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GLYCYL-L-GLUTAMINE: A DIPEPTIDE NEUROTRANSMITTER DERIVED FROM BETA-ENDORPHIN

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The objective of this research is to test the hypothesis that glycyl-L-glutamine, a dipeptide synthesized through the post-translational processing of ß-endorphin, acts as a neurotransmitter in brain and a circulating hormone in the periphery. To test this hypothesis, we established three working objectives: (1) to evaluate the physiological responses produced by glycyl-L-glutamine and test whether it modulates the opiate actions of ß-endorphin; (2) to examine its distribution in brain and pituitary and determine whether it is specifically co-localized with ß-endorphin; (3) to characterize the receptors which mediate glycyl-L-glutamine's pharmacologic effects. During the current project period, we found that glycyl-L-glutamine produces three markedly different pharmacologic responses: it stimulates a trophic response in cardiac myocytes, inducing expression of the asymmetric form of acetylcholinesterase, a classical marker for synaptic innervation; it produces neuroimmune regulatory effects in T-lymphocytes, enhancing c-myc oncogene expression, a component of the T-lymphocyte proliferative

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response to antigens; and it inhibits ß-endorphin induced antinociception, ß-endorphin's most characteristic centrally mediated action. In addition, substantial progress has been made toward establishing immunoassay and immunohistochemical methods for detecting endogenous glycyl-L-glutamine. Subsequent experiments will apply these methods to map glycyl-L-glutamine's regional distribution in brain, determining whether it is selectively localized in ß-endorphin neurons, and to identify the factors which regulate its synthesis and release. The present studies have thus demonstrated that glycyl-L-glutamine produces pharmacologic effects in both brain and peripheral tissues, supporting the concept that it acts as both a neurotransmitter and a circulating hormone, and have developed the methods required to conclusively establish glycyl-L-glutamine's role in neural and endocrine communication.

FOREWORD

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INTRODUCTION

Glycyl-L-glutamine is a dipeptide synthesized through the post-translational processing of β-endorphin. β-endorphin processing has been intensely studied in recent years because it profoundly alters the peptide's analgetic activity, transforming β-endorphin-1-31 from a highly potent opiate receptor agonist to an antagonist, β-endorphin-1-27, and to opiate inactive forms, β-endorphin-1-26 and the α-N-acetyl forms of all three peptides (Deakin et al., 1980; Akil et al., 1981; Nicolas and Li, 1985). Glycyl-L-glutamine, co-synthesized with β-endorphin-1-27 when β-endorphin-1-31 is endoproteolytically cleaved, has not been as thoroughly evaluated as the larger β-endorphin forms, although existing evidence indicates that it, too, may participate in synaptic transmission. Indeed, many of its known effects counterpose the opiate actions of β-endorphin-1-31 (Hirsch and O'Donohue, 1986; McCain et al., 1986; Koelle et al., 1988; Lotwick et al., 1990). The objective of our research is to establish whether glycyl-L-glutamine acts as a neurotransmitter in brain and a circulating hormone in the periphery.

Evidence that glycyl-L-glutamine functions in synaptic transmission first arose from electrophysiologic studies by Parish et al. (1983) showing that iontophoretic glycyl-L-glutamine application inhibited the firing frequencies of brainstem neurons. This activity was not reversed by naloxone, an opiate antagonist, or by strychnine, which blocks receptors for glycine, one of glycyl-L-glutamine's constituent amino acids. In addition, these investigators isolated glycyl-L-glutamine from sheep brainstem, demonstrating that it is present in amounts equivalent to the sum of β-endorphin-1-27 and -1-26, as one would predict. Immunohistochemical studies also showed that glycyl-L-glutamine is localized in the intermediate, but not in the anterior lobe of the pituitary (Plishka et al., 1985) where β-endorphin does not undergo C-terminal cleavage. (Eipper and Mains, 1980; O'Donohue and Dorsa, 1982).

Several additional lines of evidence further support the concept that glycyl-L-glutamine acts as a neurotransmitter. Behavioral studies revealed that glycyl-L-glutamine inhibits β-endorphin-1-31 induced grooming in rats, a response thought to reflect mechanisms of attention and arousal (Hirsch and O'Donohue, 1985). Glycyl-L-glutamine is also thought to function as a trophic agent at the neuromuscular junction (Lotwick et al., 1990) and in autonomic ganglia (Koelle et al., 1988). In both tissues, neuronal innervation induces synaptic acetylcholinesterase (AChE) activity; Gly-Gln produces a comparable effect, suggesting that it may be the neurotrophic agent mediating the response. β-endorphin-1-31 (but not -1-27) has the opposite effect, reducing AChE, and may be responsible for the subsequent lowering of AChE activity observed during synaptic reorganization.

Glycyl-L-glutamine may also play a role in the neuroendocrine regulation of the immune system. A large body of evidence supports

the concept that the central nervous system influences immune response and much of this work focuses on the role of POMC peptides as neuroimmune mediators (Weber and Pert, 1984; Morley et al., 1987). McCain et al. showed that very low concentrations of glycyl-L-glutamine enhance phytohemagglutinin (PHA) induced T-lymphocyte proliferation (McCain et al., 1986; 1987). This was a key finding because the PHA response, which mimics antigen induced lymphocyte activation, is often used as a measure of immune competence. Once again, β-endorphin-1-31 has the opposite effect, suppressing PHA-induced proliferation (McCain et al., 1987). These observations suggest that glycyl-L-glutamine release from the intermediate pituitary may partially counteract stress-induced suppression of immune function.

