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The opioid specificity of beta-endorphin enhancement of murine lymphocyte proliferation

Wendy Gilmore and Leslie P. Weiner

Grant Neurosciences Laboratory, Department of Neurology and Microbiology, USC School of Medicine, Los Angeles, CA 90033, U.S.A.

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Abstract: Beta-endorphin (β-end) is a potent analgesic peptide which exhibits a variety of pharmacological activities in the central nervous system (CNS) following binding of its N-terminus to specific opioid receptors. Although C-terminal binding sites for this 31-amino-acid peptide have been characterized in CNS tissue, identification of their possible function has been facilitated by studies of β-end effects on lymphocyte activities. In this communication, we report a detailed analysis of the opioid specificity of the ability of β-end to enhance T cell mitogen-induced proliferation in unfractionated murine splenocytes. Intact 31-amino-acid β-end peptides from several species, including human, camel and rat, enhanced concanavalin A-stimulated [^{3}H]thymidine uptake 50–640% in a dose-dependent, naloxone-irreversible fashion. The presence of the C-terminal amino acids was required for the enhancement activity, since met-enkephalin, α- and γ-endorphin, and human β-end 1–27 were ineffective. Accordingly, the truncated peptides, human β-end 6–31 and 18–31, were also able to enhance the Con A response. However, human β-end 18–31 was consistently not as effective as β-end 6–31 or the intact 31-residue peptide. These data suggest that although the C-terminus contains the primary active sequence, the N-terminus contributes to the overall potency of the effect. In support of this assertion, *N*-acetylation, which abolishes opioid binding activity, resulted in a reduced magnitude of enhancement. The data suggest that β-end interacts with a non-opioid receptor which has specificity characteristics strikingly similar to non-opioid receptors characterized in CNS tissue.

Key words: Opioid peptide; Opioid receptor; Neuroimmunomodulation; Beta-endorphin; Opioid specificity

Introduction

Beta-endorphin (β-end) is a naturally occurring, 31-amino-acid opioid peptide that is derived in its resident tissues by post-translational processing of the precursor protein, proopiomelanocortin (POMC; Mains et al., 1977; Nakanishi et al., 1979). It is a member of a large group of opioid peptides similarly derived from two additional precursors, known as proenkephalin and prodynorphin. Endorphins, enkephalins and dynorphins share the N-terminal amino acid sequence, TyrGlyGlyPhe, and differ in the number and sequence of their C-terminal residues. It is generally accepted that many of the classical biological activities of opioid peptides, including that of analgesia, follow binding of the N-terminal amino acids to specific opiate receptors (Deakin et al., 1980; DeWeid and Jolles, 1982). For this reason, the TyrGlyGlyPhe sequence is considered to be the opiate end of each peptide molecule. Interestingly, functional studies have established that the C-terminal amino acids of the β-end molecule are responsible for the high analgesic potency

Correspondence: Wendy Gilmore, Department of Neurology, MCH 142, USC School of Medicine, 2025 Zonal Ave, Los Angeles, CA 90033, U.S.A.

Abbreviations: Con A, concanavalin A; β-end, beta-endorphin; PHA, phytohemagglutinin; CNS, central nervous system; cpm, counts per minute; met-enk, met-enkephalin; α-end, alpha endorphin, IL-2, interleukin 2; NK, natural killer.

TABLE I

Comparative amino acid sequences of the N- and C-terminus of various opioid peptides

Opioid peptide	Amino acid sequence[a]	
βh-endorphin (human)	NH_2-TyrGlyGlyPheMet 1 2 3 4 5	AlaTyrLysLysGlyGlu-COOH 26 27 28 29 30 31
βc-endorphin (camel)	NH_2-TyrGlyGlyPheMet 1 2 3 4 5	Ala**His**LysLysGly**Gln**-COOH 26 27 28 29 30 31
βr-endorphin (rat)	NH_2-TyrGlyGlyPheMet 1 2 3 4 5	**ValHis**LysLysGly**Gln**-COOH 26 27 28 29 30 31
γ-endorphin (1–17)	NH_2-TyrGlyGlyPheMet 1 2 3 4 5	ThrLeu-COOH 16 17
α-endorphin (1–16)	NH_2-TyrGlyGlyPheMet 1 2 3 4 5	Thr-COOH 16
Met-enkephalin	NH_2-TyrGlyGlyPheMet-COOH 1 2 3 4 5	
Leu-enkephalin	NH_2-TyrGlyGlyPheLeu-COOH 1 2 3 4 5	

[a] Amino acid residues differing from the human sequence are indicated in bold type.

characteristically observed with this peptide (Bradbury et al., 1977; Deakin et al., 1980). Subsequent binding studies utilizing rabbit spinal cord membrane preparations (Ferrara and Li, 1980) and several cell lines derived from human brain tumors (Westphal and Li, 1984a,b) have identified two distinct binding activities for β-end, located at both the N- and C-termini of its primary structure. Since the C-terminal binding activity is not effectively displaced by peptides with shorter C-terminal tails, this end of the molecule is considered its non-opiate end.

