naloxone at 1 μM.

Perhaps these latter results relate to the observation of Plotnikoff et al. (43) that BDF mice inoculated with L1210 leukemia cells, and treated with leu-enkephalin or met-enkephalin, had a lower mortality than untreated animals. Inasmuch as NK activity may be a control mechanism in tumor growth, it is possible that the lower mortality was related to an enhanced NK activity. Unfortunately, immunological tests were not performed on these animals.

Johnson et al. (28) have reported that ACTH and α-endorphin were potent inhibitors ($\geq$ 80% suppression) of the antibody response to the T-cell-dependent antigen, sheep erythrocytes, at a concentration of 0.5 μM. Met- and leu-enkephalin were moderate inhibitors (approximately 60% suppression) at 0.2 - 2 μM, and β- and γ-endorphin were minimal inhibitors (approximately 20% suppression) at 5-6 μM.

Brown et al. (6) demonstrated that β-endorphin and met-enkephalin inhibited the production of a T lymphocyte chemotactic factor (LCF) by Con A-stimulated peripheral blood mononuclear cells. Inhibition of LCF production was observed by using concentrations of 10^{-11} - 10^{-6} M β-endorphin or met-enkephalin but not α-endorphin.

These results are summarized in Table II which exhibit conflicting reports about the effect of some opioids and endogenous opioid-like

TABLE II

EFFECT OF OPIOIDS ON SEVERAL IMMUNE PARAMETERS

Immune Parameter	Species	Author (Reference)	Compound	Effect
Lymphocyte Proliferation	Rat	Gilman (20)	β-Endorphin	↑
	Human	McCain (33)	β-Endorphin	↓
	Murine	Plotnikoff (41)	Met-enkephalin	↑
			Leu-enkephalin	↑
	Human	Wybran (61)	Met-enkephalin	→
			Leu-enkephalin	→
			Morphine	→
Active T-rosette	Human	Plotnikoff (35)	Met-enkephalin	↑
			Leu-enkephalin	→
	Human	Wybran (62)	Met-enkephalin	↑
			Morphine	↓
Plaque Forming Cells (PFC's)	Murine	Johnson (28)	α-Endorphin	↑
			β-Endorphin	→
			γ-Endorphin	→
Natural Killer (NK) Cell Activity	Human	Mathews (32)	β-Endorphin	↑
			Met-enkephalin	↑
			Leu-enkephalin	→
			Morphine	→
			α-Endorphin	→
	Human	Wybran (61)	Met-enkephalin	↑
			Leu-enkephalin	↑
			Morphine	↑
Lymphocyte Chemotactic Factor (LCF) Production	Human	Brown (6)	β-Endorphin	↓
			α-Endorphin	→
			Met-enkephalin	↓

Key: ↑ = enhanced; ↓ = inhibited; → = no effect.

peptides. Even within one parameter, there is much variability and, indeed, contrasting results. In lymphocyte proliferation, for example, β-endorphin exerted two opposite effects (20, 33). Also met-enkephalin and leu-enkephalin did not exhibit consistent effects on lymphocyte proliferation (41, 61). Therefore, it is difficult to draw conclusions using these results. Evidently, there is a need for more investigation into the effects of these compounds, in several immune parameters, for us to be able to understand the available results.

Opioid Receptors on Immune Cells

These various effects on the immune system suggested the presence of opioid receptors on immune cells. Indeed, a few studies reported the presence of these receptors on different immune cells using direct binding assays. Lopker et al. (31) demonstrated the presence of opioid receptors on human phagocytic leukocytes. Both human granulocytes and monocytes exhibited stereospecific, high-affinity binding of 3-quinuclidinyl benzilate (QNB) and dihydromorphine. When using QNB, the apparent Kd to monocytes was approximately 20 nM, while the apparent Kd to granulocytes was approximately 16 nM.

Hazum et al. (26), using ^{125}I-labelled $^{\beta}$H[D-ala^{2}] endorphin, reported the presence of specific non-opioid receptors for β-endorphin in cultured human lymphocytes. They suggested that the C-terminal region of β-endorphin is essential for this binding activity, since they found that α-endorphin was not active.

Mehrishi et al. (34) have also reported the presence of naloxone and morphine receptors on human peripheral blood lymphocytes and platelets.

Ausiallo et al. (2) reported the existence of leu-enkephalin binding activity on the surface of a human lymphoid T cell line. They suggested that binding was due to receptors made up of Lipoproteins. Their suggestion was based on the finding that phospholipase A_2 and proteinase K caused a marked inhibition of the enkephalin binding.

Lastly, Johnson and coworkers (28) reported the presence of opioid receptors on mouse spleen cells using [^{3}H] met-enkephalin. They suggested the existence of at least one type of binding site for [^{3}H] met-enkephalin with Kd = 5.9×10^{-10} although they presented no experimental data to verify that. All results are summarized in Table III.

Considering the reports of functional actions (Table II), it seems clear that opioids and endogenous opioid-like peptides have effects on the immune system of some mammalian species including mice. These functions should be mediated by receptors on immune cells, the presence of which has been reported (Table III). Nevertheless, these results are still few, and are not conclusive. The reported presence of receptors by Mehrishi (34) was neither clear nor very convincing. Furthermore, the Kd reported by Johnson (28) for a binding between met-enkephalin and opioid receptors on mice spleen cells was not substantiated by data to verify that.

Facing a scarcity of information and a need to clarify some reported results, it was important to look for the presence of opioid receptors on immune cells to demonstrate that opioids and endogenous opioid-like peptides actually bind to these cells to carry on their functions. My hypothesis was that there are opioid receptors of at least one type on mice spleen cells. A binding assay would provide a direct method when

TABLE III

BINDING STUDIES TO IMMUNE AND BLOOD COMPONENTS

Type of Cells	Species	Author (Reference)	Ligand	Possible Receptor
Monocytes	Human	Lopker (31)	[^{3}H]dihydromorphin	μ
Granulocytes	Human	Lopker (31)	[^{3}H]dihydromorphin	μ
Cultured Lymphocytes	Human	Hazum (26)	$^{\beta}$H-[^{125}I]-(D-ala^{2})-endorphin	non-opioid
Peripheral Blood Lymphocytes	Human	Mehrishi (34)	[^{3}H] naloxone	μ
Platelets	Human	Mehrishi (34)	[^{3}H] naloxone	μ
Cloned T Cells	Human	Ausiallo (2)	[^{3}H] leu-enkephalin	δ
Mouse Spleen Cells	Murine	Johnson (28)	[^{3}H] met-enkephalin	δ

looking for the presence of such receptors. Mice were available to us through our colony at Oklahoma State University. Consequently, it was feasible to use their spleen cells for our research. Two reasons prompted me to use spleen cells; the first was lack of binding studies to these cells using opioids and endogenous opioid-like peptides, and the second was the availability of immunological tools related to the immune cells of mice.

CHAPTER II

MATERIALS AND METHODS

Animals

One to four month old male and female BALB/c mice were used. These mice were obtained from a colony that originated from the Jackson Laboratory (Bar Harbor, ME) and maintained at Oklahoma State University. All mice were housed at a maximum density of six animals per cage and were maintained on Lab Blox (Ellison's Seed and Supply, OK) and water ad libitum. The light/dark cycle was at 12 hr intervals.

Reagents

[^{3}H] naloxone (54 Ci/m mole) and [^{3}H](D-ala^2, D-leu^5) enkephalin (29.1 Ci/m mole) were purchased from Amersham International (Amersham, U.K.). β-endorphin, α-endorphin, (D-ala^2, D-leu^5)-enkephalin, (D-ala^2)-methionine enkephalin, naloxone, and Phytohemagglutinin (PHA-P) were purchased from Sigma Chemical Company (St. Louis, MO). [methyl-^{3}H] thymidine (2.0 Ci/m mole), econofluor, and aquasol were purchased from New England Nuclear (Boston, MA). Whatman glass microfibre filters, AH-934, GF-A, -B, -C, -D, and -F (2.4 cm diam.) and the Metrical Membrane Filter, GA-6 (2.4 cm diam.), were purchased from Fisher Scientific Company (Pittsburgh, PA). Trichloroacetic acid (TCA) was purchased from MC/B Manufacturing Chemists, Inc. (Norwood, OH).

Buffer and Media

Enriched RPMI 1640 and 10% FCS (pH 7.4) was used as a suspending medium for cells in lymphocyte proliferation assay. They have the following ingredients per liter: RPMI 1640 medium (1 pack), sodium pyruvate (110.0 mg), L-glutamine (292.0 mg), MEM amino acids, 50x (20.0 ml), MEM non essential amino acids, 100x (10.0 ml), MEM vitamins solution, 100x (4.0 ml), penicillin (100 IU/ml), streptomycin 74% (135 mg/l), and 10% FCS (100 ml) purchased from GIBCO (Grand Island, NY). Amphotericin B (2.5 mg), deoxycholate (2.05 mg), and 2-mercaptoethanol 0.35% (1.0 ml) were purchased from Sigma Chemical Company (St. Louis, MO).

Hank's balanced salt solution (HBSS), pH 7.4, was purchased in powder form from GIBCO (Grand Island, NY) and rehydrated according to instructions.

Phosphate buffer saline (PBS), pH 7.4, 296 mOsM contained 7.9 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, and 154 mM NaCl.

Tris-ammonium chloride (Tris/Cl) contained 0.17 M Tris [(hydroxymethyl) amino methane] and 0.16 M ammonium chloride.

Spleen Cell Preparation

The method of Ortez (38) was modified as described for preparing mice spleen cells. The mice were anaesthetized with chloroform and killed by cervical dislocation. They were pinned down to a cardboard, the abdominal cavity of each was opened, and the spleens were cut loose from the surrounding mesentery and fat. Each spleen was cut into 2-4 pieces which were disrupted in a dual 22 homogenizer in HBSS.

The spleen cells were dispensed in HBSS (5 mls/spleen) and centrifuged (CRU-5000 (IEC)) at 1800 RPM for 12 min. The supernatant was discarded and the spleen cells were washed again in HBSS (3 mls/spleen). After the second wash, the spleen cells were suspended in Tris/Cl (2.5 mls/spleen) to lyse RBC's. After an incubation period of 10 min at room temperature, the cells were centrifuged at the same conditions as above. They were resuspended in HBSS (2-3 mls/spleen) and counted under the microscope using a hemocytometer. Cell viability, determined by Trypan blue (0.2%) exclusion, ranged between 90-95%. The number of spleen cells collected from each mouse averaged between 1 and $2x10^8$. The number of mice used in each experiment ranged between 1 and 4. If the cells were to be used in a proliferation assay, this process was carried out under sterile conditions and the cells were suspended in enriched RPMI 1640 and 10% FCS at the final stage.

Brain Membrane Preparation

A mouse used as a spleen donor was also used to prepare brain membranes. After removing the spleen, the skull was opened with the help of a pair of scissors and forceps. The brain was removed, and homogenized between the frosted ends of two microscope slides. Brain membranes were washed two times and resuspended in HBSS (5 mls/brain).

Peritoneal Exudate Cells (PEC's) Preparation

In PEC's preparation, the method of Gallily and Feldman (19) was modified as described. Three mice were injected intraperitoneally (i.p.) with 2-2.5 mls of 3% thioglycollate using a 21 g needle. The variation

in the amount of injected thioglycollate was related to the size of mice used.

Four days later, PEC's were harvested. The mice were anaesthetized with chloroform. Each mouse was pinned down to a cardboard with the abdomen up. An incision was made through the skin, and up the center line. The skin was pulled to the sides exposing the abdominal wall. HBSS (4 mls) were injected into the peritoneal cavity, using a 26 g needle. The HBSS injected contained 5 U/ml of heparin to prevent blood clotting. The injection was made along the mid-anterior line. The abdomen area was then massaged to allow the HBSS to circulate throughout the abdominal cavity. A lateral pull on the abdomen was applied to create a pocket of fluid at the flank area. After 5 min, a needle was inserted, and suction was applied to retrieve the peritoneal fluid; 3-3.5 mls were usually harvested. This procedure was done for all three mice used in each experiment.

PEC's from the injected mice were added together and were centrifuged for 15 min at 2400 RPM. After the cells were washed again, the supernatant was aspirated, and the pellet was suspended in 2 mls of Tris/Cl. After an incubation time of 10 min at room temperature, the cells were centrifuged for 15 min at 2400 RPM. The supernatant was aspirated, and the cells were resuspended in 3 mls of HBSS.

The PEC's tended to clump due to thioglycollate stimulation. Consequently, counting the cells under the microscope using a hemocytometer, gave an underestimate of the amount of cells that are there. The PEC's recovered ranged between 2 and 5 x 10^7 cells for three mice.

Cell Proliferation Assay

The method of Gilman (20) for cell proliferation was modified as described. A sterile suspension of spleen cells was used in each experiment. The mitogen PHA and one of the endorphins or the enkephalins (test compounds) were added to spleen cells in culture tubes. A number of spleen cells (varying with the experiment) were suspended in 800 µl of enriched RPMI and 10% FCS. 100 µl of 100 µg/ml PHA was added to the spleen cells. Immediately after the addition of PHA, 100 µl of enriched RPMI and 10% FCS or test compound was added. The compound tested and its concentration varied with the experiment. For the most part, they were endorphins and enkephalins compared over concentration ranges of 1×10^{-10} to 1×10^{-4} M. All test compounds as well as PHA were prepared as 10x stock solutions in enriched RPMI and 10% FCS.