These intriguing findings support the concept that glycyl-L-glutamine, like other β-endorphin peptides, functions both as a neurotransmitter in brain and as a circulating hormone in the periphery; however, the basic studies required to firmly establish such a role for glycyl-L-glutamine have not been performed. Several criteria must be fulfilled. First, glycyl-L-glutamine's pharmacologic spectrum of activity must be definitively established, emphasizing its interactions with β-endorphin-1-31 and opiate drugs. Second, it must be demonstrated that glycyl-L-glutamine is actually present in, and only in, β-endorphin releasing neurons and endocrine cells. And third, receptors for glycyl-L-glutamine must be unequivocally identified and thoroughly characterized. We predict that glycyl-L-glutamine receptors exhibit 'synaptic specificity', meaning they are found only in β-endorphin neuronal synapses. The concept of synaptic specificity is important because it means that drugs targeted on glycyl-L-glutamine receptors will act only at β-endorphin neuronal synapses, unlike all existing opiate drugs which interact with different opioid receptor subtypes present in all three opioid peptide systems. Thus, the longer term objective of this research is to establish the data base necessary to design therapeutic agents targeted on glycyl-L-glutamine receptors to selectively modify the biological effects of both glycyl-L-glutamine and β-endorphin.

RESULTS AND DISCUSSION

I. Pharmacologic Effects:

The initial objective of our research was to further evaluate the physiological responses produced by glycyl-L-glutamine. We reasoned that knowledge about glycyl-L-glutamine's physiological effects would guide subsequent efforts to localize glycyl-L-glutamine and its receptors, to determine whether it functions alone in neural and endocrine communication or specifically modulates β-endorphin's actions, and ultimately, to predict the effects of pharmacologic agents targeted on glycyl-L-glutamine receptors. To initiate these studies, we selected three

experimental paradigms, evaluating both peripheral and central responses to glycyl-L-glutamine, including; its trophic effect on cardiac myocytes, its neuroimmunomodulatory activity on T-lymphocyte proliferation and its central action on β-endorphin-induced antinociception.

A. Trophic Effects on Cardiac Myocytes: The initial objective of this project period was to test whether glycyl-L-glutamine produces trophic effects on cardiac myocytes, extending our ongoing interest in the cardiovascular effects of β-endorphin peptides (Hirsch and Millington, 1991). The rational for the study was based on an earlier report that glycyl-L-glutamine induces the expression of acetylcholinesterase (AChE) at the neuromuscular junction (Lotwick wt al., 1990) and in sympathetic ganglia (Koelle et al., 1988). Multiple molecular forms of AChE exist in both cardiac and skeletal muscle, as well as in ganglia and other tissues, but only one, the asymmetric A_{12} form is specifically associated with neuronal synapses (Rieger et al., 1980). A marked increase in A_{12} AChE activity occurs during the developmental period when skeletal muscle is innervated; however, the neuronal agent which induces A_{12} AChE has not been identified. Recently, Lotwick et al. reported that very low glycyl-L-glutamine concentrations (10 nM - 10 μM) induce A_{12} AChE expression in rat and chick muscle cells in vitro suggesting to the authors that it may be the trophic substance normally responsible for regulating the AChE response to innervation (Lotwick et al., 1990). Although glycyl-L-glutamine has not as yet been identified in motor neurons, it is known that β-endorphin peptides, including β-endorphin-1-27, are transiently expressed during the critical developmental period (Haynes et al., 1984) inferring that glycyl-L-glutamine must also be present (Parish et al., 1983). Interestingly, β-endorphin-1-31 has the opposite effect on AChE, reducing expression of the A_{12} form (Haynes et al., 1984).

Our results demonstrate that glycyl-L-glutamine also produces trophic effects on cardiac myocytes (Battie et al., 1991). Myocyte cultures were prepared by enzymatically dissociating ventricles from 2-4 day old rats; the myocytes were confluent and beating for at least 48 h prior to experimentation (Hagler and Nyquist-Battie, 1990). Separating AChE molecular forms by sucrose density gradient fractionation revealed that cultured myocytes, like skeletal muscle cells, normally produce very little A_{12} AChE. Only about 3% of total AChE activity was attributable to the A_{12} form; globular forms, including monomeric, G_1 (56%), and tetrameric, G_4 (42%), forms predominate (Fig. 1). Co-incubation with glycyl-L-glutamine (1 μM) for 72 h produced a dramatic increase in the A_{12} form, elevating it from 3% to 21% of total AChE activity. Corresponding decreases occurred in the G_1 (36%) form, but there was no change in G_4 AChE or in total AChE activity. Glycyl-L-glutamine did not change the specific activity of total cellular acetylcholinesterase or its rate of secretion. The response to glycyl-L-glutamine appeared to be relatively specific to the extent that neither glycyl-L-glutamate nor glycyl-D-glutamine had any effect on AchE

expression. These results provide the first evidence that glycyl-L-glutamine acts as a trophic agent in the heart.

β-Endorphin Processing in Rat Heart: Yet to be determined, is whether glycyl-L-glutamine is normally present in heart although the recent identification of immunoreactive β-endorphin in rat heart (Forman et al., 1989) suggests that it may, indeed, be present. We plan to address this question directly, once we have developed methods for quantifying tissue glycyl-L-glutamine concentrations; however, the identification of C-terminally shortened β-endorphin peptides in heart would provide compelling inferential evidence that glycyl-L-glutamine is also present and techniques for isolating the molecular forms of β-endorphin are routinely used in our laboratory.