Although the precise function of the C-terminal amino acids of β-end has not been identified in the CNS, there is considerable evidence to indicate that they play a role in modulating immune function in the periphery. For instance, β-end enhances PHA- and Con A-induced proliferation in rat splenocytes, an effect that does not occur in the presence of the related opioid peptides, met-enkephalin (met-enk) and α-endorphin (α-end)(Gilman et al., 1982). Met-enk and α-end lack the C-terminal residues, but share, respectively, the first 5 and 16 amino acids of the β-end sequence (Table I). In addition, we have recently demonstrated that β-end enhances production of a lymphokine with interleukin-2 (Il-2)-like activity in mitogen-stimulated murine T cell preparations (Gilmore and Weiner, 1988). The β-end enhancement of both proliferation and IL-2 production was not blocked by the specific opiate antagonist, naloxone, which inhibits many of the biological activities attributed to the N-terminal end of the opioid peptides. Finally, binding studies using the transformed human B cell line, RPMI 6237 (Hazum et al., 1979), murine EL4 thymoma cells (Schweigerer et al., 1985) and the terminal complex of human complement (Schweigerer et al., 1982, 1983) have identified C-terminal-specific binding sites on lymphocytes.

Although the preceding data appear to emphasize the role of the β-end C-terminus in modulation of lymphocyte activities, there is also evidence that met-enk and α-end influence immune function. Both peptides inhibit in vitro antigen-specific antibody responses in mouse (Johnson et al., 1982) and human (Heijnen et al., 1986) model systems, while β-end has minimal or no effect on this response. In addition, β-end and met-enk potentiate the cytolytic activity of natural killer (NK) cells (Mathews et al., 1983) as well as their ability to produce γ-interferon (Mandler et al., 1986; Brown and van Epps, 1986) in naloxone-reversible fashion. This indicates that the N-terminal amino acids of opioid peptides are also capable of immunomodulation, and suggests the participation of a classical opiate receptor.

In this communication, we report that β-end enhances Con A-stimulated proliferation in unfractionated murine splenocytes in naloxone-irreversible fashion. A detailed analysis of the opioid peptide specificity of this suggests that both ends of the β-end molecule interact with the target cell or cells in a manner similar to that described for the analgesic activity of β-end in the CNS. The data indicate that although the C-terminal amino acids contain the active or primary signalling moiety, the N-terminus contributes to the overall potency of the opioid peptides.

Materials and Methods

Animals

Male 4–6-week-old C56B1/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). They were housed under standard lighting conditions (12:12 h light:dark cycle) and allowed to adapt to animal room conditions at least one week prior to use.

Reagents

All reagents were diluted in RPMI 1640 medium (Gibco; Grand Island, NY) supplemented with 20 mM HEPES, 0.1 mM MEM nonessential amino acids, 4 mM L-glutamine, 1 mM sodium pyruvate and 5×10^{-5} M 2-mercaptoethanol (assay medium), filter-sterilized and stored at –20° or –70°C prior to use. Concanavalin A (Con A) was obtained from Pharmacia (Piscataway, NJ) and naloxone was from Sigma Chemical Co. (St. Louis, MO). All opioid peptides were purchased from Peninsula Laboratories (Belmont, CA), who provide proof of purity in the form of HPLC profiles with each lot. The peptides are designated as follows: βh-end 1–31, human β-endorphin 1–31; βh-end (1–5)(16–31), human β-endorphin (1–5)(16–31); D-Ala^2-βh-end 1–31, D-Ala^2-human β-endorphin 1–31; NAcβh-end 1–31, *N*-acetylated human β-endorphin 1–31; βc-end 1–31, camel β-endorphin 1–31; βr-end 1–31, rat β-endorphin 1–31; βh-end 1–27; human β-endorphin 1–27; βc-end 1–27, camel β-endorphin 1–27; α-end, human alpha-endorphin; γ-end, human gamma-endorphin; βh-end 6–31, human β-endorphin 6–31; and βh-end 18–31, human β-endorphin 18–31. Met and leu-enkephalin were also from Peninsula Laboratories. A comparison of the amino acid sequences of some of these peptides is presented in Table I.

Assay procedures

Single cell suspensions were prepared from the spleens of mice killed by cervical dislocation. Without depleting for red blood cells, the unfractionated spleen cells were washed, resuspended to 2×10^6 cell/ml in assay medium, and added to the wells of 96-well plates (Linbro, Flow Laboratories, McLean, VA) at 100 μl per well. The cells were cultured in the presence and absence of Con A and opioid peptide in a total volume of 250 μl/well for 72 h in a humidified atmosphere at 37°C, 7% CO_2. During the last 24 h, cells were pulsed with 1 μCi/well [^{3}H]thymidine (ICN; 10 Ci/mmol). Harvesting onto glass-fiber filter paper was accomplished using an automated sample harvester (MASH II; Whittaker M.A. Bioproducts, Walkersville, MD). Counts per minute (cpm) incorporated [^{3}H]thymidine were determined by liquid scintillation spectroscopy. In some experiments, individual opioid peptides were added daily throughout the 72 h culture period in an attempt to compensate for cell-associated peptidase activity. In other experiments, bacitracin (Sigma Chemical Co., St. Louis, MO) was added to the assay medium at 50 μg/ml. Results were evaluated for statistical significance using Student's *t* test, comparing the mean cpm of triplicate determinations obtained in the absence of opioid peptide (Con A alone) with that observed in the presence of opioid peptide.