Two kinds of controls were used. The first control represents the basic proliferation of spleen cells without using a mitogen. These control tubes contained only spleen cells in 1 ml of the suspending medium. The second control represented cell proliferation of spleen cells due to PHA only, without the use of a modulator (an endorphin or an enkephalin). These control tubes contained spleen cells and PHA in 1 ml of the suspending medium. In all experiments, the first control and experimental cultures were run in triplicate, while the second (mitogen positive) control cultures were run in sextuplicate (six times).

The control and experimental culture tubes were incubated in a humidified water-jacketed chamber (National, a Heinacke company) of 3% CO_2 in air, at 37° C. They were pulsed with 200 µl containing 0.5 µCi of [methyl-^{3}H] thymidine, at 56 hour of culture.

Cell culture was terminated by centrifugation of culture tubes at 74 hour, in a CRU-5000 centrifuge (IEC) at 2400 RPM, for 15 min. Supernatants were aspirated and the spleen cells were resuspended in 100 µl of PBS. The contents of each culture tube were transferred to 13 x 26 mm pieces of 3 MM filter papers and kept overnight to air dry.

The next day, the filter papers were immersed in 10% TCA (10 ml/filter paper) for 20 min. Then they were transferred to another batch of 10% TCA (10 ml/filter paper) for 20 min. Each filter paper was then dipped four times in chloroform, and placed under a heated lamp to dry for approximately 15 min. The filter papers were left for 5 min to cool, and then each was transferred to a glass vial having 10 mls of econofluor scintillation fluid. Radioactivity was measured using Beckman LS 7500 liquid scintillation counter.

Receptor Binding Assay

In receptor binding assays, the method of Mehrishi (34) was adopted as modified. Radioactive opioid compounds were used to look for opioid receptors on mouse spleen cells. Mouse brain cell membranes were used as positive controls because they carry both µ and δ opioid receptors. The radioactive compounds used were either [^{3}H] naloxone or [^{3}H](D-ala^2, D-leu^5)-enkephalin. Binding assays were conducted in duplicate, triplicate, or sometimes pentuplicate, using culture tubes (12x75) for incubation. Binding experiments were carried out in glass tubes when [^{3}H] naloxone was the radioactive compound used. In contrast, plastic tubes were employed when [^{3}H](D-ala^2, D-leu^5)-enkephalin was used, because of the potential binding of peptides to glass. Mouse spleen cells (1 x 10^7) (or brain membranes) were suspended in a final

volume of 300 µl of HBSS and added to each test tube. Either 1.1×10^5 DPM of [^{3}H] naloxone or 5.5×10^5 DPM of [^{3}H](D-ala^2, D-leu^5)-enkephalin, dissolved in 50 µl of HBSS, were quickly added to spleen cells (or brain membranes) unless otherwise mentioned.

Specificity of the binding was demonstrated by adding 50 µl of a 1×10^{-4} M solution of unlabelled naloxone or (D-ala^2, D-leu^5)-enkephalin (depending on the radiolabel used) to compete with the radioactive compound, while 50 µl of HBSS were added to other samples. Specific binding was the difference in binding in the presence and the absence of unlabelled ligands. After addition of the [^{3}H] compound, the samples were incubated at room temperature for 1 hr, unless otherwise stated.

In competitive binding assays, 50 µl of unlabelled naloxone at a concentration range between 1×10^{-4} to 1×10^{-9} M competed with [^{3}H] naloxone, while 50 µl of HBSS were added to other samples. The culture tubes were then treated similar to those in other binding assays.

In saturation assays, 50 µl of unlabelled (D-ala^2, D-leu^5)-enkephalin were added to 50 µl of [^{3}H](D-ala^2, D-leu^5)-enkephalin ranging in concentration between 5.5×10^3 to 5.5×10^5 DPM, while 50 µl of HBSS were added to other samples. The culture tubes were treated similar to those in other binding assays.

A filtration technique was employed to separate the bound and free radioactive compounds. A filtration box, having 12 wells connected to a vacuum pump, was used (Millipore). In each well, one fiberglass filter (Whatman AH-934) was positioned. Each filter was sealed with a polyethylene ring to prevent any leaks to the outside.

At the end of the incubation time, each filter paper was quickly wetted with 2 mls of HBSS having 1% bovine serum albumin (BSA). Bovine

serum albumin reduced the amount of non-specific binding to filter papers. The samples were then quickly rinsed with cold HBSS (4 washes, 2 mls each wash) which was applied to the filters. The samples were treated one at a time. The total elapsed time for filtration and washing was approximately 45 sec per sample. The vacuum was applied at a level that would keep the filter wet between each wash, but buffer would not remain standing on the filter.

After finishing all the samples, the filters were placed in plastic vials containing 6 mls of aquasol. The contents of the vials were then mixed well by shaking. The vials were stored for a few hours, and then counted in a Beckman LS 7500 liquid scintillation counter.

Protein Determination Assay

The Coomassie Blue microassay was used to estimate protein. Biorad reagent (0.4 ml) was added to 1.6 ml of protein solutions (BSA standard or unknown samples). After 10 min, absorbance was read on a spectrophotometer (spectronic 20) at $\lambda 595$. A curve relating absorbance to protein content of the standard BSA solution was generated. The protein content of each sample was estimated from this curve.

Statistical Analysis

Results of cell proliferation assays were expressed as the mean cpm of PHA stimulated cultures minus the mean of control (basal cell proliferation) cultures $\pm$ standard error mean (SEM). The paired Student's t test was used in determining significant differences between experimental cultures (PHA and an endorphin or an enkephalin) and cultures having only PHA. $p \leq 0.05$ was considered statistically

significant (53). Stimulation index (SI) was the ratio of the mean cpm of experimental cultures to the mean cpm of cultures having only PHA.

Results of binding assays were expressed as mean cpm minus nonspecific binding to the AH-934 filters. The Paired Student's t test was used to determine the presence of specific opioid receptors by comparing treatments having unlabelled ligands with those lacking them. $p \leq 0.05$ represented a significant difference between the two treatments.

CHAPTER III

RESULTS

Standardization of Binding

Choosing an Appropriate Filter

Before beginning to study opioid receptors on lymphocytes, it was necessary to develop a suitable radioligand binding assay. The first component of the assay to be tested was the step involving separation of bound and free radioligands. Because of its speed of action, a filtration method was selected. Table IV shows the results of a study to determine the most appropriate filter type. Several grades of fiberglass filters and a single pore size membrane filter were tested. Each filter type was tested in triplicates and triplicate determination was made on each filter.

In this experiment, 6×10^6 or 9×10^6 (depending on experiment) spleen cells were suspended in 600 µl of PBS and added to the filter. The filter was then washed 4 times (2 mls each) with PBS. The filtrate was collected and measured 8.6 ml. The protein content of three 1.6 ml aliquots of each filtrate was estimated as described in Materials and Methods. The average absorbance and calculated protein content for each filtrate are presented in Table IV. The protein content of the entire 600 µl spleen cell suspension was determined using cells disrupted in deionized water. The percentage of protein passing through the filter

TABLE IV

PERCENTAGE OF PROTEIN PASSING THROUGH A FILTER

Filter Type (pore size)	Filter 1		Filter 2		Filter 3		Protein Avg. (µg)	Protein Total (µg)	Protein Passed (%)
	Absorbance[a] ± SEM	Protein (µg)	Absorbance[a] ± SEM	Protein (µg)	Absorbance[a] ± SEM	Protein (µg)			
GA-G (0.45 µm)	0.095 ± 0.002	4.9	0.092 ± 0.004	4.77	ND	ND	4.84	26.02	22.9[b]
GF-F (0.7 µm)	0.038 ± 0.006	1.47	0.030 ± 0.001	1.13	0.015 ± 0.001	0.40	1.00	5.38	7.1[c]
GF-B (1.0 µm)	0.031 ± 0.004	1.18	0.040 ± 0.001	1.60	0.025 ± 0.009	0.89	1.22	6.56	11.5[c]
GF-C (1.2 µm)	0.054 ± 0.002	2.80	0.054 ± 0.003	2.80	0.049 ± 0.005	2.50	2.70	14.51	12.8[b]
AH-934 (1.5 µm)	0.036 ± 0.005	1.94	0.056 ± 0.003	2.90	0.054 ± 0.003	2.80	2.55	13.71	12.1[b]
GF-A (1.6 µm)	0.080 ± 0.001	3.48	0.037 ± 0.005	1.45	0.058 ± 0.004	2.41	2.45	13.17	17.5[c]
GF-D (2.7 µm)	0.083 ± 0.003	3.62	0.079 ± 0.007	3.42	0.086 ± 0.004	3.81	3.62	19.46	25.8[c]

[a]Absorbance at λ 595.

[b]Amount of protein poured on the filter was 113.52 µg.

[c]Amount of protein poured on the filter was 75.37 µg.

was calculated as the ratio of the protein passing through the filter to the amount added. These values are expressed in the last column of Table IV.

Based upon the study, filter GF-F showed the best protein retention value (7.1%) and would have been our first choice. However, another factor, equally important in filter choice, was the amount of non-specific binding of radioligand to filters. Therefore, the four best filters by protein retention (GF-F, GF-B, GF-A, and AH-934) were selected for evaluation of [^{3}H] naloxone retention. The filters were prewashed with 2 mls of HBSS. Then 250 µl of HBSS having 1.1 x 10^5 DPM of [^{3}H] naloxone, without spleen cells, was quickly added to the prewashed filters. The results are shown in Table V. A definite pattern was clear from these results. The larger the pore size, the less [^{3}H] naloxone was retained. Consequently, given radiolabelled compound binding alone in choosing a filter, AH-934 would have been our optimum choice. AH-934 was ultimately selected for use throughout the rest of the studies because it allowed the least amount of nonspecific binding to filter (66.8 $\pm$ 5.4), and at the same time, a moderate amount of proteins to pass through (12.1%).

Effect of Washing on [^{3}H] Naloxone Retention

In binding assays, it is important to keep nonspecific binding to a minimum. One source of nonspecific binding is radioligand that is not effectively washed away. Therefore, an experiment was performed to determine the number of washes of a filter necessary to effectively reduce nonspecific binding (see Table VI). Filtration was done using

TABLE V

NON-SPECIFIC RETENTION OF [^{3}H] NALOXONE ON FILTERS

Filter Type (pore size)	[^{3}H] Naloxone Retention on Filter Paper (CPM ± SEM)
AH-934 (1.5 μm)	66.8 ± 5.4
GF-B (1.0 μm)	78.6 ± 3.5
GF-C (1.2 μm)	79.1 ± 10.1
GF-F (0.7 μm)	93.9 ± 3.6

Note: No spleen cells were incubated with [^{3}H] naloxone, or added to filters. The experiment was run in triplicates.

TABLE VI

EFFECT OF WASHING ON [^{3}H] NALOXONE RETENTION BY AH-934 FILTERS

Number of Washes	[^{3}H] Naloxone Retention on Filter Paper (CPM ± SEM)
2	379.2 ± 61.1
4	223.8 ± 28.9
6	169.1 ± 7.4
8	135.7 ± 34.9
10	85.6 ± 0.4

Note: Results are expressed as cpm excluding non-specific binding to filters AH-934. The experiment was run in duplicates.

AH-934 filters which were washed with 2, 4, 6, 8, or 10 washes (2 mls/wash). The results clearly showed the more washes applied to a filter, the less [^{3}H] naloxone was being retained on that filter. Notwithstanding these results, it was assumed that too many washes might be reducing specific binding as well. Therefore, it was decided that four washes were enough to reduce nonspecific binding, and still be able to maintain specific binding.

Role of BSA as an Antiadsorbent

Bovine serum albumin (BSA), being an antiadsorbent, was chosen to prewash the filters. Different concentrations of BSA ranging between 0.0 to 4.0% were used. [^{3}H] naloxone (1.1×10^5 DPM) was added to the prewashed filters without incubation with spleen cells. The results have shown that BSA decreased the amount of nonspecific binding to filters when used at concentrations ranging between 1 and 2% by about 33% (Figure 2). When the amount of BSA was increased up to 4.0%, there was a substantial increase in binding (306%). Thus, it was decided to prewash filters with 1% BSA in all future experiments.

Effect of Number of Spleen Cells on Binding

A variable number of spleen cells ranging between 0.75×10^6 and 1×10^8 were suspended in HBSS and incubated with 1.1×10^5 DPM of [^{3}H] naloxone. Figure 3 shows an increase in binding as the number of spleen cells was increased. These results represented a total of both specific and nonspecific binding. It was felt that at a high concentration of spleen cells, nonspecific binding would be substantial, a condition unfavorable in binding assays. Also, at very high

Figure 2. Prewash of AH-934 with bovine serum albumin. No spleen cells were incubated with 1.1 x 10^5 DPM of [^{3}H] naloxone. The experiment was run in duplicate.