We, therefore, initiated efforts to characterize the post-translational processing of β-endorphin in rat heart. The methodologic approach consists of three steps: extraction using Sep-Pak C-18 cartridges; gel filtration high performance liquid chromatography (HPLC) to separate β-lipotropin from β-endorphin-sized peptides (Eipper et al., 1983) and ion exchange HPLC to identify the individual β-endorphin forms (Millington et al., 1987). Gel filtration HPLC demonstrated that β-endorphin immunoreactivity (iβ-endorphin) in the heart is primarily attributable to β-endorphin sized molecules and not their immediate precursor, β-lipotropin; β-lipotropin constituted only 22 % of total immunoreactivity (Fig. 2) (Evans et al., 1991), similar to that of the intermediate pituitary and brain rather than the anterior pituitary where β-lipotropin predominates (Eipper and Mains, 1980; O'Donohue and Dorsa, 1982). Further analysis of β-endorphin sized peptides by ion exchange HPLC revealed that β-endorphin-1-31 is the predominant form expressed in heart, constituting 49.6% of total iβ-endorphin but that α-N-acetylated, C-terminally shortened forms were also produced, including β-endorphin-1-27 (14.3%), α-N-acetyl-β-endorphin-1-27 (8.4%), β-endorphin-1-26 (7.9%) and N-acetyl-β-endorphin-1-26 (4.7%) (Fig. 3). Thus, approximately 35% of the β-endorphin-1-31 produced in heart is further cleaved to glycyl-L-glutamine and C-terminally shortened forms, β-endorphin-1-27 and β-endorphin-1-26.

β-Endorphin's precise cellular localization in heart tissue remains to be determined. But recently, however, our collaborators have found, using *in situ* hybridization histochemistry, that POMC mRNA is expressed by cardiac ventricular cells, suggesting that POMC peptides are synthesized by heart tissue, rather than autonomic neurons innervating the heart (personal communication, Dr. Lloyd Forman). Consistent with this finding, our studies also revealed that, in addition to β-endorphin, rat heart contains additional products of POMC processing, including ACTH and α-melanocyte stimulating hormone (α-MSH). Thus, gel filtration HPLC showed that, as in the case of β-lipotropin's conversion to β-endorphin, ACTH was almost entirely cleaved to α-MSH; the α-MSH:ACTH ratio was 6.1:1, similar to that for β-endorphin:β-lipotropin (4.6:1). Ongoing studies will use reverse phase HPLC to further

identify the specific α-MSH forms present in heart extracts (des-acetyl-α-MSH, α-MSH or di-acetyl-α-MSH). Identification of ACTH and α-MSH in rat heart further supports the conclusion that the immunoreactive β-endorphin found in heart is an authentic product of POMC processing. Moreover, these studies are the first to identify ACTH and α-MSH, as well as the individual molecular forms of β-endorphin, in rat heart and they provide strong evidence that glycyl-L-glutamine is also expressed.

B. Neuroimmune Effects: A rapidly growing body of evidence documents the role of β-endorphin peptides in mediating the effects of stress on the immune system (Weber and Pert, 1984). β-endorphin-1-31 produces a variety of actions on immune cell function, many of which are mediated by receptors recognizing the non-opioid forms of β-endorphin released from the intermediate lobe. Little is known about the role of glycyl-L-glutamine, although McCain et al. have shown that glycyl-L-glutamine enhances phytohemagglutinin (PHA) induced T-lymphocyte proliferation (McCain et al., 1987), a measure of the ability of lymphocytes to respond to antigenic stimuli which is often used to test immune competence. The effect of glycyl-L-glutamine on T-lymphocytes appears to be indirect, however, resulting from inhibition of T-cell suppressor cell activity. β-endorphin-1-31 produces the opposite response, enhancing suppressor cell activity, which in turn inhibits T-lymphocyte proliferation. Thus, anterior and intermediate lobe secretory products produce opposing actions on suppressor cells; β-endorphin-1-31, released almost exclusively from the anterior lobe, activates suppressor cells while glycyl-L-glutamine, released from the intermediate lobe, inhibits the response.

β-endorphin-1-31 also acts directly on T-lymphocytes, stimulating PHA-induced proliferation (Gilman et al., 1982; Gilmore and Weiner, 1988, 1989; Hemmick and Bidlack, 1990). The exact identity of the receptor mediating this, and other immune responses to β-endorphin remains somewhat controversial, however, and there is evidence for the involvement of both opioid (Madden et al., 1987; Avadia et al., 1989) and non-opioid (Hazum et al., 1979) β-endorphin binding sites. Interestingly, in lymphocyte proliferation assays, β-endorphin-1-27 act as an agonist with a potency similar to β-endorphin-1-31, suggesting the involvement of a non-opioid receptor, similar to our findings on central cardioregulation (Hirsch and Millington, 1991). Yet to be examined, is whether glycyl-L-glutamine also directly stimulates T-lymphocyte proliferation; if so, then one might hypothesize that C-terminal cleavage of β-endorphin-1-31 to β-endorphin-1-27 and glycyl-L-glutamine amplifies the response by producing two stimulatory peptides.