Results

Characteristics of the β-endorphin enhancement of lymphocyte proliferation

The ability of β-endorphin to influence in vitro lymphocyte proliferation was initially examined using the intact 31-amino-acid opioid peptides, βh-end 1–31 and βc-end 1–31, which were added to unfractionated murine splenocytes in the presence and absence of Con A. βc-end 1–31 differs from βh-end 1–31 at two C-terminal amino acid residues (Tyr^{27} Glu^{31} in βh-end 1–31; His^{27} Gln^{31} in βc-end 1–31; see Table I). Both peptides consistently enhanced the Con A-stimulated proliferative response, measured as counts per minute (cpm) incorporation of [^{3}H]thymidine. Figs. 1 and 2 demonstrate that the enhancement occurred in dose-dependent fashion at concentrations of βh-end 1–31 (Fig. 1) and βc-end 1–31 (Fig. 2) ranging from pharmacological levels at 10^{-6} M, to physiological levels at 10^{-10} M. Significant enhancement was infrequently observed at endorphin concentrations lower than 10^{-10} M. The magnitude of the enhancement, expressed as the % increase relative to control wells lacking peptide, was dependent not only upon endorphin dose, but also upon the concentration of Con A added to the cells. The greatest enhancement was typically observed at Con A concentrations inducing a suboptimal proliferative response. Thus, at 0.15 μg/ml Con A, the magnitude of the enhancement observed at 10^{-6} M peptide was greatest, and varied in indi-

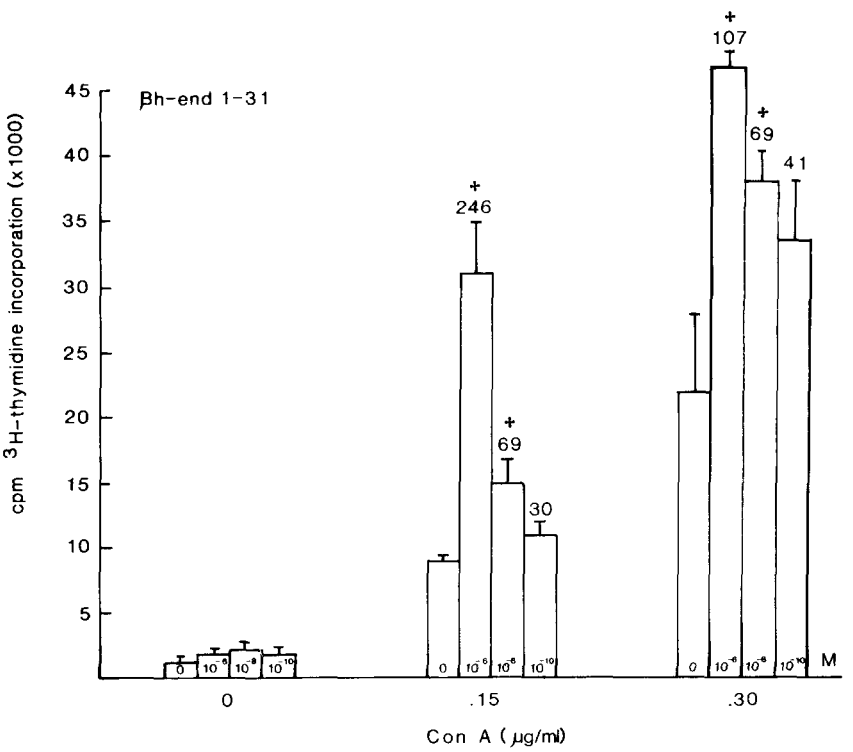

Fig. 1. βh-end 1–31 enhancement of Con A-stimulated proliferation in C57Bl/6J splenocytes. Unfractionated spleen cells (2×10^6 cells/ml) were incubated in the presence and absence of Con A (0.15–0.30 μg/ml) and varying concentrations of βh-end 1–31 for 72 h in 96-well plates. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) during the last 24 h of culture and harvested using an automated cell harvester as described in Materials and Methods. The mean [^{3}H]thymidine uptake (cpm) for triplicate cultures and standard deviations are illustrated. Statistical significance was calculated using Student's *t* test, comparing the mean cpm [^{3}H]thymidine uptake in wells with β-end with that obtained in wells without β-end. The percent magnitude of enhancement is indicated above the standard deviation bars, with *p* values <0.01 designated by an asterisk. The experiments were repeated a minimum of four times with similar results.