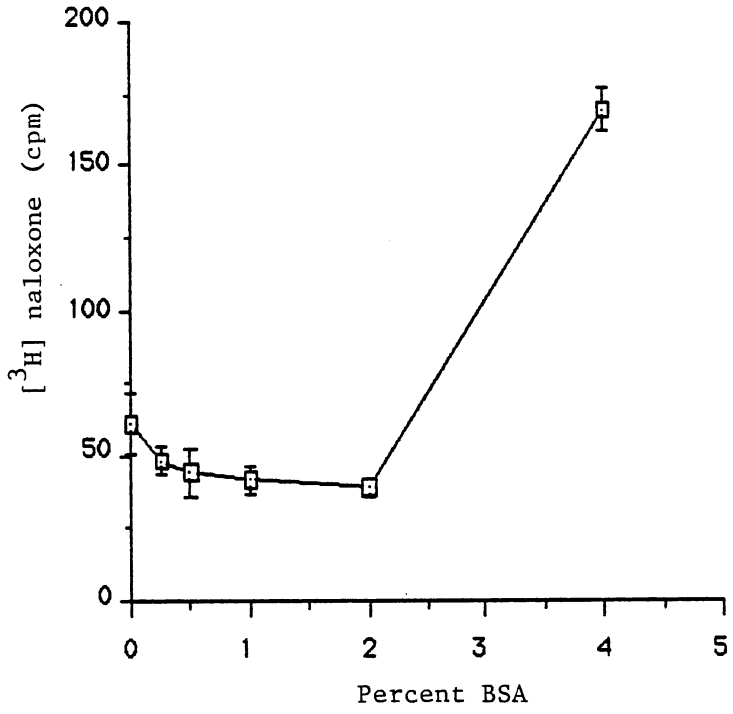
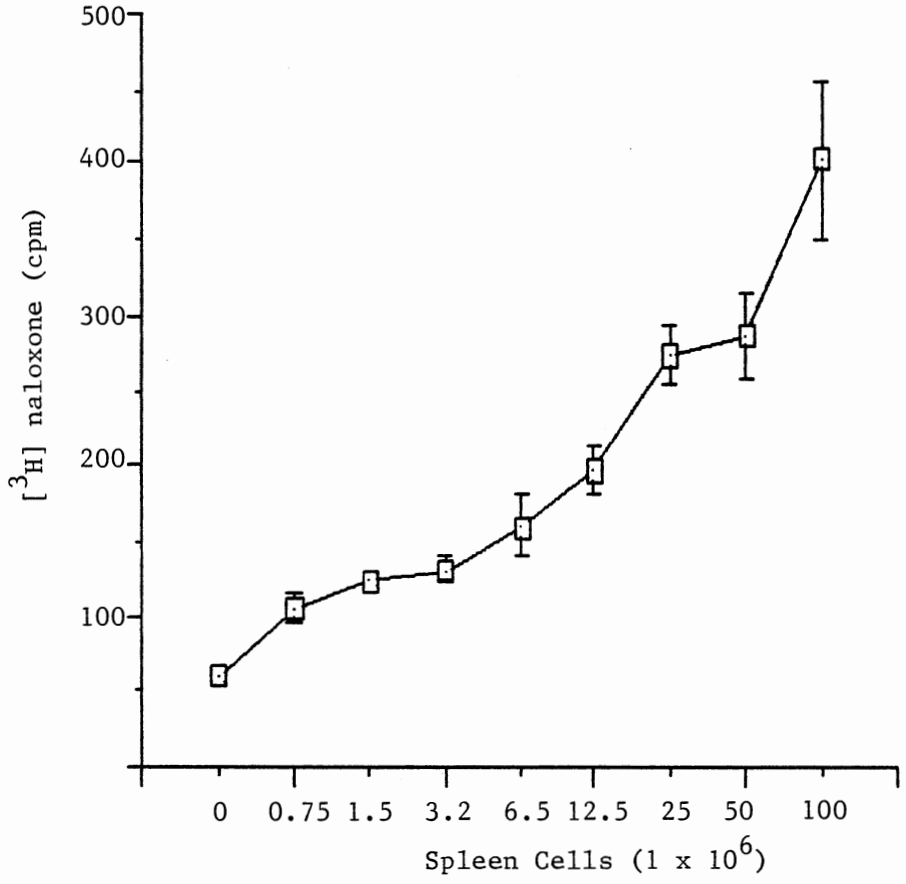

200
150
100
50
0
[3H] naloxone (cpm)
0
1
2
3
4
5
Percent BSA

Figure 3. Effect of spleen cell number on binding. Spleen cells ranging between 0.75 x 10^6 and 1 x 10^8 were incubated with 1.1 x 10^5 DPM of [^{3}H] naloxone, for 30 min at 37°C. Experiment was run in triplicate.


500
400
300
200
100
[3H] naloxone (cpm)
0
0.75
1.5
3.2
6.5
12.5
25
50
100
Spleen Cells (1 x 10^6)

concentrations (5×10^7 - 1×10^8) spleen cells were not suspended well in the medium. Therefore, we decided to use 1×10^7 spleen cells in future binding assays.

[^{3}H] Naloxone Binding to Spleen, Brain and PEC

Competitive Inhibition Using Spleen Cells

[^{3}H] naloxone was incubated with 1×10^7 spleen cells. Then, variable concentrations of unlabelled naloxone ranging between 1.7×10^{-9} and 1.7×10^{-5} M were added to the incubated cells (Figure 4). Although there was a difference between control and experimental tubes, this difference was not statistically significant ($p \leq 0.05$). This was an indication that there was probably no specific receptors of the μ type. Nevertheless, this experiment had been done at a single condition (room temperature, 1 hr) and it could not be ruled out that other conditions might reveal receptor specific binding.

Relation Between Incubation Time and Binding

Spleen cells were incubated with [^{3}H] naloxone at room temperature for different incubation periods ranging between 30 min and 3.0 hrs. At each incubation time, specific binding was tested by adding unlabelled naloxone (Table VII). There was no significant difference between the two treatments (with and without unlabelled naloxone) at all incubation times. Nevertheless, a small difference between the two treatments persisted.

Figure 4. Competitive inhibition of naloxone: Spleen cells. Variable concentrations of unlabelled naloxone ranging between 1.7×10^{-8} and 1.7×10^{-5} M were added to the incubated cells. Cells were incubated for 1 hr at room temperature. Results are expressed as cpm excluding nonspecific binding to AH-934 filters. Experiment was run in duplicate.

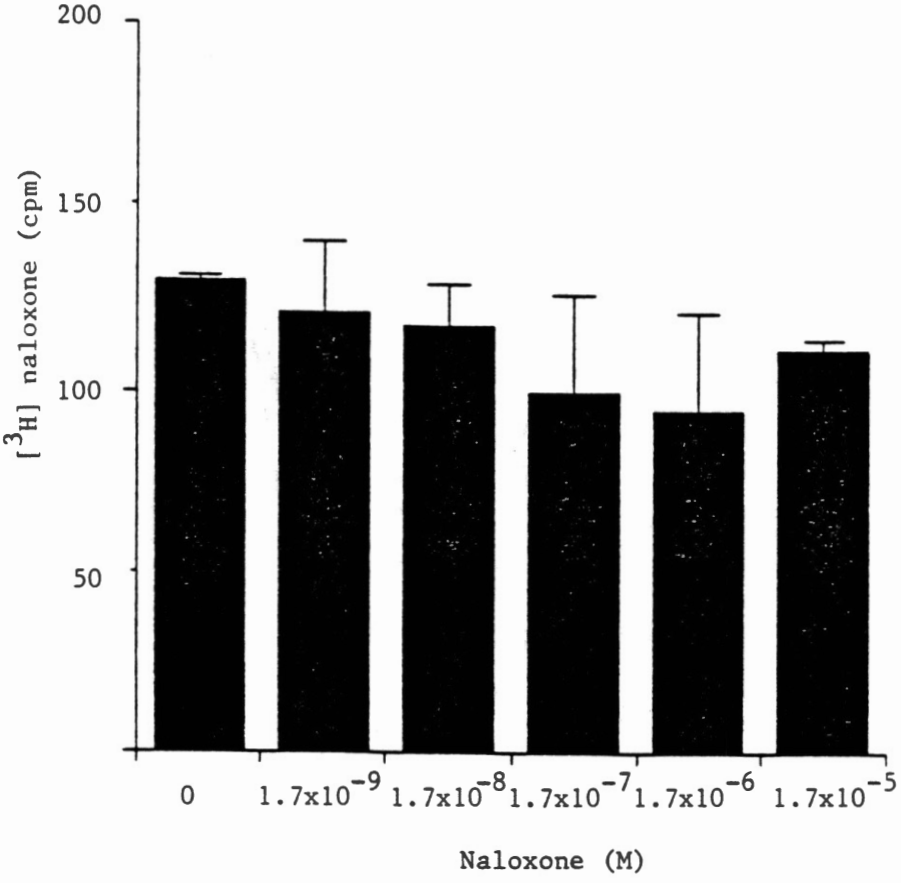

200
150
100
50
[3H] naloxone (cpm)
0
1.7x10-9
1.7x10-8
1.7x10-7
1.7x10-6
1.7x10-5
Naloxone (M)

TABLE VII

EFFECT OF INCUBATION TIME ON SPECIFIC BINDING

Incubation Time	(-) Cold Naloxone (CPM ± SEM)	(+) Cold Naloxone (CPM ± SEM)
30 min	190.3 ± 12.8	173.5 ± 3.2
50 min	158.0 ± 14.1	142.5 ± 16.4
1 hr	200.6 ± 41.4	101.0 ± 28.4
1.5 hrs	144.8 ± 13.2	97.2 ± 33.6
2 hrs	156.9 ± 62.6	93.1 ± 14.8
3 hrs	191.0 ± 27.3	166.9 ± 13.8

Note: Spleen cells were incubated with [^{3}H] naloxone at room temperature for incubation periods ranging between 30 min and 3.0 hrs. 1.7×10^{-5} M naloxone was added for some tubes for the indicated incubation time. The experiment was done in duplicate. Results were expressed as cpm excluding nonspecific binding to AH-934 filters.

Specificity of Binding to Brain Cell Membranes

There was the possibility that the binding assay was not working right. A competitive inhibition assay using brain cell membranes would serve as a proper control, since it was known that they contain opioid receptors of the μ type. Brain cell membranes were incubated, with [^{3}H] naloxone and variable concentrations of unlabelled naloxone between 1.7×10^{-9} and 1.7×10^{-6} as mentioned in the materials and methods. Figure 5 shows that an increase in the concentration of unlabelled naloxone led to the decrease of [^{3}H] naloxone binding. The competitive inhibition was significant ($p \leq 0.05$) at all concentrations of unlabelled naloxone used. At 1.7×10^{-6} M unlabelled naloxone there was a 80.2% decrease in binding, and still the curve did not level off. This competitive inhibition was a clear demonstration of specific binding. Thus our assay system was working well.

Specificity of Binding: Brain and Spleen

It was important, therefore, to use brain cell membranes (positive control) and spleen cells in the same experiment. They were incubated with 1.1×10^5 DPM of [^{3}H] naloxone for 1 hr at room temperature. 1.7×10^{-5} M unlabelled naloxone was used to inhibit [^{3}H] naloxone binding. It is clear from Figure 6 that brain membranes contained specific receptors for naloxone. On the other hand, the spleen cells again failed to show any significant ($p \leq 0.05$) specific binding (111.3 ± 10.8 compared to 89.7 ± 9.9). This further suggested that either there are no specific μ type opioid receptors in spleen cells or that the receptors were too few to be detected by our assay procedure. In another experiment, 5.5×10^5 DPM of [^{3}H] naloxone were incubated with

Figure 5. Competitive inhibition of naloxone: Brain cell membranes. Variable concentrations of unlabeled naloxone ranging between 1.7×10^{-9} and 1.7×10^{-6} were added to brain cell membranes which were incubated with 1.1×10^{5} DPM of [^{3}H] naloxone for 1 hr at room temperature. Experiment was run in triplicate. Results are expressed as counts excluding nonspecific binding to AH-934 filters. (*) indicates a significant difference ($p \leq 0.05$) from control.