To test this hypothesis, we examined the effect of β-endorphin and glycyl-L-glutamine on c-myc protooncogene expression in a human T-lymphocyte cell line, Jurkat E6-1. Mitogenic stimulation induces a rapid increase in c-myc mRNA levels, both in normal lymphocytes (Reed et al., 1986) and in Jurkat E6-1 cells (Hough et al., 1990); c-myc mRNA is thought to encode a transcriptional activating factor

which is a necessary intermediary in the proliferative response. Jurkat E6-1 cells (1 x 10^6) were grown in RPMI medium containing 10% fetal calf serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml) and mitogenesis was stimulated with concanavalin A (con A). Total RNA was isolated by centrifugation through 5.7 M CsCl followed by phenol/chloroform extraction and ethanol precipitation. RNA was then electrophoresed on formaldehyde agarose gels, transferred to nitrocellulose by vacuum blotting and hybridized with a ^{32}P-labeled nick-translated 2.5 kb ecoRI fragment of human c-myc cDNA. Quantitation was performed by densitometric analysis of the resulting autoradiograms and standardized against rRNA.

We found that incubating Jurkat E6-1 cells with both β-endorphin-1-31 (30 nM) and glycyl-L-glutamine (30 nM) produced a 2.5-fold increase in con A (50 ng/ml) stimulated c-myc mRNA expression; when added to the cell cultures alone, neither β-endorphin-1-31 nor glycyl-L-glutamine had any significant effect at the dose tested (30 nM) (Fig 4). As expected, con A alone elevated c-myc mRNA to 200% of control levels. Final interpretation of these results awaits full individual dose-response evaluations of both peptides. However, while yet preliminary, the results suggest that glycyl-L-glutamine potentiates β-endorphin-1-31 induced c-myc mRNA expression. It will be of particular interest to test whether β-endorphin-1-27 produces the same response and, if so, to evaluate the structural determinants of the β-endorphin receptor involved to determine whether glycyl-L-glutamine acts through the same or a different binding site.

β-Endorphin Processing in Human Pituitary: The finding that glycyl-L-glutamine potentiates β-endorphin-induced c-myc gene expression in a human lymphocyte cell line raised the question as to the source of glycyl-L-glutamine which normally regulates immune cell function. One likely source may be the lymphocytes themselves, which are known to express β-endorphin and other POMC derived peptides (Morley et al., 1987), a possibility which we are now beginning to examine experimentally. A second source, of course, is the intermediate pituitary which, at least in the rat, produces high levels of glycyl-L-glutamine. But adult humans lack an intact intermediate lobe questioning whether β-endorphin-1-31 undergoes significant post-translational processing to glycyl-L-glutamine and C-terminally shortened β-endorphin peptides. Previous studies have identified small amounts of N-acetyl-β-endorphin-1-27 immunoreactivity in extracts of fetal (Facchinetti et al., 1989) and adult (Smith et al., 1985) human pituitaries, but N-acetyl-β-endorphin-1-26 was not produced, suggesting that species differences in β-endorphin's primary sequence may prevent its synthesis in the human. These studies were limited in scope, however, and did not examine non-acetylated β-endorphin forms, nor did they identify the cell type, whether corticotroph or melanotroph, containing acetylated β-endorphin peptides.

To address these questions, we analyzed the molecular forms of β-endorphin in human pituitary using both chromatographic analysis and immunohistochemistry (Bernard et al., 1991). Our

previous studies showed that β-endorphin processing is quite stable in post-mortem rat brain, remaining essentially the same as control values for up to 24 hours (Millington and Smith, 1991). Gel filtration HPLC analysis of human pituitaries revealed that the major portion of total iβ-endorphin is attributable to β-lipotropin; β-endorphin sized peptides constituted only 17.6% of total iβ-endorphin (Fig. 5). Further ion exchange HPLC analysis showed that β-endorphin-1-31 was the predominate β-endorphin peptide, composing 85.0% of total iβ-endorphin. β-endorphin-1-27 (4.5%) and β-endorphin-1-26 (6.6%) were also present along with very low levels of α-N-acetyl-β-endorphin peptides, together comprising less than 4% of total iβ-endorphin (Fig. 6). Thus, approximately 15% of total pituitary β-endorphin-1-31 is further processed to glycyl-L-glutamine and C-terminally shortened β-endorphin peptides.

We also employed immunohistochemical experiments using an antiserum which specifically recognizes α-N-acetyl-β-endorphin peptides to map the distribution of cells which process β-endorphin-1-31. The antiserum stained a small number of cells localized along the border between the anterior and neural lobes suggesting that they may be derived from the fetal intermediate lobe; however, α-N-acetyl-β-endorphin immunoreactive cells were also dispersed throughout the anterior lobe. α-MSH immunoreactive cells are similarly distributed, suggesting that cells resembling melanotrophs, biochemically, are distributed throughout the human pituitary. In summary, these results demonstrate that, despite important differences in primary sequence, β-endorphin is processed to both β-endorphin-1-27 and -1-26 the human pituitary.