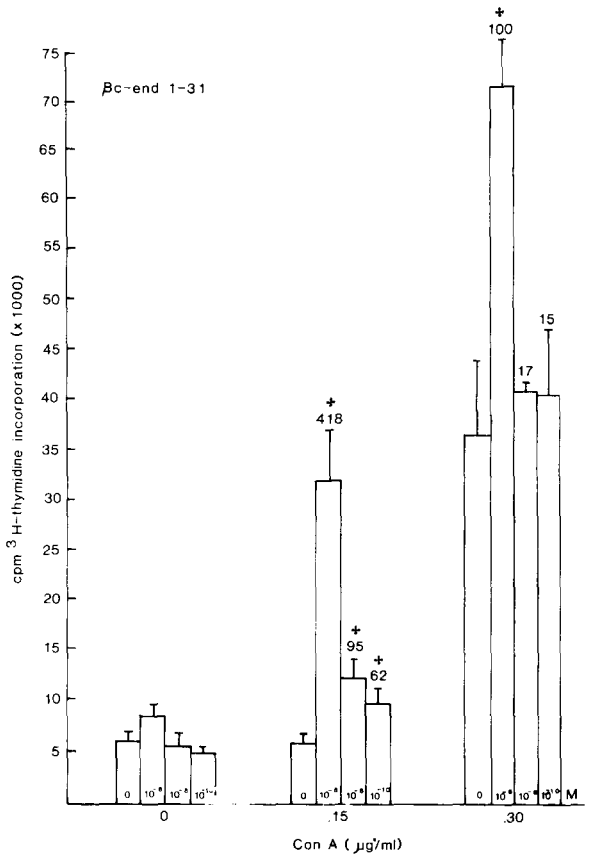

Fig. 2. Enhancement of splenocyte proliferation by βc-end 1–31. Experimental conditions were as described for Fig. 1. Percent enhancement is indicated above the standard deviation bars ($P < 0.01$). Results are again representative of at least four different experiments.

vidual mice from 50 to 640%. As the concentration of Con A was increased to 0.30 μg/ml, the magnitude of the enhancement at 10^{-6} M decreased at least two-fold (see Fig. 1) and frequently more (see Fig. 2). At 0.60 μg/ml Con A, enhancement was minimal or undetectable (data not shown). In addition, the effective dose of βh-end 1–31 and βc-end 1–31 varied with Con A concentration, such that enhancement was observed from 10^{-6} to 10^{-10} M peptide at 0.15 μg/ml Con A, while the enhancement occurred only at 10^{-6}–10^{-7} M peptide at higher Con A concentrations. This suggests that the magnitude of the enhancement depends upon the state of activation of the cell. Similar, if not identical, results were obtained if PHA was substituted for Con A (data not shown). In most experiments (70%), neither βh-end 1–31 nor βc-end 1–31 stimulated proliferation in the absence of mitogen, which also suggests that these opioid peptides have a preference for activated lymphocytes. These data are in agreement with our previous observations concerning βh-end 1–31 and βc-end 1–31 enhancement of IL-2 production (Gilmore and Weiner, 1988).

Subsequent experiments tested additional opioid peptides for their ability to enhance Con A-induced proliferation. Representative data are illustrated in Table II. Rat β-end 1–31 (βr-end 1–31), which differs from βc-end 1–31 at one C-terminal amino acid

TABLE II

Enhancement of Con A-induced proliferation in C57Bl/6J splenocytes by various β-end preparations

Opioid peptide (10^{-6} M)	Con A (μg/ml)		
	0	0.15	0.30
0	1.0 ± 0.5[a]	5.4 ± 0.5	33.7 ± 2.8
βh-end 1–31	1.1 ± 0.09	23.7 ± 5.4 (337)[b]*	41.5 ± 4.1 (23)
βc-end 1–31	1.0 ± 0.05	32.5 ± 2.8 (498)*	46.5 ± 1.9 (37)*
βr-end 1–31	1.8 ± 0.4	31.0 ± 1.1 (470)*	47.4 ± 5.3 (40)*
βh-(1–5)(16–31)	1.2 ± 0.2	34.1 ± 3.1 (528)*	47.7 ± 4.6 (32)
NAcβh-end 1–31	0.6 ± 0.08	16.6 ± 2.7 (106)*	35.7 ± 7.6

[a] Results are expressed as the mean cpm ± standard deviation × 1000 in triplicate determinations. These experiments were repeated a minimum of three times with similar results.

[b] Numbers in parentheses represent % enhancement over control wells lacking opioid peptide. Asterisks represent statistically significant differences at p values <0.01 (Student's t test).

residue (Ala^{26} in βc-end 1–31; Val^{26} in βr-end 1–31), was fully capable of the enhancement effect. Human β-end (1–5)(16–31), an opioid peptide lacking the intermediate amino acids and possessing the N- and C-terminal ends of the intact molecule, was also an effective enhancer of the Con A proliferative response. Interestingly, the differences in amino acid sequence existing among the four peptides tested did not affect the incidence of the enhancement. However, the magnitude of enhancement observed with βc-end 1–31 tended to be 50–250% greater than that observed with equimolar concentrations of the human hormone at low Con A doses. Greater enhancement or potency was also frequently associated with βh-end (1–5)(16–31) and was occasionally observed with βr-end 1–31. These data suggest that a moderate degree of species selectivity exists in the specificity of the β-end receptor expressed on the target splenocyte, and that this selectivity is directed toward the C-terminus of the molecule. In addition, a higher magnitude of enhancement was observed when the βh-end 1–31 analogue, D-Ala^2-βh-end 1–31, was tested (data not shown). D-Ala^2 substitution at the N-terminus confers greater resistance to proteolytic enzymes and increases the potency of opioid peptides in other biological assays (Deakin et al., 1980). All of these peptides exhibited dose-response curves similar to those illustrated in Figs. 1 and 2. Finally, *N*-acetylation of the human β-end molecule (NAc-βh-end 1–31) resulted in a reduced, but nevertheless significant magnitude of enhancement at 10^{-6} M peptide (Table II). Lower concentrations of this modified peptide had no effect on the Con A response. These data indicate that modification of either the N- or the C-terminus of the β-end molecule can result in changes in biological activity, and suggest that both termini interact with receptors on the target splenocytes.