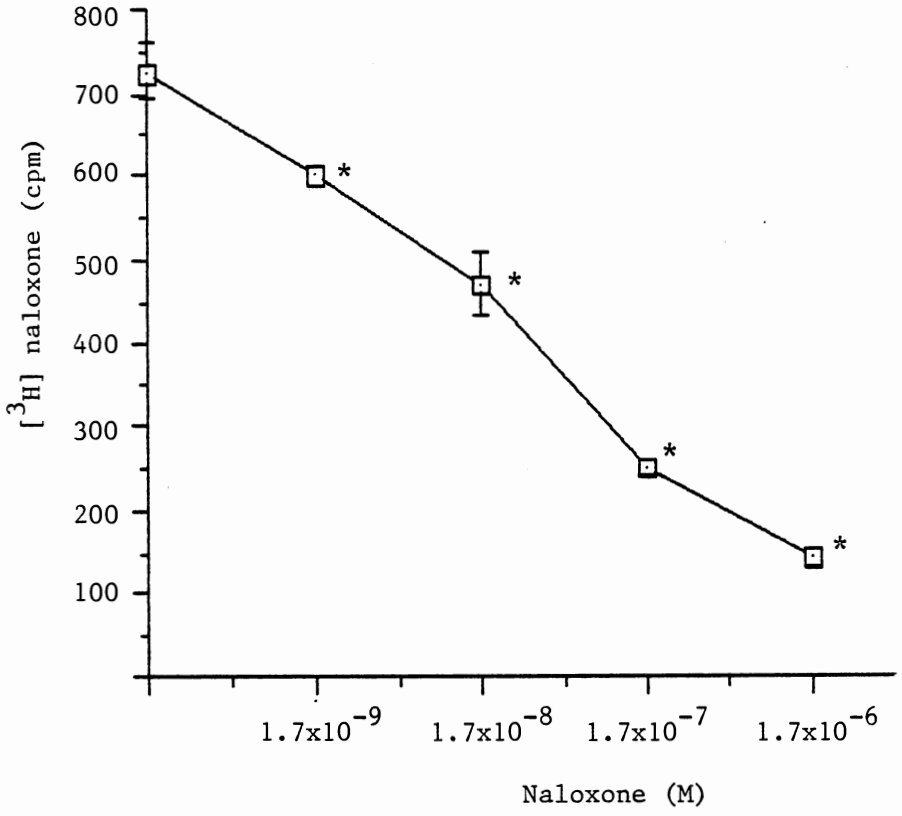
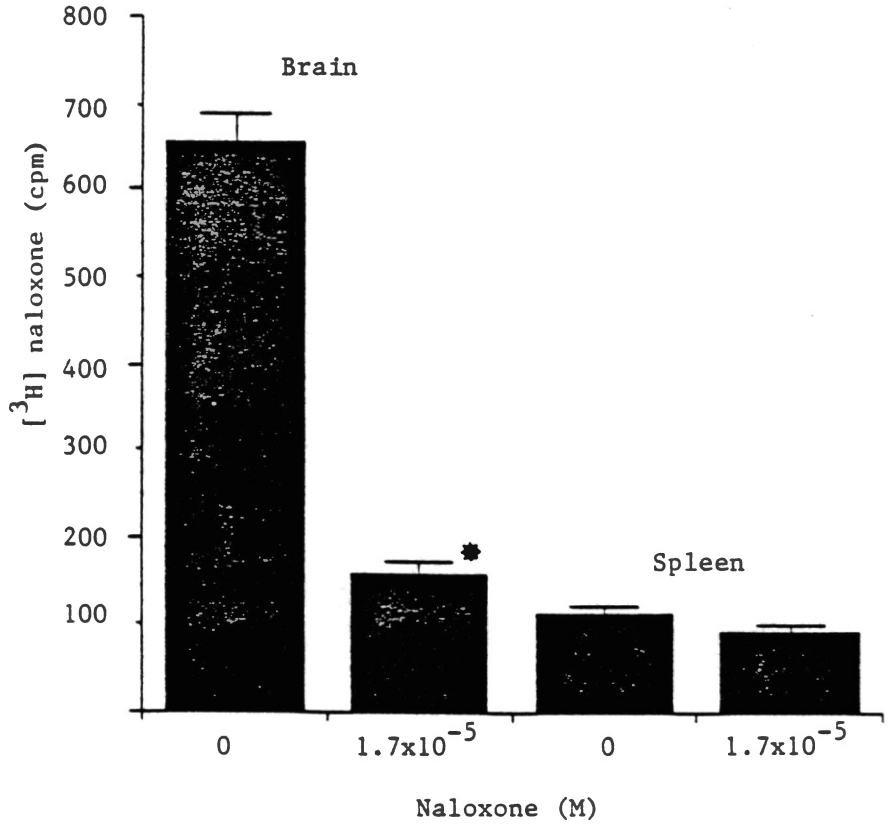

800
700
600
500
400
300
200
100
[3H] naloxone (cpm)
*
*
*
*
1.7x10-9
1.7x10-8
1.7x10-7
1.7x10-6
Naloxone (M)

Figure 6. Specificity of [^{3}H] naloxone binding using 1.1 x 10^5 DPM: Brain and spleen. The cells were incubated with [^{3}H] naloxone for 1 hr at room temperature with and without 1.7 x 10^{-5} M naloxone. Experiment was run in triplicate. Results are expressed as cpm excluding nonspecific binding to AH-934 filters. (*) indicates a significant difference ($p \leq 0.05$) from control.


800
700
600
500
400
300
200
100
[3H] naloxone (cpm)
Brain
Spleen
*
0
1.7x10-5
0
1.7x10-5
Naloxone (M)

spleen cells with or without 1.7×10^{-5} M naloxone for 1 hr at room temperature. Figure 7 shows that there was no significant difference between the two treatments for spleen cells. This meant that increasing the concentration of [^{3}H] naloxone did not provide any evidence of a significant ($p \leq 0.05$) specific binding to μ receptors. On the other hand, 1.7×10^{-5} M naloxone inhibited [^{3}H] naloxone binding to brain cell membranes, demonstrating specific binding to μ receptors.

Competitive Inhibition: Brain and PEC

The persistent observation of a small amount of specific binding to spleen cells prompted the speculation that there may be a small population of cells possessing a high number of receptors. One minor component of spleen cells are macrophages. Therefore a competitive inhibition assay using both PEC's, a source of macrophages, and brain cell membranes was performed to determine the presence of specific opioid receptors on PEC's. It is clear from the data in Figure 8 that PEC's, like spleen cells, failed to show a decrease in binding upon the addition of 1.7×10^{-5} M unlabelled naloxone. This is an indication of the absence of specific μ receptors on PEC's. Brain cell membranes, serving as a positive control, clearly showed specific binding. A second experiment also using PEC's gave comparable results.

Standardization of Proliferative Assay

Effect of PHA Concentration

Phytohemagglutinin (PHA), a predominantly T cell activator, was used as a mitogen. Different concentrations of PHA ranging between 5 and 30 μg/ml PHA were added to 1.0 x 10 spleen cells (Figure 9a). The dose

Figure 7. Specificity of [^{3}H] naloxone binding using 5.5 x 10^5 DPM: Brain and spleen. The cells were incubated with [^{3}H] naloxone for 1 hr at room temperature with and without 1.7 x 10^{-5} M naloxone. Brain cell membranes were run in singlet. Spleen cells were run in pentuplicate. Results are expressed as counts excluding nonspecific binding to filters.

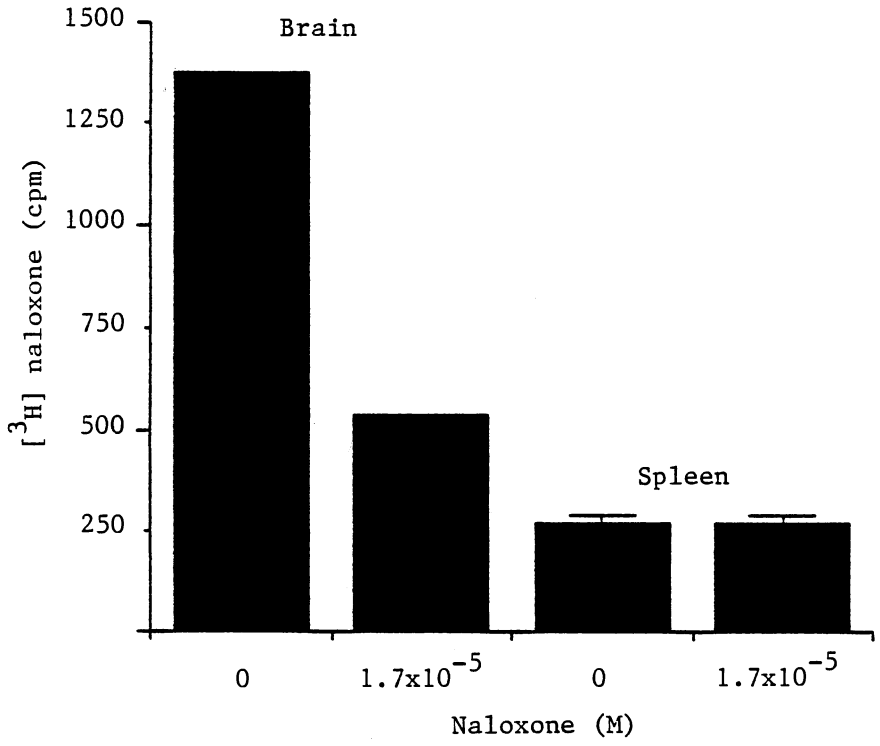

1500
1250
1000
750
500
250
[3H] naloxone (cpm)
Brain
Spleen
0
1.7x10-5
0
1.7x10-5
Naloxone (M)

Figure 8. Specificity of binding: Brain and PEC. 0.8×10^7 PEC's were incubated with [^{3}H] naloxone for 1 hr at room temperature with and without 1.7×10^{-5} M naloxone. Experiment was run in triplicate. Results are expressed as counts including nonspecific binding to filters. (*) indicates a significant difference ($p \leq 0.05$) from control.

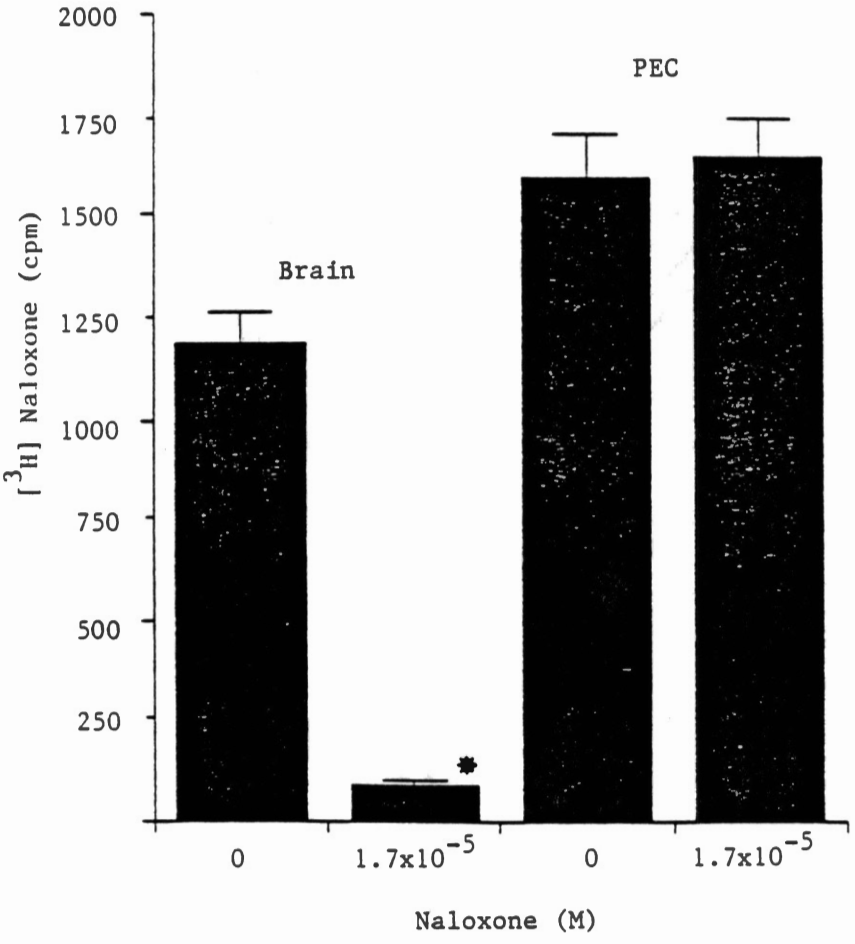
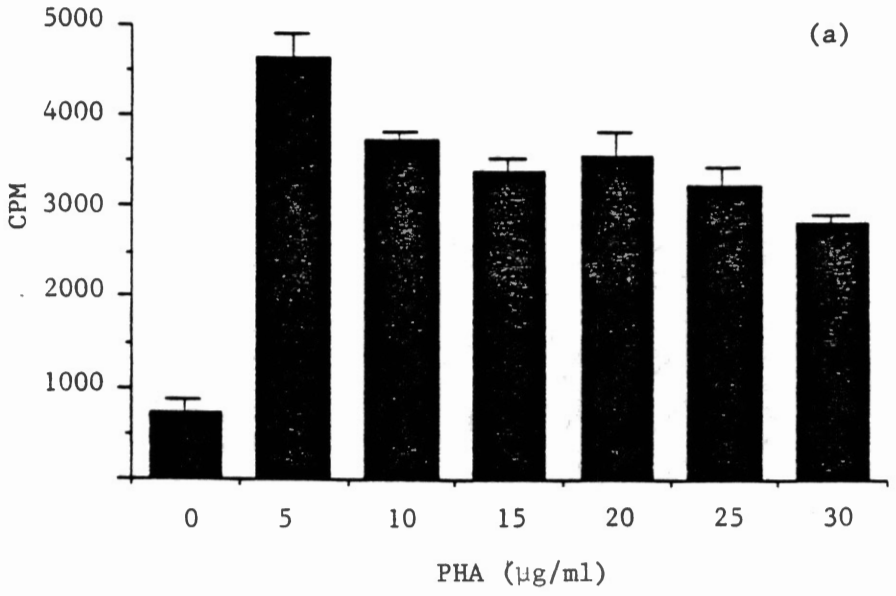
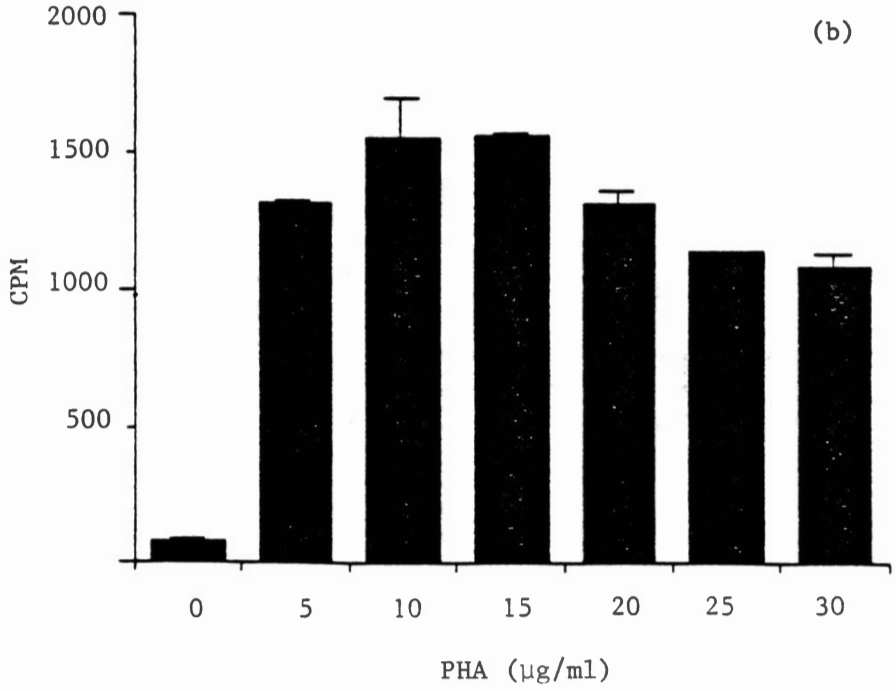

2000
1750
1500
1250
1000
750
500
250
[3H] Naloxone (cpm)
Brain
PEC
*
0
1.7x10-5
0
1.7x10-5
Naloxone (M)

Figure 9. Effect of PHA concentration on PHA-induced lymphocyte proliferation. Spleen cells were incubated with variable PHA concentrations ranging between 5 and 30 μg/ml. (a) Spleen cells used were 1.0×10^6. (b) Spleen cells used were 2.5×10^6.