C. Antinociception: The profound analgesia produced by β-endorphin-1-31 is, of course, its most characteristic physiologic action. Thus, it seemed logical to us to begin our evaluation of glycyl-L-glutamine's central effects by testing whether it modifies β-endorphin-1-31-induced antinociception. Although glycyl-L-glutamine biosynthesis within endogenous pain control pathways has not, as yet, been demonstrated directly, it can be inferred from the fact that β-endorphin-1-27, co-synthesized with glycyl-L-glutamine when β-endorphin-1-31 is post-translationally processed, is present within the periaqueductal grey and other brain structures known to involved in endogenous pain control (Berglund et al., 1989; Zakarian and Smyth, 1979; Basbaum and Fields, 1984). The C-terminal proteolysis of β-endorphin-1-31 within the pain control system is particularly intriguing because β-endorphin-1-27 has been shown to be a potent antagonist of β-endorphin-1-31 antinociception (Nicholas and Li, 1985). Previous studies have demonstrated that glycyl-L-glutamine antagonizes certain behavioral responses produced by β-endorphin-1-31; specifically, glycyl-L-glutamine (22 nmol) inhibits β-endorphin-1-31 induced grooming and stretch-yawn syndrome, behaviors thought to reflect states of attention and arousal while producing no overt behavioral response when administered alone (Hirsch and O'Donohue, 1986). In light of these findings, we hypothesize that glycyl-L-glutamine may inhibit the antinociceptive action of β-endorphin-1-31.

To test this hypothesis, we initiated studies of glycyl-L-glutamine's effect on β-endorphin-1-31 antinociception using the tail flick reflex as an antinociception test paradigm. The tail flick test measures the response time for a rat to remove its tail from a beam of light irradiating the distal tail surface (D'Amour and Smith, 1941). Rats are briefly restrained during the test, the heat intensity of the light is adjusted to produce a baseline reflex of 3.5 seconds, and for animals that do not respond, the test is terminated after 8.5 seconds. Baseline values are calculated from the mean of three trials and rats are tested once every fifteen minutes for two hours. The data are expressed as percent of maximum possible effect (%MPE) using the conventional equation: %MPE = [test latency - baseline/cutoff - baseline] x 100. Intracerebroventricular (icv) injections are performed by implanting a guide cannula in the lateral ventricle (stereotaxic coordinates, from lambda: A=6.7 mm, L=1.5 mm, D=4.3 mm) under ketamine/xylazine anesthesia and held in place by dental acrylic. Rats are allowed to recover from surgery for one week prior to each experiment and peptide injections are administered to conscious animals over a period of 60-90 seconds using a 10 μl syringe attached to an injection cannula with PE-10 tubing. Cannula placements are verified by dye injection at the end of each experiment.

Our initial objectives were to establish this nociceptive test paradigm in our laboratory, ensuring that the method generated reproducible data in our hands, and to produce initial dose-response data. We first tested the antinociceptive effect of intraperitoneal morphine sulfate (3.5 mg/kg) and as expected, found that morphine produced a prolonged increase in nociceptive latency, approximately doubling the response time for up to 60 min; naltrexone (10 mg/kg, i.p.) completely blocked the response. Next, after implanting chronic intraventricular (icv) cannulae in rats, we further showed that central morphine administration (10 and 50 μg) produced a dose dependent increase in nociceptive latency. The response followed a similar time-course, being maximal at 40 min and returning toward baseline values after 60 min. The response to β-endorphin-1-31 administration (3, 5 or 8 μg) was also dose dependent but of longer duration, persisting for up to two hours.

After establishing consistent baseline data, we then tested whether glycyl-L-glutamine modulates the antinociceptive response to β-endorphin-1-31. Our initial results indicate that, indeed, it does. β-Endorphin-1-31 (1.5 nmol; 5 μg, icv) alone prolonged the response latency to a maximum of 53 %MPE sixty minutes after icv injection (Fig. 7). Co-administration of glycyl-L-glutamine (15 nmol) completely abolished the antinociceptive response, returning the response latency to baseline values; the inhibitory response was initially apparent 30 minutes after injection and remained significantly reduced for the remainder of the 120 minute test session. Higher glycyl-L-glutamine doses (50, 500 and 1500 nmol) also effectively inhibited the response to β-endorphin-1-31. Glycyl-L-glutamine, administered alone (50 or 500 nmol, icv) produced no effect whatsoever, consistent with previous reports on glycyl-L-glutamine's behavioral effects (Hirsch and O'Donohue,

1986). While yet preliminary, these results support the concept that glycyl-L-glutamine antagonizes the central response to β-endorphin-1-31. Thus, C-terminal proteolysis of β-endorphin-1-31 produces two peptides, β-endorphin-1-27 and glycyl-L-glutamine, both of which antagonize its antinociceptive potency.

II. Analytical Methods:

The results discussed thus far demonstrate that glycyl-L-glutamine produces pharmacological responses both in the periphery, on cardiac myocytes and immune cells, and in brain, modulating β-endorphin-1-31 antinociception. These experiments provide clear evidence that glycyl-L-glutamine subserves a physiological role as both an endocrine hormone and brain neurotransmitter; however, to conclusively substantiate such a role, it is also necessary to demonstrate that glycyl-L-glutamine is contained in and released from neural and endocrine cells. We have further hypothesized that glycyl-L-glutamine is specifically synthesized through β-endorphin-1-31 processing, and therefore, is selectively localized within β-endorphin neural and endocrine cells. This is important concept because, if so, then one might predict that drugs targeted on glycyl-L-glutamine receptors will be quite specific in their action, selectively modulating neurotransmission at β-endorphin neuronal synapses, unlike existing drugs which act at opiate receptors which mediate the effects of not only β-endorphin, but other opioid peptides, as well. To test these hypotheses, however, it is first necessary to develop analytical techniques to isolate and quantify glycyl-L-glutamine and map its distribution in brain. Toward this objective, we have initiated efforts to develop high performance liquid chromatography (HPLC) methods to isolate glycyl-L-glutamine and both immunoassay and immunohistochemical methods to quantify and map its localization in brain.