The effect of naloxone on β-endorphin induced enhancement of proliferation

To investigate the role of the N-terminus in the enhancement of proliferation, and to identify the in-

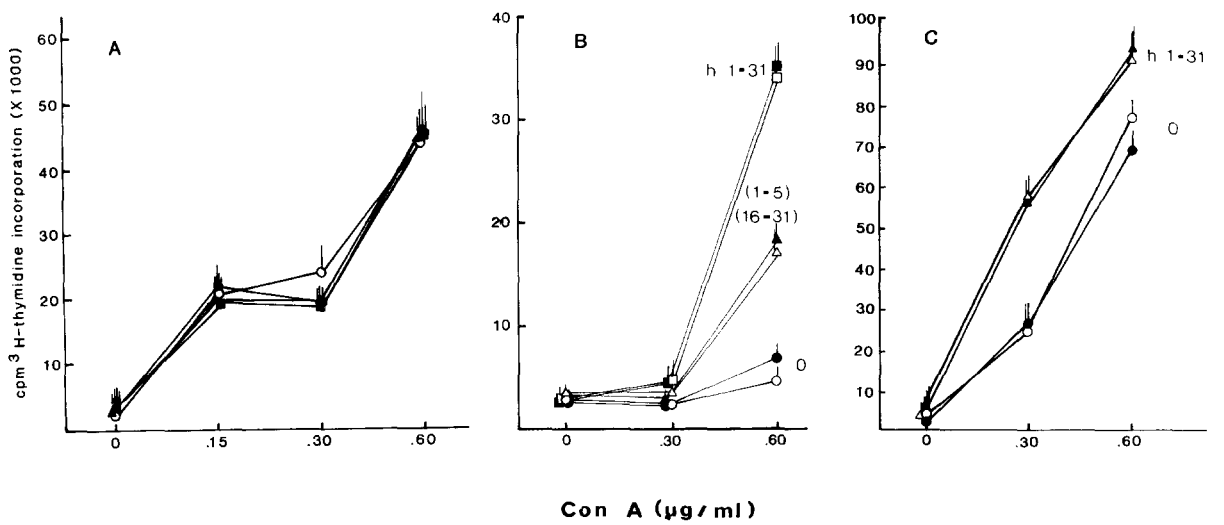

Fig. 3. The effect of naloxone on Con A-stimulated proliferation and β-end-induced enhancement of splenocyte proliferation. Results are expressed as mean [^{3}H]thymidine uptake (cpm) in triplicate cultures, with standard deviation represented by error bars. A. Naloxone was added at varying concentrations to splenocytes in the presence and absence of Con A (added at 0.15–0.60 µg/ml) and in the absence of opioid peptide. Concentrations of naloxone are represented by the following: ○, no naloxone; ●, 10^{-4} M; ■, 10^{-5} M; ▲, 10^{-6} M. B. Naloxone (10^{-5} M; solid symbols) was added to cells in the presence of βh-end (1–5)(16–31), represented by the square symbols, or βh-end 1–31, represented by the triangular symbols. Control wells included naloxone added in the absence of peptide (circular symbols). C. Naloxone (10^{-4} M, solid symbols) was added to the cells in the presence (triangles) and absence (circles) of 10^{-6} M βh-end 1–31. Results are representative of four individual experiments.

volvement or lack of involvement of opiate receptors, naloxone was added to the spleen cells in the presence and absence of β-end and Con A. As shown in Fig. 3A, the addition of 10^{-4} to 10^{-6} M concentrations of naloxone in the absence of β-end had no effect on the Con A-induced proliferative response. Naloxone (10^{-5} M) did not interfere with the enhancement induced by 10^{-6} M βh-end (1–5)(16–31) or βh-end 1–31 (Fig. 3B). Similarly, increasing the concentration of naloxone to 100-fold molar excess (10^{-4} M) had no effect on the enhancement observed in the presence of βh-end 1–31 (Fig. 3C). These data indicate that β-endorphin does not interact with a typical opiate receptor on the target splenocytes. They further suggest that while the N-terminus probably contributes to the overall enhancement effect, the primary activity appears to reside in the C-terminal amino acids.