(a)
5000
4000
3000
2000
1000
CPM
0
5
10
15
20
25
30
PHA (µg/ml)


(b)
2000
1500
1000
500
CPM
0
5
10
15
20
25
30
PHA (µg/ml)

response curve generated, peaked at 5 μg/ml and dropped down steadily as the concentration of PHA increased. At 5 μg/ml, PHA caused about 540% increase over basal cell proliferation. This increase dropped down to 410% at 10 μg/ml. A slightly different dose response curve was generated using 2.5×10^6 spleen cells and the same concentration range of PHA (Figure 9b). The curve peaked at between 10 and 15 μg/ml of PHA and then dropped down steadily at higher concentrations.

Effect of Cell Number

A variable number of spleen cells ranging between 0.5×10^6 and 10×10^6 were tested to determine the effect of spleen cell number on PHA-induced proliferation. Figure 10 shows that the lymphocyte proliferation increased with an increase in cell number, reaching a peak of 832.6 ± 72.7 cpm at 2.0×10^6 cells, and then began to decline steadily at higher concentrations of cells. It was interesting to notice that lymphocyte proliferation dropped to -96.7 ± 12.1 cpm at 10×10^6 spleen cells, which was significantly below basal cell proliferation ($p \leq 0.05$).

Endogenous Opioid-Like Peptide Modulation of PHA Induced Proliferation

β-Endorphin

β-endorphin was used at concentrations ranging between 2×10^{-11} and 2×10^{-6} M to modulate PHA-induced proliferation. It was added to spleen cells (1×10^6) induced to proliferate by 10 μg/ml of PHA. It is clear from Figure 11a that there had been a consistent increase in activation as the concentration of β-endorphin was decreased. At 2×10^{-10} and 2×10^{-11} M, which are rather physiological concentrations, the

Figure 10. Effect of cell number on PHA induced lymphocyte proliferation. Spleen cells ranging between 0.5×10^6 and 10×10^6 were incubated with 10 µg/ml of PHA.

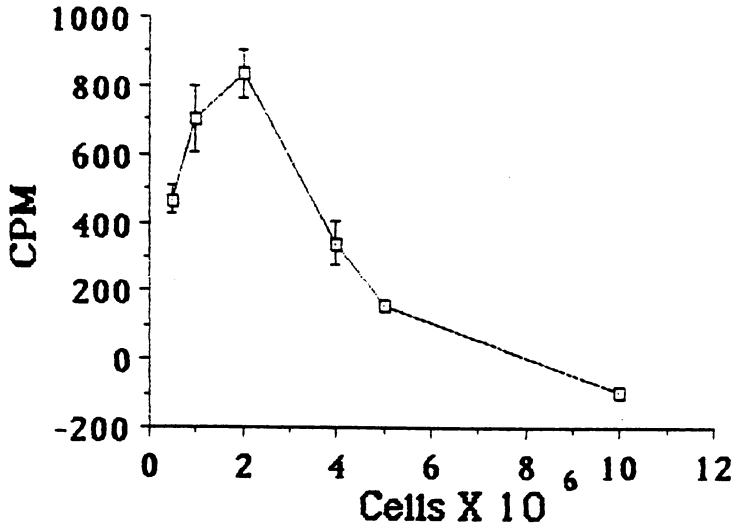
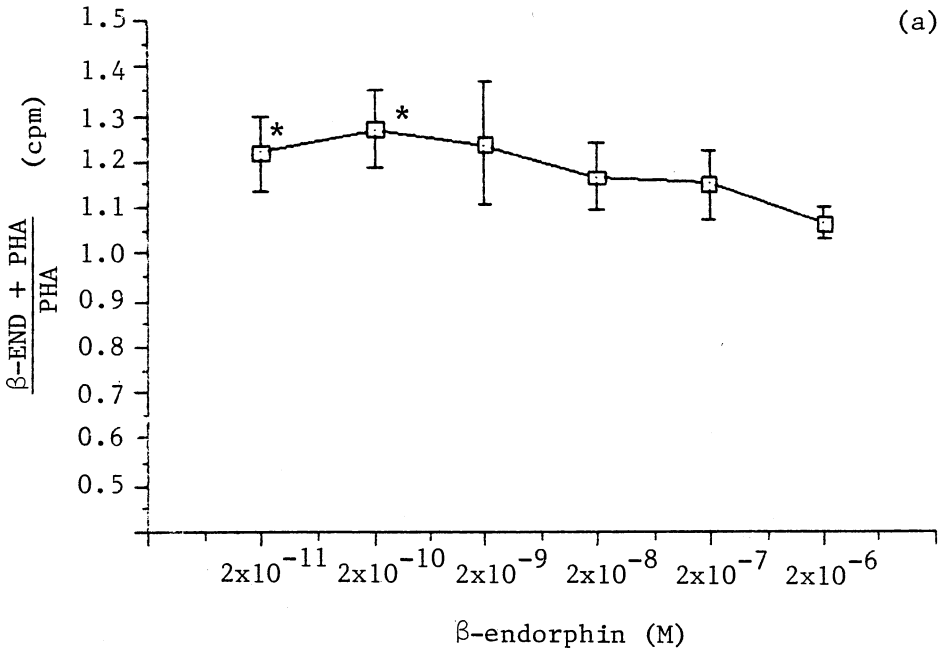
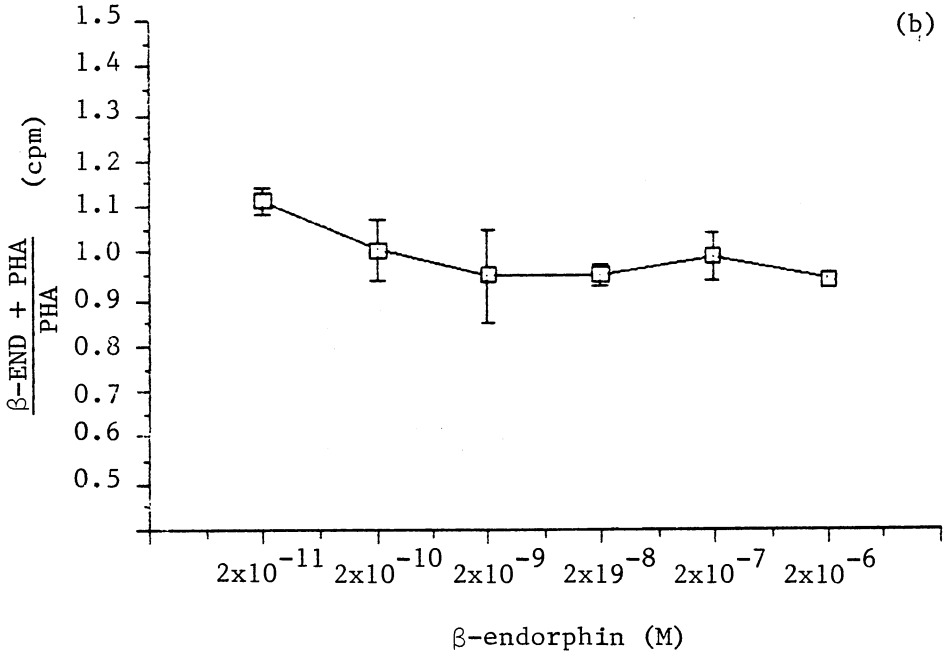

1000
800
600
400
200
0
-200
CPM
0
2
4
6
8
10
12
Cells X 10 6

Figure 11. β-endorphin modulation of PHA-induced proliferation. It was added, at concentrations ranging between 2×10^{-11} and 2×10^{-6} M, to 1×10^{6} spleen cells induced to proliferate by 10 μg/ml of PHA. Two experiments (a) and (b) were performed. Control cultures having only PHA were run in sextuplicate, while β-endorphin modulated cultures were run in triplicate. (*) means a significant difference ($p \leq 0.05$) between cultures having β-endorphin + PHA and cultures having PHA only.


(a)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
β-END + PHA / PHA (cpm)
*
*
2x10^-11 2x10^-10 2x10^-9 2x10^-8 2x10^-7 2x10^-6
β-endorphin (M)


(b)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
β-END + PHA / PHA (cpm)
2x10^-11 2x10^-10 2x10^-9 2x19^-8 2x10^-7 2x10^-6
β-endorphin (M)

activation was significant ($p \leq 0.05$). Unfortunately, another experiment using the same range of concentrations of β-endorphin did not show a clear modulatory effect for β-endorphin (Figure 11b).

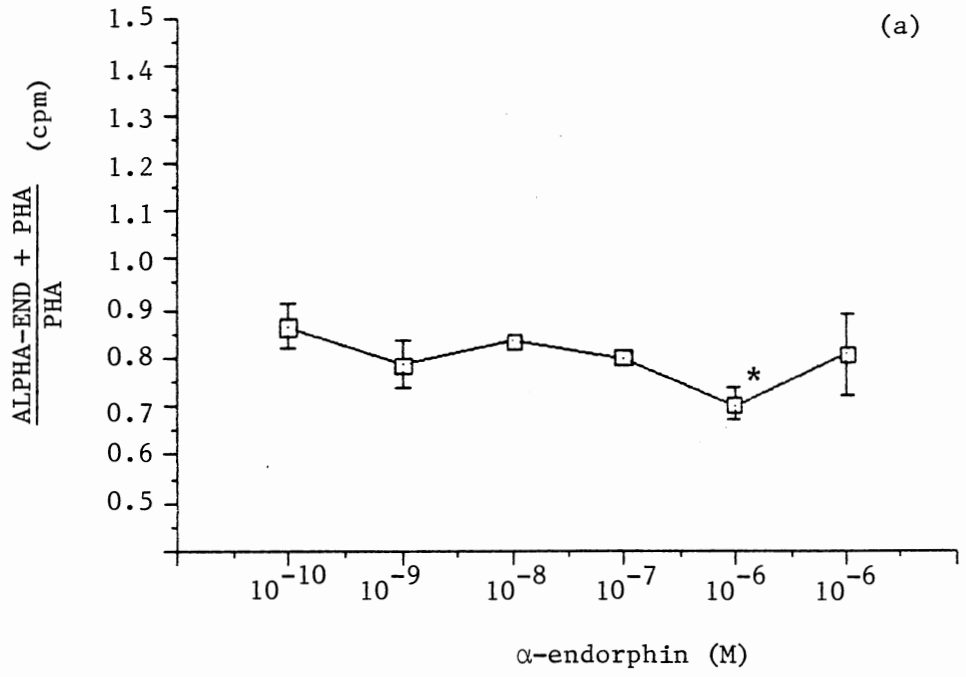
α-Endorphin

α-endorphin was used at concentrations ranging between 1×10^{-10} and 1×10^{-5} M to modulate PHA-induced proliferation. It was added to spleen cells (1×10^{6}) induced by 10 μg/ml PHA. Figure 12a shows that α-endorphin was suppressing PHA-induced cell proliferation (an activation index < 1.0) at all concentrations used. This suppression tended to increase with higher α-endorphin concentrations. Suppression was significant ($p \leq 0.05$) at 1×10^{-6} M. Figure 12b showed the same tendency of increased suppression of cell proliferation as the concentration of α-endorphin was increased. Suppression was significant ($p \leq 0.05$) at 10^{-5} M.