A. HPLC Analysis: Our initial objective, was to establish methods for separating glycyl-L-glutamine from related peptides; this is important for demonstrating the specificity of subsequent quantitation methods and for documenting the purity of radiolabeled glycyl-L-glutamine, to be used for radioimmunoassay (RIA) and/or receptor binding studies. Both of our methods utilize reverse phase HPLC; the first, using an acetonitrile gradient in 0.05% trifluoroacetic acid, produces baseline separation between glycyl-L-glutamine, glycyl-L-glutamate and glycyl-L-asparagine, as well as structurally unrelated dipeptides, tripeptides, and the opioid peptides, met-enkephalin and β-endorphin. The broad range of peptides separated by this method is ideal for demonstrating RIA specificity; however, it is less optimum for purification purposes because glycyl-L-glutamine elutes relatively close to the solvent front (within 5 minutes). We therefore developed a second separation method, using a sodium phosphate mobile phase containing ion pairing agents, which extends the dipeptide's retention time to 18 minutes.

B. Glycyl-L-glutamine Assay: We next initiated pilot studies to establish a method for measuring glycyl-L-glutamine in brain. Peptides are typically measured by RIA, which requires both a specific antisera and a radiolabeled peptide. But small molecules, such as glycyl-L-glutamine, present two potential difficulties. First, antisera raised against small molecules coupled to larger proteins, such as albumin, typically work well for immunohistochemistry, in which the antigen is fixed to tissue proteins, but do not always recognize the free antigen by RIA. Secondly, many small peptides, including glycyl-L-glutamine, cannot be iodinated. Therefore, we began by identifying four potential alternatives for measuring glycyl-L-glutamine (with increasing feasibility, as well as difficulty): (i) RIA, labeling glycyl-L-glutamine with Bolton-Hunter reagent (an ^{125}I-labeled molecule which can be coupled to the peptide's N-terminal); (ii) RIA, using ^{3}H-glycyl-L-glutamine; (iii) ELISA assay, in which glycyl-L-glutamine is first coupled to albumin; (iv) chromatographic isolation and quantitation by HPLC with fluorescent derivitization (Parish et al., 1983).

First, using an antisera which specifically recognizes glycyl-L-glutamine by immunohistochemistry (Plishka et al., 1985), we tested whether it would bind [^{125}I] Bolton-Hunter labeled glycyl-L-glutamine in an RIA. We succeeded in labeling glycyl-L-glutamine with Bolton-Hunter reagent, which we verified by HPLC, but found that the antisera did not recognize the Bolton-Hunter-glycyl-L-glutamine complex. This was not entirely unexpected; Bolton-Hunter reagent, a relatively large molecule, blocks the dipeptide's N-terminal, a likely antigenic determinant. Initial attempts to develop a RIA using ^{3}H-glycyl-L-glutamine, were also unsuccessful, suggesting that, like many antisera raised against amino acids and small peptides (Buijs et al., 1989), the essential immunogenic epitope of our antiserum included, not only glycyl-L-glutamine, but also a portion of the protein and/or coupling agent incorporated in the immunoreactive complex used to immunize the host animals. Indeed, our earlier immunohistochemical studies established that the antiserum did recognize endogenous glycyl-L-glutamine in pituitary tissue sections in which, presumably, the dipeptide undergoes fixative-induce coupling to tissue proteins. Moreover, this immunohistochemical staining could be completely blocked by preincubating the antiserum with free glycyl-L-glutamine. These results suggested to us that perhaps a similar strategy could be used to quantitatively analyze endogenous glycyl-L-glutamine by ELISA assay.

To test this idea, we first coupled glycyl-L-glutamine to bovine serum albumin (BSA), using either carbodiimide (1-ethyl-3(3-dimethyl-aminopropyl hydrochloride) or glutaraldehyde as coupling agents, then removed the remaining coupling agent and free glycyl-L-glutamine by dialysis. We found that the glycyl-L-glutamine antiserum did, indeed, recognize the glycyl-L-glutamine-BSA conjugate. Initially, we demonstrated this by isolating the glycyl-L-glutamine-BSA conjugate using SDS polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting to ensure that no free glycyl-L-glutamine or other interfering components were present,

then identified the immunogen using a second antiserum coupled to alkaline phosphatase. Once establishing that the antiserum specifically recognized the conjugate by SDS PAGE, subsequent studies were conducted by applying the conjugate directly to nitrocellulose filters. Both carbodiimide and glutaraldehyde were efficient coupling agents although the sensitivity of the antiserum for glutaraldehyde conjugated glycyl-L-glutamine was nearly ten fold higher than for the carbodiimide coupled dipeptide. Dilution studies indicated that glycyl-L-glutamine immunoreactivity was concentration dependent with respect to both antigen and antiserum; less than 1 nmol glycyl-L-glutamine could readily be detected at an antiserum dilution of 1:2,000. Consistent with our hypothesis, the antiserum did not recognize free glycyl-L-glutamine applied to the nitrocellulose filter, although in control experiments, our β-endorphin antiserum readily stained free β-endorphin similarly applied to the membrane. Hence, these studies demonstrate that protein coupling is required for the antiserum to recognize glycyl-L-glutamine. Moreover, they established the feasibility of using BSA-glycyl-L-glutamine conjugation to quantitatively analyze the endogenous dipeptide.