The effect of opioids composed of varying lengths of N-terminal sequences

If the C-terminus of β-endorphin contains the active or enhancement-inducing peptide sequence, it can be argued that the removal of C-terminal amino acids would either decrease the incidence or potency of the enhancement, or abolish it altogether. Such a possibility was tested by the addition of opioid peptides lacking C-terminal amino acids, which are referred to as N-terminal opioid peptides. In most experiments designed to test this possibility, the N-terminal peptides βh-end 1–27, βc-end 1–27 (Table III, experiment 1) met- and leu-enk (Table III, experiment 2) and α-end (Table III, experiment 3) had no significant effect on the Con A-induced proliferative response. By contrast, βh-end 1–31, βc-end 1–31 and βh-end (1–5)(16–31) showed the typical pattern of enhancement with a magnitude of 118, 67 and 155%, respectively, at 0.15 μg/ml Con A. However, in some experiments, met-enk, α-end and βh-end 1–27 enhanced the Con A response 50–100% at 10^{-6} M peptide, a phenomenon that seemed to be associated with a high magnitude of enhancement (300–600%) observed in the same assays in which any of the intact 31-amino-acid peptides were tested (data not shown). βh-end 1–27 and α-end were more likely to enhance the response than met-enk, and leu-enk was not effective in any of the

TABLE III

The effect of N-terminal opioid peptides on Con A-induced proliferation in C57Bl/6J splenocytes

Expt.	Opioid peptide (10^{-6} M)	Con A (μg/ml)		
		0	0.15	0.30
1	0	2.9 ± 0.3[a]	8.5 ± 0.07	28.6 ± 7.1
	βh-end 1–31	4.9 ± 0.8	18.7 ± 0.7 (118)[b]*	43.1 ± 3.8 (50)*
	βh-end 1–27	2.5 ± 0.7	7.7 ± 1.2	26.0 ± 3.3
	βc-end 1–27	2.5 ± 0.3	7.3 ± 1.2	28.4 ± 3.6
2	0	3.4 ± 0.1	25.3 ± 1.5	40.7 ± 2.8
	βc-end 1–31	6.1 ± 0.9	42.5 ± 2.6 (67)*	46.7 ± 1.4
	met-enk	3.0 ± 0.7	23.1 ± 0.6	36.9 ± 3.9
	leu-enk	4.4 ± 0.6	29.4 ± 1.8	35.9 ± 6.4
3	0	5.7 ± 1.2	12.6 ± 1.7	51.2 ± 6.7
	βh-end (1–5)(16–31)	6.1 ± 1.9	32.3 ± 0.2 (155)*	86.3 ± 1.5 (68)*
	α-end	4.1 ± 0.4	14.2 ± 1.0	50.5 ± 7.1
	met-enk	6.5 ± 1.7	15.2 ± 3.7	43.2 ± 5.5

[a] Results are expressed as the mean cpm ± S.D. × 1000. These experiments were repeated a minimum of four times with similar results.

[b] Numbers in parentheses represent % enhancement over control wells lacking opioid peptide. Asterisks represent statistical significance at $p < 0.01$.

experiments in which it was tested. These data suggest that the fewer β-end C-terminal amino acids are present, the less likely it is that the opioid peptide will enhance the Con A proliferative response in the target splenocyte. Such activity is highly reminiscent of studies demonstrating that the sequential removal of C-terminal amino acids from the 31-amino-acid human peptide is associated with decreasing potency in the induction of analgesia (Deakin et al., 1980). However, these data may also indicate the activity of cell-associated peptidases, which are more likely to inactivate the shorter peptides. Experiments in which the cultures were replenished with the original dose of each peptide on a daily basis did not demonstrate a more reliable enhancement; nor did the substitution of D-Ala^2-met-enk for met-enk or the addition of the broad-spectrum peptidase inhibitor, bacitracin (data not shown). These results argue against peptidase activity being the sole determinant of the incidence of enhancement. Finally, the lack of naloxone irreversibility of the enhancement observed with peptides in which both the N- and C-termini are intact again argues for an active role of the C-terminus in mediating this biological effect.

The effect of opioid peptides composed of various β-end C-terminal amino acids.

In order to investigate further the role of the C-terminus of βh-end 1–31, two opioid peptides lacking N-terminal amino acids were tested. As illustrated in Fig. 4, βh-end 6–31 enhanced the proliferative response induced by 0.15 μg/ml Con A in dose-dependent fashion, and at a magnitude similar to that observed with the intact βh-end 1–31 peptide. At the same mitogen and peptide concentration, βh-end 18–31 was also capable of enhancement, but at approximately half the magnitude observed with the longer peptides. In addition, both βh-end 1–31 and βh-end 6–31, but not βh-end 18–31, were capable of enhancing the response induced by 0.30 μg/ml Con A (data not shown). This suggests that the longer the N-terminal extension of the