(D-Ala^2, D-Leu^5) Enkephalin

(D-ala^2, D-leu^5) enkephalin was also tested at concentrations ranging between 1×10^{-10} and 1×10^{-5} M, to determine its effect on PHA-induced proliferation. It was added to spleen cells (1×10^{6}) induced by 10 μg/ml PHA. Figure 13a showed that cell proliferation peaked at 1×10^{-7} M of (D-ala^2, D-leu^5) enkephalin, reaching an activation index (A.I.) of 1.21 ± 0.03. When the concentration was further decreased, proliferation began to drop, reaching an A.I. of 1.0 between 10^{-8} and 10^{-9} M. Suppression of cell proliferation was significant ($p \leq 0.05$) at 10^{-9} and 10^{-10} M, reaching A.I.'s of 0.76 ± 0.02 and 0.57 ± 0.04 respectively. Figure 13b shows a similar

Figure 12. α-endorphin modulation of PHA-induced proliferation. It was added, at concentrations ranging between 1×10^{-10} and 1×10^{-5} M, to cultures of 1×10^{6} spleen cells induced to proliferate by 10 μg/ml of PHA. Two experiments (a) and (b) were performed. Control cultures having only PHA were run in sextuplicate, while α-endorphin modulated cultures were run in triplicate. (*) means significant difference ($p \leq 0.05$) between cultures having α-endorphin and PHA, and cultures having PHA only.


(a)
ALPHA-END + PHA / PHA (cpm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
10^-10
10^-9
10^-8
10^-7
10^-6
10^-6
α-endorphin (M)

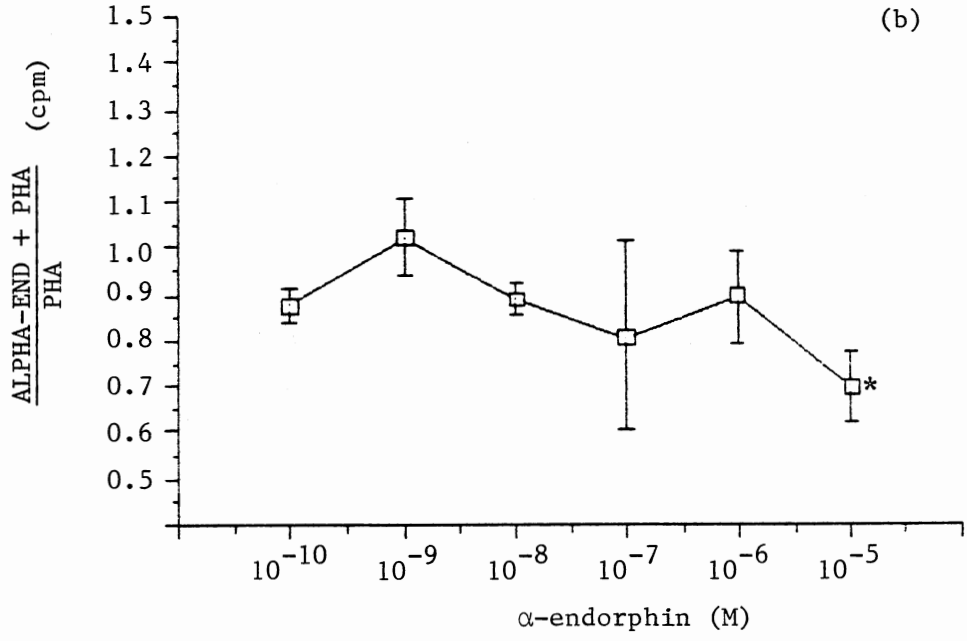
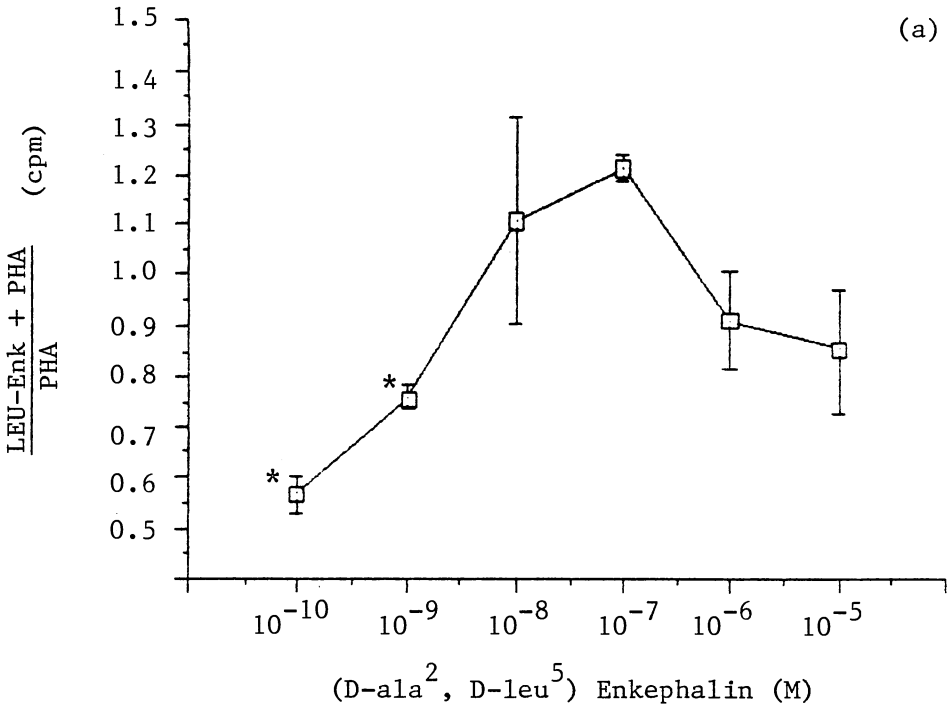
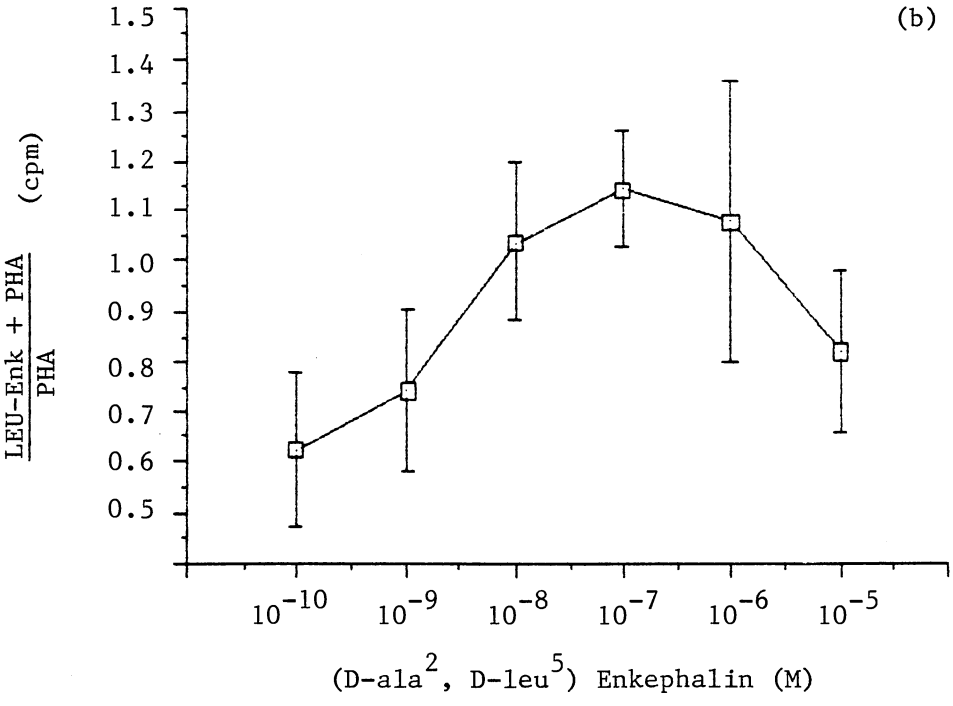

(b)
ALPHA-END + PHA / PHA (cpm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
α-endorphin (M)

Figure 13. (D-ala^2, D-leu^5) enkephalin modulation of PHA-induced proliferation. It was added, at concentrations ranging between 1×10^{-10} and 1×10^{-5} M, to cultures having 1×10^6 spleen cells induced to proliferate by 10 μg/ml of PHA. Two experiments (a) and (b) were performed. Control cultures having only PHA were run in sextuplicate, while (D-ala^2, D-leu^5) enkephalin modulated cultures were run in triplicate. (*) means a significant difference ($p < 0.05$) between cultures having (D-ala^2, D-leu^5) enkephalin + PHA and cultures having PHA only.


(a)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
LEU-Enk + PHA / PHA (cpm)
*
*
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
(D-ala^2, D-leu^5) Enkephalin (M)


(b)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
LEU-Enk + PHA / PHA (cpm)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
(D-ala^2, D-leu^5) Enkephalin (M)

dose response curve reaching a maximum at 1×10^{-7} M and then suppression at 1×10^{-9} and 1×10^{-10} M.

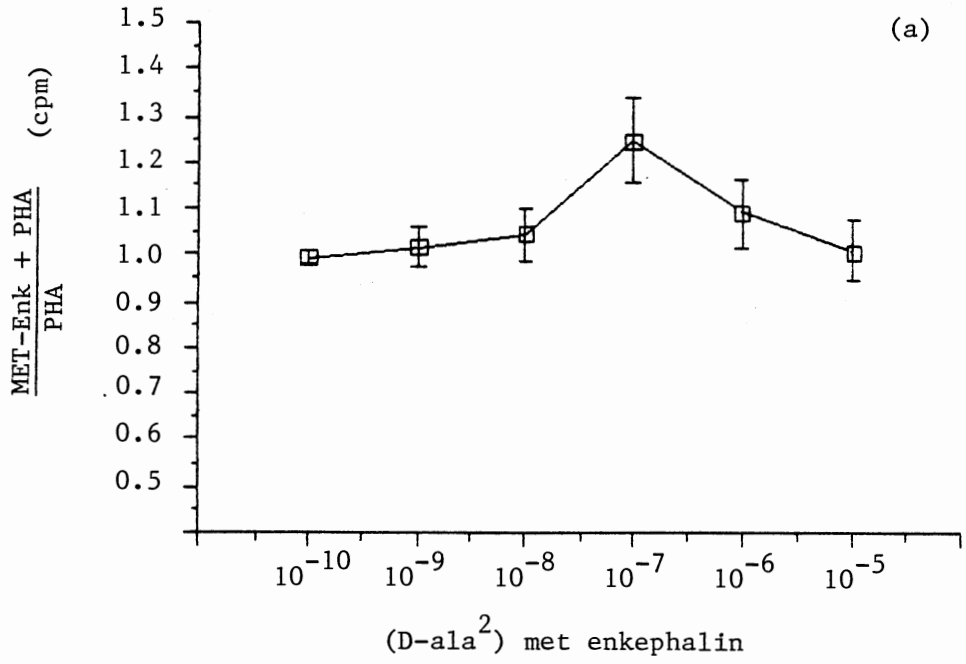
(D-Ala^2) Met Enkephalin

(D-ala^2) met enkephalin was tested at concentrations ranging between 1×10^{-10} and 1×10^{-5} M, to determine its effect on PHA-induced proliferation. It was added to spleen cells (1×10^6) induced by 10 µg/ml PHA. (D-ala^2) met enkephalin (Figures 14a and b) did not show any modulatory activity of PHA-induced cell proliferation when used at concentrations ranging between 1×10^{-10} and 1×10^{-5} M.

[^{3}H] (D-ala^2, D-leu^5) Enkephalin Binding to Spleen and Brain

In view of the effect of (D-ala^2, D-leu^5) enkephalin on PHA-induced proliferation shown in Figure 13, we decided to look for (D-ala^2, D-leu^5) enkephalin binding to spleen cells. [^{3}H] (D-ala^2, D-leu^5) enkephalin was used in binding assays to determine the presence of δ receptors in assays similar to those used to test for µ receptors. The results of a saturation binding assay using spleen cells (experimental) and brain cell membranes (positive control) is shown in Figure 15. When using the brain, total and specific binding were increasing with higher amounts of labelled enkephalin. The amount of specific binding increased from 128.9 cpm when using 55×10^3 DPM, to 804.5 cpm when using 550×10^3 DPM. There was no difference between including and excluding unlabelled (D-ala^2, D-leu^5) enkephalin (1.7×10^{-5} M) at any concentration of [^{3}H] (D-ala^2, D-leu^5) enkephalin. These results have been confirmed by a specificity binding assay using 550×10^3 DPM for the radioligand. The

Figure 14. (D-ala^2) met enkephalin modulation of PHA-induced proliferation. It was added, at concentrations ranging between 1 x 10^{-10} and 1 x 10^{-5} M, to cultures having 1 x 10^6 spleen cells induced to proliferate by 10 µg/ml of PHA. Two experiments (a) and (b) were performed. Control cultures having only PHA were run in sextuplicate, while (D-ala^2, D-leu^5) enkephalin-modulated cultures were run in triplicate. (*) means a significant difference ($p < 0.05$) between cultures having (D-ala^2, D-leu^5) enkephalin + PHA and cultures having PHA only.