This experimental strategy has also proven quite useful for characterizing the glycyl-L-glutamine antiserum specificity. We found that the antiserum did not recognize BSA-conjugated β-endorphin, conclusive evidence that the antiserum does not cross-react with the C-terminal of the intact β-endorphin molecule. Thus far, the antiserum appears to be specific for glycyl-L-glutamine; for example, it does not recognize the inverse dipeptide sequence, glutamyl-L-glycine, glycyl-D-glutamine, or either of glycyl-L-glutamine's constituent amino acids.

Subsequent experiments demonstrated that pre-incubating the antiserum with free glycyl-L-glutamine inhibited antiserum binding to the BSA-conjugated glycyl-L-glutamine in a concentration dependent manner. Thus, as shown in immunohistochemical studies, the free dipeptide inhibits antiserum binding to conjugated glycyl-L-glutamine, even though the antiserum does not appear to recognize the free, unconjugated dipeptide. Thus, using this strategy, it may yet be feasible to develop an analytical method for measuring endogenous glycyl-L-glutamine. To test this possibility, we initiated efforts to develop an ELISA method for detecting glycyl-L-glutamine in brain and pituitary extracts.

Actually developing an ELISA assay has proven to be more problematic than expected, however. But with perseverance, we have recently succeeded in generating saturable binding curves for BSA-conjugated glycyl-L-glutamine; the limit sensitivity appears to be approximately 1 pmole at an antiserum dilution of 1:10,000 (Fig. 8). Ongoing experiments will now test whether pre-incubating the antiserum with free glycyl-L-glutamine inhibits binding to BSA-conjugated glycyl-L-glutamine, as it did in our preliminary studies using nitrocellulose filters. If so, then we may yet be successful in developing an ELISA assay to quantitate endogenous glycyl-L-glutamine concentrations in brain.

C. Immunohistochemical Studies: One objective of this research is to test the hypothesis that glycyl-L-glutamine is selectively localized within β-endorphin-releasing neurons and endocrine cells. This is important to establish because, if so, then drugs targeted on glycyl-L-glutamine may be quite selective in their action. To test this hypothesis, we initiated immunohistochemical studies to map the distribution of glycyl-L-glutamine and β-endorphin. The first, critical question to be addressed, as for any immunohistochemical study, was whether the glycyl-L-glutamine antiserum specifically identifies glycyl-L-glutamine, and not β-endorphin from which it is derived, within neurons and endocrine cells. To determine this, we tested whether the antiserum would selectively stain melanotroph cells in the intermediate lobe of the rat pituitary, which are known to contain glycyl-L-glutamine, but not corticotrophs in the anterior lobe, which contain β-endorphin but not glycyl-L-glutamine.

Using a standard fluorescently-labeled second antibody method, we found that the antiserum (1:1000 dilution) produced intense staining over virtually every cell in the intermediate lobe but produced no specific staining whatsoever in the anterior lobe. The finding that melanotrophs, but not corticotrophs, are stained indicates that the antiserum does not recognize the C-terminal of β-endorphin-1-31 from which glycyl-L-glutamine is derived. Further support for this conclusion is provided by control experiments showing that pre-incubating the antiserum with glycyl-L-glutamine (1 μM) inhibited melanotroph staining, but that β-endorphin-1-31 (1 μM) did not. Thus, consistent with the results described earlier for Western blotting experiments, these results indicate that the antiserum selectively recognizes glycyl-L-glutamine and not β-endorphin-1-31 or any other anterior pituitary peptide or protein.

It remains to be determined whether the antiserum will stain glycyl-L-glutamine neurons in brain with equivalent specificity, but these experiments are now in progress. For our initial studies, we used carbodiimide-fixed coronal sections cut at the level of the arcuate nucleus, the region containing the major proportion of β-endorphin neuronal perikarya. We found that the antiserum did, in fact, stain neuronal processes, but only in the median eminence. Control experiments confirmed the staining specificity by showing that it was completely blocked by pre-incubating the antiserum with 1 μM glycyl-L-glutamine. Neuronal cell bodies were not recognized by the antiserum, which was not unexpected because the animals had not received prior treatment with colchicine. These results may suggest that glycyl-L-glutamine is specifically localized within axons projecting to the median eminence; however, a more likely explanation is that the sensitivity of our staining method is too low to detect the dipeptide in other neuronal processes within the hypothalamus. To test this possibility, we plan to test more sensitive staining methods, such as the peroxidase/antiperoxidase technique.

Testing the hypothesis that glycyl-L-glutamine is specifically localized in β-endorphin neurons and endocrine cells also requires antisera against β-endorphin and, perhaps, other pro-opiomelanc cortin-derived peptides. This has been problematic, up till now, because neither our β-endorphin radioimmunoassay antisera nor other commercially available ones adequately labeled β-endorphin containing cells. During this project period we have more aggressively tested a variety of antisera and have found both β-endorphin and α-MSH antisera which selectively label pituitary corticotroph and/or melanotroph cells with high sensitivity (Micevych and Elde, 1982; Sherry et al., 1982) (Incstar Corp., Stillwater, MN).

We now have demonstrated; (a) that our glycyl-L-glutamine antiserum specifically recognizes the dipeptide in pituitary cells; (b) that it stains neuronal processes in brain; and (c) that the necessary β-endorphin antiserum is available and operable in our laboratory. Our final objectives are to increase the staining sensitivity for glycyl-L-glutamine in brain, where glycyl-L-glutamine levels are low relative to pituitary concentrations, and to confirm that our β-endorphin antiserum specifically stains neuronal processes as previously reported (Micevych and Elde, 1982; Sherry et al., 1982). Once we have met these objectives, we will proceed with a detailed mapping study of glycyl-L-glutamine immunoreactive cell bodies and processes in brain, comparing their distribution with that of β-endorphin immunoreactive neurons to determine whether the two peptides are strictly co-localized or if glycyl-L-glutamine is expressed in non-β-endorphin neurons as well.