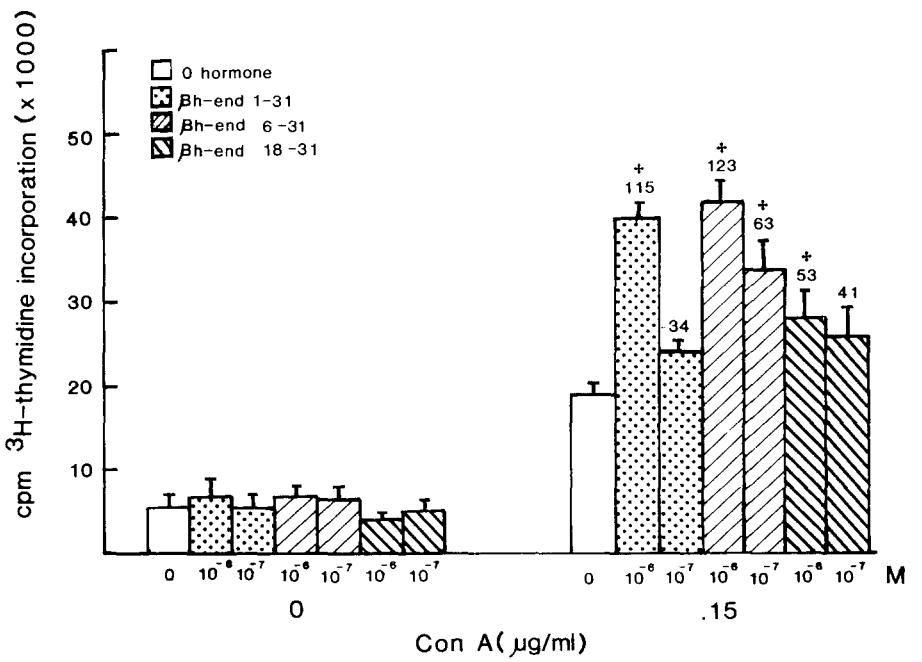

Fig. 4. The influence of C-terminal opioid peptides on the Con A proliferative response. Experimental conditions were as described for Fig. 1, as are the designations for percent enhancement. Asterisks represent statistically significant enhancement at $P < 0.01$. Experiments were repeated a minimum of four times with similar results.

C-terminus, the greater the potency of the biological activity of the opioid peptide. Daily addition of either βh-end 18–31 or βh-end 6–31 at concentrations ranging from 10^{-6} to 10^{-8} M failed to increase either the magnitude or the potency of these peptides. Thus, it is again apparent that the presence of the N-terminal amino acids contributes to the interaction of β-end with its receptor(s). These data provide further support for the notion that both ends of the β-endorphin molecule are responsible for promoting the enhancement of Con A-induced proliferation of murine splenocytes.

Discussion

In this communication we have reported a detailed examination of the ability of β-end to enhance mitogen-stimulated murine splenocyte proliferation, measured as cpm [^{3}H]thymidine incorporation. Our initial and primary goals were to identify the portion of the molecule responsible for the effect, and to infer characteristics of the receptor(s) expressed by the target splenocytes. Previous studies by ourselves (Gilmore and Weiner, 1988) and others (Gilman et al., 1982) have identified the C-terminal amino acids of the 31-residue β-end molecule as the active sequence in the enhancement of T lymphocyte or splenocyte functions. In the current studies, the most intriguing finding is that although the primary enhancement activity was associated with the C-terminus, increased magnitude and potency were observed with extension of the linear peptide sequence toward the N-terminus. Thus, the magnitude of the enhancement is greatest with the intact 31-amino-acid hormone, equal or less with the truncated βh-end 6–31, which is in turn greater than that observed with βh-end 18–31. The N-terminal tyrosine residue does not appear to be required for the effect, since naloxone did not inhibit the enhancement and since *N*-acetylation, which inactivates its analgesic and opioid binding activity, also did not abolish the enhancement. In addition, peptides possessing the N-terminal core sequence and lacking the C-terminal 4 amino acids were essentially ineffective enhancers of Con A-stimulated proliferation. The opioid specificity of this biological activity is strikingly similar to that described for opioid peptide binding in CNS tissue, including the rabbit spinal cord (Ferrara and Li, 1980) and various neuroblastoma cell lines (Westphal and Li, 1984a). In these studies, the binding of βh-end 1–31 was not inhibited by naloxone or met-enk, and βh-end 6–31 was 75% as effective as βh-end 1–31. Similar, if not identical, binding activity for βh-end 1–31 on human transformed lymphocytes was reported by Hazum et al. (1979). The binding activity of the 31-amino-acid peptide in each of these studies was heterogeneous, with high-affinity sites showing an apparent K_d of 2–4 nM and low-affinity sites with at least 10-fold lower dissociation constants. Equally intriguing are reports of C-terminal binding of βh-end 1–31 to the terminal SC5b–9 complex of human complement (Schweigerer et al., 1982, 1983). C-terminal peptides and NAc-βh-end 1–31 were able to bind this complex with high affinity, while naloxone, morphine and met-enk were not. Collectively, these reports provide more than sufficient evidence that non-opioid binding sites with similar properties are shared by cells in the CNS and immune system. The data reported in the current communication indicate that, for cells of the immune system, these non-opioid binding sites are capable of transducing a biologically significant signal at peptide concentrations that are consistent with their reported binding affinities.