(a)
MET-Enk + PHA / PHA (cpm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
(D-ala^2) met enkephalin

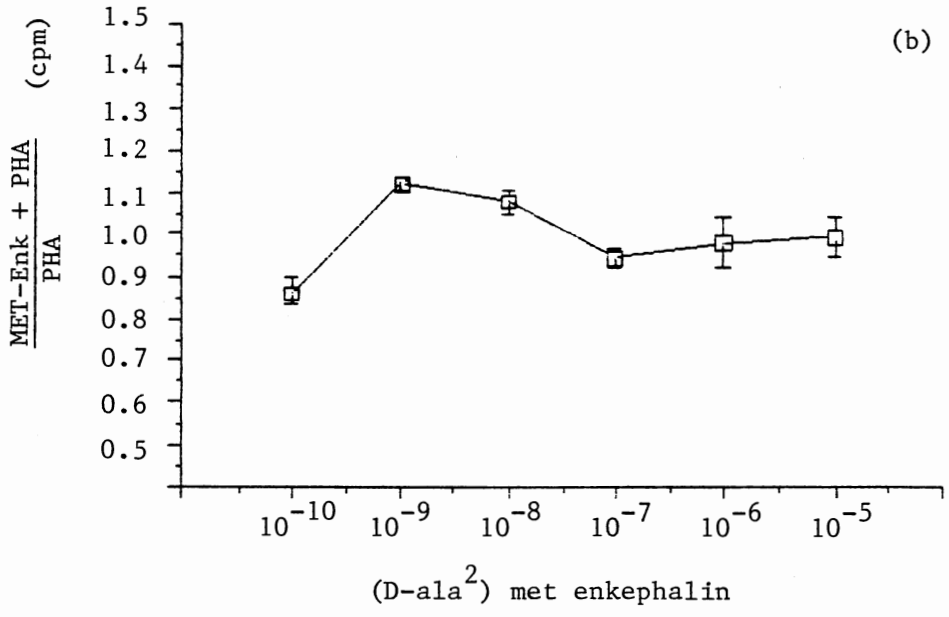
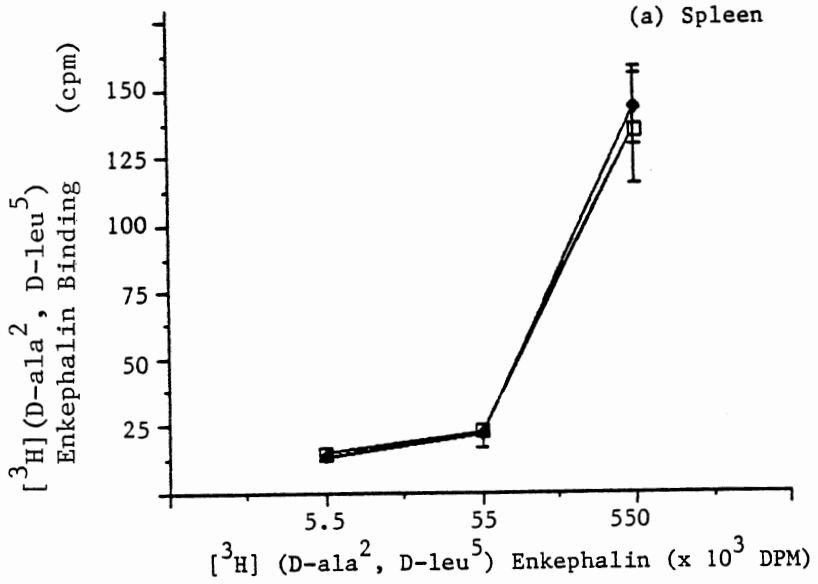
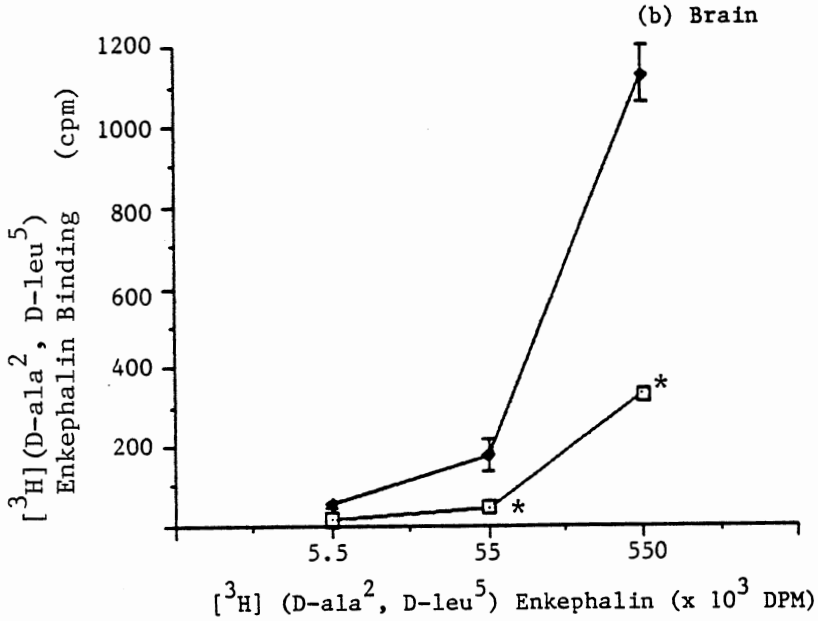

(b)
MET-Enk + PHA / PHA (cpm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
(D-ala^2) met enkephalin

Figure 15. Saturation binding assay using [^{3}H] (D-ala^{2}, D-leu^{5}) enkephalin. (a) Spleen cells were used in duplicate. (b) Brain cells membranes were used in duplicate. □ with 1.7 x 10^{-5} M (D-ala^{2}, D-leu^{5}) enkephalin; ◆ without (D-ala^{2}, D-leu^{5}) enkephalin.

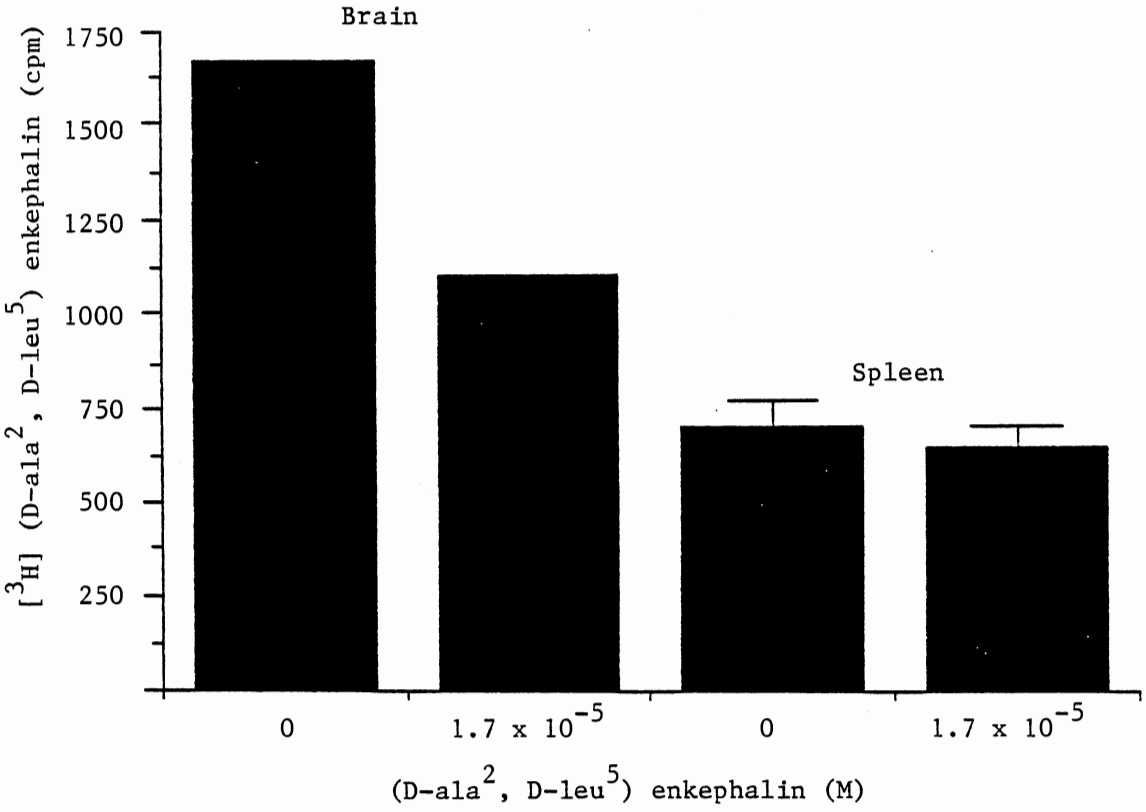

(a) Spleen
[3H](D-ala2, D-leu5) Enkephalin Binding (cpm)
150
125
100
75
50
25
5.5
55
550
[3H] (D-ala2, D-leu5) Enkephalin (x 10^3 DPM)


(b) Brain
[3H](D-ala2, D-leu5) Enkephalin Binding (cpm)
1200
1000
800
600
400
200
*
*
5.5
55
550
[3H] (D-ala2, D-leu5) Enkephalin (x 10^3 DPM)

results (Figure 16) demonstrated clearly that there was no significant difference ($p \leq 0.05$) between including and excluding 1.7×10^{-5} M (D-ala^2, D-leu^5) enkephalin. This would suggest that probably spleen cells did not have opioid receptors of δ type for (D-ala^2, D-leu^5) enkephalin.

To determine if 5.5×10^5 DPM was not enough in picking up specificity, 1.1×10^6 DPM of the radiolabelled ligand was also used. The results (Figure 17) indicated the absence of specific binding ($p \leq 0.05$), at both concentrations of [^{3}H] (D-ala^2, D-leu^5) enkephalin, when spleen cells were used. It was noticed that increasing the radioactivity had increased the total binding, and thus increasing nonspecific binding. On the contrary, brain cell membranes showed specific binding at both used concentrations of [^{3}H] (D-ala^2, D-leu^5) enkephalin. When using 1.1×10^6 DPM, it was clear that nonspecific binding also increased tremendously. Specific binding did not, essentially, change between the two concentrations suggesting that saturation had occurred.

Figure 16. Specificity of [^{3}H] (D-ala^{2}, D-leu^{5}) enkephalin binding. Spleen cells were used in pentuplicate. Brain cells membranes were used in singlet. The concentration of (D-ala^{2}, D-leu^{5}) enkephalin was 1.7×10^{-5} M.


Brain
Spleen
1750
1500
1250
1000
750
500
250
[3H] (D-ala2, D-leu5) enkephalin (cpm)
0
1.7 x 10-5
0
1.7 x 10-5
(D-ala2, D-leu5) enkephalin (M)

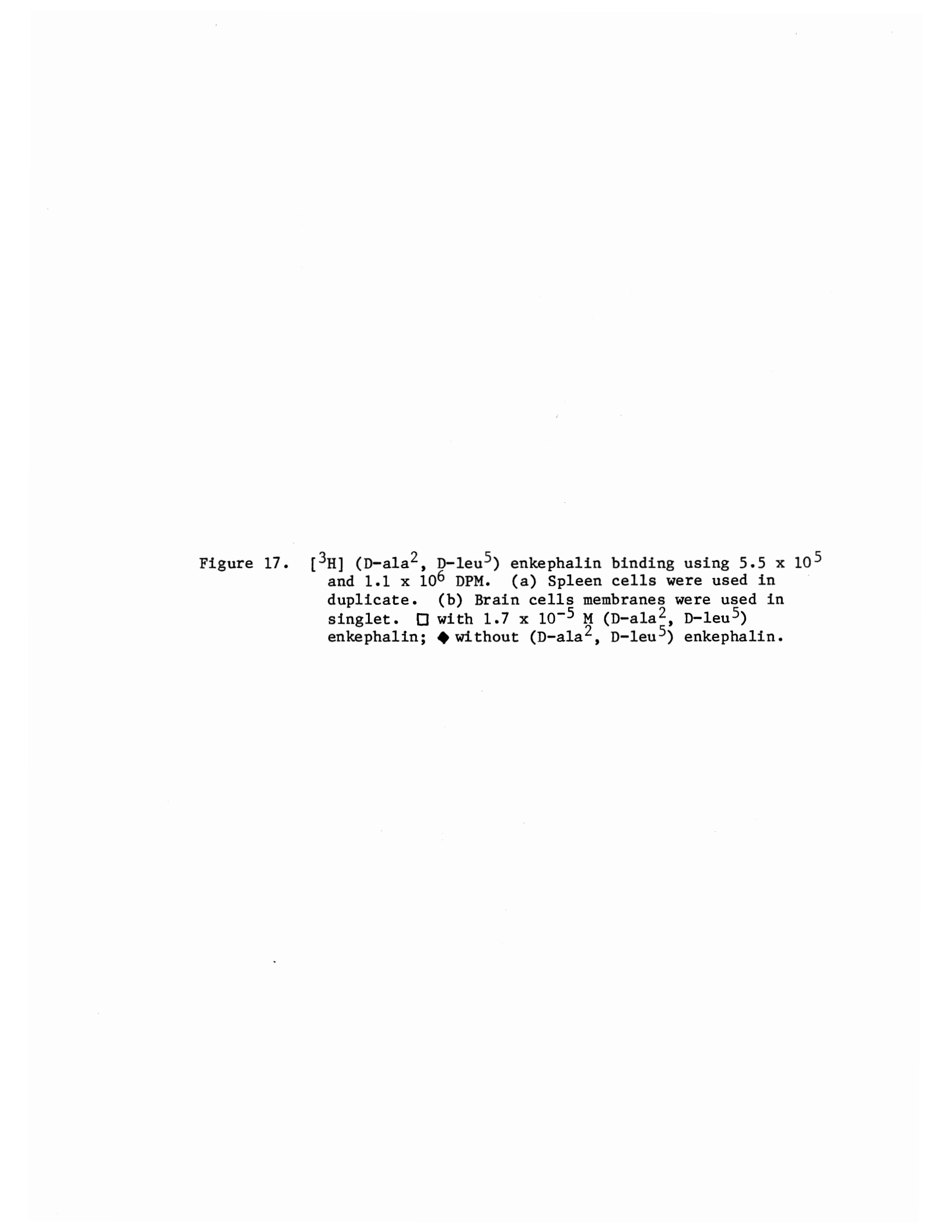
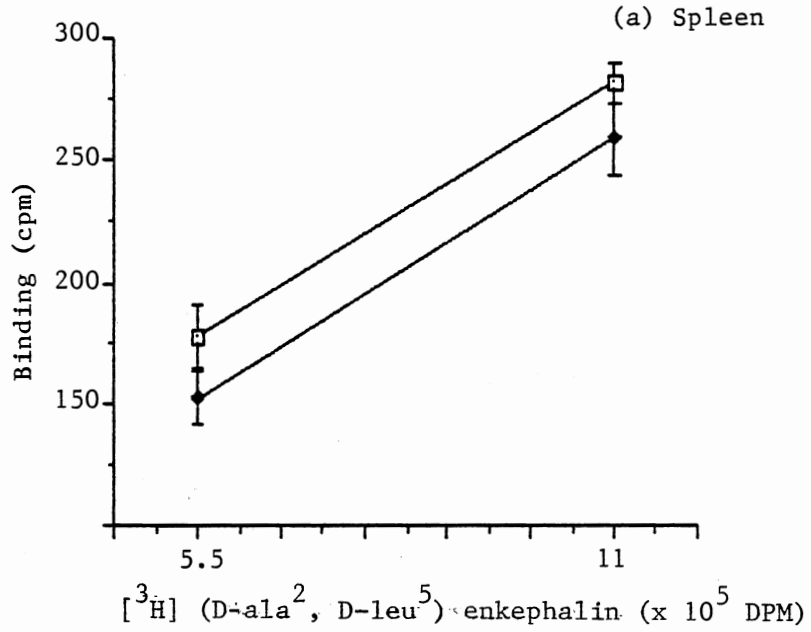
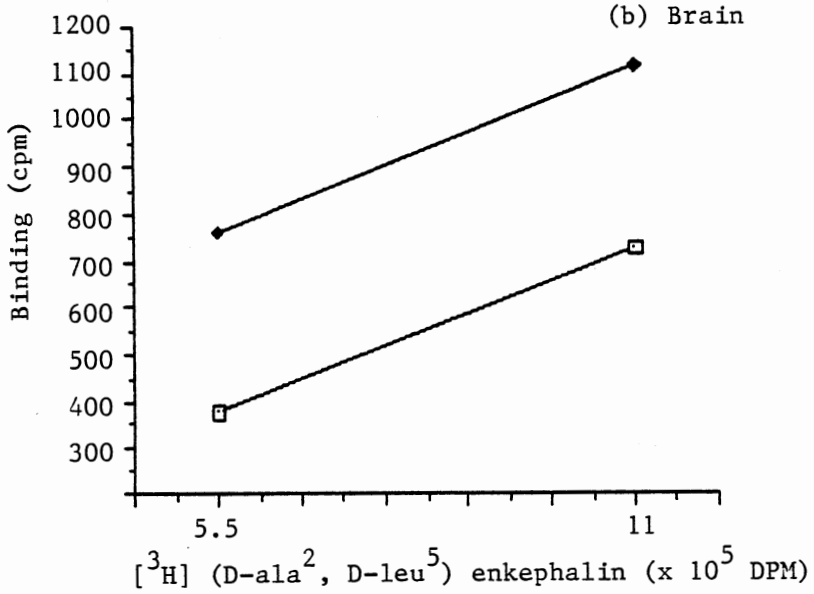


Figure 17. [^{3}H] (D-ala^{2}, D-leu^{5}) enkephalin binding using 5.5 x 10^{5} and 1.1 x 10^{6} DPM. (a) Spleen cells were used in duplicate. (b) Brain cells membranes were used in singlet. □ with 1.7 x 10^{-5} M (D-ala^{2}, D-leu^{5}) enkephalin; ◆ without (D-ala^{2}, D-leu^{5}) enkephalin.


(a) Spleen
300
250
200
150
Binding (cpm)
5.5
11
[3H] (D-ala2, D-leu5) enkephalin (x 10^5 DPM)


(b) Brain
1200
1100
1000
900
800
700
600
500
400
300
Binding (cpm)
5.5
11
[3H] (D-ala2, D-leu5) enkephalin (x 10^5 DPM)

CHAPTER IV

DISCUSSION

In this study, a standardized procedure for determining specific binding of opioids and endogenous opioid-like peptides to cell receptors was developed. A rationale for choosing a specific filter type by previous investigators was not available. The choice varied with the cell and radioligand types used. Whatman fiberglass filters GF-B were chosen by Pert et al. (40) because they are thick, and thus they are able to hold relatively large quantities of tissues. GF-F was suggested for use by others (21) without stating their reasons. In our study, Whatman filters AH-934 were found to be the best fit judging from results in Tables IV and V. They represented a reasonable compromise between tissue retention and radioligand exclusion.

In a binding assay, it is important to reduce the amount of nonspecific binding to filters. This nonspecific binding might be reduced by washing or by including an antiadsorbent into the assay system. These two criteria were confirmed in Table VI and Figure 2. We found that continuous washing removed more and more radioactivity. Nevertheless, it was entirely possible that radioactivity specifically bound to the cells was also being washed away. Therefore, we settled for a compromise of four washes. The inclusion of 0.25-2% BSA in a prewash of the filter reduced the amount of nonspecific radioligand binding. However, it was interesting to note that when the filters were prewashed

with 4% BSA, a substantial increase in binding followed. Accordingly, it was reasonable to assume that BSA, or some contaminant at high concentrations bound [^{3}H] naloxone, which was opposite to what we were looking for.

Identification of specific binding between opioids and endogenous opioid-like peptides to opioid receptors on mouse spleen cells was the main objective of our studies. The existence of such receptors had been proposed by several authors (Table III). This can best be demonstrated by competition and saturation studies. Competition of [^{3}H] naloxone was dealt with by adding unlabelled naloxone in 1000 fold excess. If there was specific binding, unlabelled naloxone should have been able to compete with [^{3}H] naloxone, thus reducing the amount of specific binding. This did not happen (Figures 4, 6, and 7) when murine spleen cells were used. However, competitive inhibition of [^{3}H] naloxone binding was demonstrated in brain cell membranes which were used as positive controls in the same experiment. These results did not support the presence of opioid receptors of the μ type on murine spleen cells.

Competition and saturation of [^{3}H] (D-ala^2, D-leu^5) enkephalin binding were the subject of Figures 15, 16, and 17. These results did not support the presence of opioid receptors of the δ type. The results suggested that at 1.1 x 10^6 DPM of [^{3}H] (D-ala^2, D-leu^5) enkephalin, nonspecific binding to spleen cells and to brain cell membranes was very high (Figure 17). We found that increasing the amount of radioactivity, from 5.5 x 10^5 to 1.1 x 10^6 DPM, did not help to demonstrate specific binding. The use of 5.5 x 10^5 DPM was judged to be providing enough radioactivity to detect specific binding.

It was noticed that addition of naloxone or (D-ala^2, D-leu^5) enkephalin to its corresponding [^{3}H] compound gave a small, but consistent decrease in binding with spleen cells (Table VII and Figures 6, 15, 16, and 17). There is more than one plausible explanation for this decrease. One possibility is that there are good numbers of opioid receptors on a small subpopulation of spleen cells. This possibility is supported by the studies of Ausiallo et al. (2) and Hazum et al. (26) who reported the presence of opioid receptors on cultured human T lymphocytes and by Lopker (31) who reported the presence of μ receptors on monocytes. However, we could not detect specific binding in PEC which contain a high percentage of macrophages. Another possibility is that there are small numbers of receptors on all or several subpopulations of spleen cells. A third possibility is that expression of opioid receptors may require an induction signal. The presence of such an induction signal in their culture systems could explain the positive findings of Ausiallo et al. (2) and Hazum et al. (26).

Our results are not in congruence with Mehrishi (34) who reported that at least some of the [^{3}H] naloxone binding sites on human peripheral blood lymphocytes (HPBL) were opioid receptors of the μ type. One reason may be the fact that the system that they were working with, HPBL, is different from mice spleen cells. In addition, there was no mention whether the decrease in [^{3}H] naloxone binding upon addition of morphine hydrochloride was statistically significant. On the other hand, Johnson (28) reported [^{3}H] leu-enkephalin binding to mouse spleen cells. Unfortunately, these results were not accompanied by experimental data which makes it difficult for us to evaluate them. In addition,

[^{3}H] (D-ala^2, D-leu^5) enkephalin, which is the radioligand we used, is different from theirs.

A second problem we dealt with was the modulatory effects of endogenous opioid-like peptides on PHA-induced proliferation. PHA, being primarily a T cell activator, binds to T lymphocyte membrane receptors and initiates a series of metabolic events which include marked stimulation of lymphoblast formation and cell proliferation (59). PHA-induced proliferation, as extrapolated from DNA synthesis, is thought representative of in vivo competence. The analogous physiologic phenomenon, antigen stimulated blastogenesis, is considered to be an important step in activation of host immune defense. Therefore, modulation of PHA-induced proliferation may represent an in vivo modulation. β-endorphin exerted a significant enhancing effect at 2×10^{-10} and 2×10^{-11} M (Figure 11). The normal circulating levels of β-endorphin in rat spleen ranges between 10^{-10} and 10^{-11} M (11). Therefore, β-endorphin can exert its effect at rather physiological concentrations. An enhancement of lymphocyte proliferation using β-endorphin was also reported by Gilman (20) using rat spleenocytes in their proliferation assay. Yet, a modulatory effect using β-endorphin does not necessarily mean that it is mediated by opioid receptors. In fact, there are suggestions that β-endorphin exerts its effect through non-opioid receptors (26).

Unfortunately, those results were not maintained in a second experiment using β-endorphin. Indeed, a difference between results is not uncommon in modulation of mitogen-induced proliferation. For example, Gilman (20) reported an enhancement of rat lymphocyte proliferation in only half of the experiments they have performed. They

also reported a difference in the β-endorphin concentration effective in modulating PHA-induced proliferation between different experiments. Part of this variation in results is simply due to differences in the responsiveness of different spleen cell preparations to PHA. In addition, the effect of β-endorphin is probably seen only when the cells are suboptimally stimulated by mitogen. If a particular dose of PHA is maximally stimulating the cells, no additional effect of the compound used will be detected. In fact, a reverse effect may arise. Part of the experimental variation in the effect of these endogenous opioid-like peptides may also arise from differences between individual animals of the same species. These differences are a reflection of differences in housing conditions, in stress levels, in health state, and in genetic makeup between different mice. These factors may, in turn, have an effect on the composition and the responsiveness of spleen cells to these endogenous opioid-like peptides.

We also reported that α-endorphin suppressed PHA-induced proliferation of spleen cells at high concentrations (10^{-6} - 10^{-5} M). Gilman et al. (20) reported that α-endorphin had no significant effect at 3×10^{-8} M but they did not try higher concentrations. Thus, we found no contradiction between our results and theirs. In fact, the only reported α-endorphin activity is its inhibitory role on PFC response (28).

(D-ala^2, D-met^5) enkephalin did not have any significant effect on PHA-induced proliferation (Figure 14). Similar results have been reported by Wybran (61) using HPBL and by Gilman (20) using rat spleenocytes, where they could not find any significant activation or suppression effects. On the other hand, in our experiment, (D-ala^2, D-leu^5) enkephalin inhibited PHA-induced lymphocyte

proliferation at 10^{-9} and 10^{-10} M (Figure 13). These results are not in agreement with Plotnikoff (41) who reported an enhancement in [^{3}H] thymidine uptake over a wider range of concentrations of leu-enkephalin. However, this enhancement was dependent on PHA concentrations.

It becomes clear that variation in the results between different laboratories dealing with endogenous opioid-like peptides may be due to different aspects in the proliferation assay. This includes the endogenous opioid-like peptide, and the concentration over which it is used. It also includes the type of cells and the species from which the cells are taken. Also, the mitogen and the range of concentrations over which it is used are important factors. The culturing medium and length of culturing time may be additional factors involved.

There appears to be a discrepancy between the results of our binding studies and those of modulation of PHA-induced proliferation. (D-ala^2, D-leu^5) enkephalin, for example, suppressed PHA-induced proliferation of spleen cells, but we did not detect any opioid receptors of the δ type. This apparent discrepancy can be resolved considering the difference between spleen cells used in the two assays. In binding assays, naive spleen cells were used, while in the cell proliferation assay, the spleen cells used were activated lymphocytes. Therefore, there is a possibility that stimulation of lymphocyte proliferation may have induced opioid receptors on the surface of spleen cells.

The proliferative response of lymphocytes to T cell mitogens is a complex process involving the activation of existing T cells into activated T cells, with the participation of macrophages and soluble factors produced by these cells (14). Our results do not delineate the cellular target for the endogenous opioid-like peptides. A peptide may

act directly upon the proliferating T cell lymphoblast. Alternatively, it can exert its effect indirectly by acting primarily on macrophages, or the resting subset of T lymphocytes responsible for interleukin-2 production. There is the possibility also that each of the endogenous opioid-like peptides exert its effect on a different subpopulation of spleen cells. Therefore, cell fractionation and reconstitution experiments should allow us to determine the cellular targets of these peptides.

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