The precise identity of the receptor(s) present on the activated or proliferating splenocyte is currently unknown. Three major opioid receptor types have been identified, including mu, delta and kappa (for review, see Simon, 1987; Civelli et al., 1987). β-end binds both mu and delta receptors with similar affinity, and also binds kappa receptors, albeit with lesser affinity. In addition, β-end appears to interact with a fourth non-opioid or naloxone-insensitive receptor, designated sigma. The balance of current evidence indicates that the major opioid receptor types represent distinct molecular entities which show selectivity for opioid peptides derived from each of the three precursor molecules. Thus, opioid peptides offer a unique opportunity to study receptor selectivity, a phenomenon which is important in

the regulation of CNS and endocrine function (Schwyzer, 1986). One of the hypotheses that has emerged in the study of opioid peptide binding is that the N-terminus represents the 'message domain' and its ability to bind preferentially to different receptors or receptor subtypes is determined by the various C-terminal tails, or 'address segments' (Schwyzer, 1986). In this scheme, receptor selectivity is also influenced by peptide interactions in the membrane. Our data suggest, however, that the C-terminus is capable of transducing an intracellular message in cells of the immune system, an assertion that is supported by studies of both CNS and lymphocyte functions. For example, the C-terminal tetrapeptide of βh-end 1–31, LysLysGlyGlu, initiates forelimb regeneration in the adult newt (Morley and Ensor, 1986). The C-terminal dipeptide GlyGlu has several biological activities, including the regulation of endplate acetylcholinesterase in rat and chick muscle cells (Haynes and Smith, 1985), inhibition of neuronal firing in the reticular formation (Smyth et al., 1983) and inhibition of human suppressor T cell activity (McCain et al., 1986). Although the intracellular signalling mechanisms responsible for these activities are incompletely understood, it is known that opioid binding in CNS tissue is linked to inhibition of adenylate cyclase (AC) (Collier and Roy, 1974; Wolleman, 1981). Farrar (1986) has reported that β-end inhibits basal and prostaglandin E_2-stimulated AC activity in the cloned T cell line, CT6. A more recent report indicates that β-end enhances Con A-stimulated $^{45}Ca^{2+}$ uptake in rat thymocytes, an effect that was not inhibited by naloxone (Hemmick and Bidlack, 1985). Both of these effects are consistent with conditions favoring T cell activation by mitogens or antigens (Gilbert and Hoffman, 1985; Ledbetter et al., 1986; Weiss et al., 1986).

The possibillity that the lack of or decrease in the magnitude of the enhancement observed with the shorter peptides may be due to cell-associated peptidase activity is worthy of consideration, especially since D-Ala^2-βh-end 1–31 was frequently capable of an increased magnitude of enhancement. Our attempts to control for peptidase activity by the use of the more stable analogue, D-Ala^2-met-enk, by daily addition of this peptide as well as all of the remaining N- or C-terminal fragments of β-end, and by the use of bacitracin in the assay medium, failed to increase the incidence or magnitude of the enhancement. In addition, preliminary experiments in which a cocktail of peptidase inhibitors was included in the assays show that the intact 31-amino-acid opioid peptides are capable of at least 400% greater potency than several of the smaller peptides, including met-enk. As previously mentioned, these results argue against peptidase activity being the only determinant of enhancement activity in the current experimental model. They further suggest that the alternative hypothesis, i.e., that the lack of enhancement reflects the unique properties of the receptor(s) expressed on the target splenocyte, is valid.

The identity of the splenocyte expressing these β-end receptors is currently unknown. Since β-end enhances Con A- and PHA-induced IL-2 production in murine splenocytes and in the cloned T cell line, LBRM-33, in similar fashion (Gilmore and Weiner, 1988), it seems likely that the target cell is a helper T cell. It is unlikely that it is a B cell, since β-end does not affect LPS-stimulated rat splenocyte proliferation (Gilman et al., 1982). Interestingly, Farrar (1986) reported data in which classical mu and delta opioid receptor agonists show selective binding to human T and B cells. Whether this selectivity extends to phenotypically distinct T cell subsets is a topic of investigatory interest in our laboratory. It is also possible that β-end influences macrophage activities to enhance the overall proliferative response. Preliminary experiments indicate that this does not occur in our assay system, though final verification depends upon additional analyses.

One of the more interesting observations in the current study is the preference of β-end for activated lymphocytes; i.e., β-end does not stimulate proliferation in the absence of Con A or PHA. Similarly, β-end does not stimulate proliferation in rat splenocytes (Gilman et al., 1982) or IL-2 production in either mouse splenocytes or LBRM-33 cells (Gilmore and Weiner, 1988). Additional data reported by Cross et al. (1987) indicate that 6-hydroxydopamine, injected into the cisterna mag-

na of mice, is capable of inducing suppressor T cells only in animals that have been immunized with specific antigen. The most plausible explanation for these phenomena is that resting lymphocytes do not express high-affinity receptors for neuropeptides or neurotransmitters. It is well documented that high-affinity receptors for insulin and growth hormone are expressed on activated, and not resting, lymphocytes (Helderman and Strom, 1978; Snow et al., 1981). In addition, the binding affinity of dopamine to rat lymphocyte membranes increased significantly in Con A-stimulated cells relative to unstimulated cells (Ovadia and Abramsky, 1987). Finally, Hazum et al. (1979) found that low-affinity receptors for β-end were present on normal human lymphocytes, but that only transformed lymphocytes expressed both high- and low-affinity receptors. These data provide compelling evidence for the existence of an immunomodulatory role for neuropeptides. Furthermore, the data presented in the current communication suggest not only that opioid peptides display significant immunomodulatory activity, but that they may do so in much the same manner as they modify neural activity.

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