SUMMARY AND CONCLUSIONS

A. Pharmacologic Responses: To date, we have demonstrated that glycyl-L-glutamine produces three markedly different pharmacologic responses: it induces a trophic response, stimulating A_{12} AChE expression in cardiac myocytes; produces neuroimmune regulatory effects on T-lymphocytes, enhancing c-myc oncogene expression; and inhibits a centrally mediated response to β-endorphin-1-31, antinociception. Thus, glycyl-L-glutamine produces pharmacologic effects in both brain and peripheral tissues, supporting the concept that it acts as both a neurotransmitter and a circulating hormone.

1. Trophic Effects on Cardiac Myocytes: Neonatal ventricular myocytes loose the ability to synthesize A_{12} AChE when grown in culture. The mechanism is uncertain, although the fact that myocytes continue to produce regular contractions in culture suggests that it does not result from the lack of contractile activity but rather from the absence of either neuronal innervation or, perhaps, a trophic substance. Here, we have shown that glycyl-L-glutamine restores A_{12} AChE expression to essentially the same level as observed *in vivo* (Nyquist-Battie, 1991), suggesting the possibility that it may be the responsible regulatory agent. This observation

was predicated on similar results in skeletal muscle (Lotwick et al., 1990) and sympathetic ganglia (Koelle et al., 1988); together, these finding suggest that glycyl-L-glutamine's trophic action on AChE expression is widespread and not specific for cardiac tissue. The concurrent finding that heart tissue post-translationally processes β-endorphin-1-31 to β-endorphin-1-27, and presumably glycyl-L-glutamine, supports the physiologic relevance of the observed effects. Furthermore, evidence that ventricular cells, themselves, express POMC mRNA (personal communication, Dr. Lloyd Forman) raises the intriguing possibility glycyl-L-glutamine, as well as other POMC peptides, acts as an autocrine factor to regulate AChE expression and, perhaps, other aspects of cardiac function. This concept is supported by evidence that ventricular cells express other peptide precursors, including pro-atrial natriuretic peptide (Gu, 1991) and pro-enkephalin (Springhorn and Claycomb, 1989), yet we have not, as yet, ruled out the possibility that glycyl-L-glutamine may also be released from autonomic neurons innervating the heart.

These studies are now complete and the resulting manuscript is currently in preparation. However, our interest in the cardiovascular effects of glycyl-L-glutamine continues. Studies are now being initiated to test whether glycyl-L-glutamine participates in the central regulation of cardiovascular function. This hypothesis arises from our prior studies showing that C-terminal cleavage of β-endorphin-1-31 to β-endorphin-1-27 enhances its hypotensive potency when centrally administered (Hirsch and Millington, 1991), *in marked contrast* to antinociception in which β-endorphin-1-27 acts as a highly potent antagonist of β-endorphin-1-31. Thus, we hypothesize that glycyl-L-glutamine will also produce hypotension, based on the concept that C-terminal proteolysis of β-endorphin-1-31 produces two peptides, glycyl-L-glutamine and β-endorphin-1-27, with the same physiological action, thereby potentiating the response to neuronally released β-endorphin peptides.

2. Neuroimmune Effects: An extensive literature now documents the role of β-endorphin and other POMC peptides in regulating immune cell function (Weber and Pert, 1984; Blalock et al., 1985; Morley et al., 1987). Consistent with these findings, glycyl-L-glutamine has been shown to inhibit T-cell suppressor cell activity, thereby indirectly enhancing antigen induced T-lymphocyte proliferation (McCain et al., 1987). Our studies demonstrate that, in addition, glycyl-L-glutamine, produces direct effects on T-lymphocytes, enhancing conconavalin A (con A) induced c-myc oncogene expression, a measure of T-lymphocyte proliferation. Together, these results indicate that glycyl-L-glutamine enhances T-lymphocyte proliferation through both direct and indirect mechanisms.

Nevertheless, our initial finding, that glycyl-L-glutamine stimulates c-myc oncogene expression only in the presence of equivalent β-endorphin concentrations, needs to be extended, testing complete dose response curves to determine whether the effect can be duplicated by either peptide alone or if they act

only in concert. In addition to measuring c-myc expression, it will also be necessary to demonstrate directly that glycyl-L-glutamine does, indeed, enhance T-lymphocyte proliferation. We plan to accomplish this using ^{3}H-thymidine incorporation, a well established method for measuring the T-lymphocyte proliferative response to immune modulators.

We also plan to continue investigating the source of glycyl-L-glutamine which modulates T-lymphocytes. As discussed previously, one likely source is the intermediate pituitary which synthesizes high levels of glycyl-L-glutamine (Plishka et al., 1985), at least in the rat, and based on our studies, to a small extent in the human pituitary as well (Bernard et al., 1991). But it is also quite possible that macrophages or other immune cells synthesize and secrete glycyl-L-glutamine to regulate T-lymphocyte function locally. Indeed, there is considerable evidence that other POMC peptides are so produced (Blalock, 1985). To evaluate this question, we are initiating experiments, using Northern blot analysis, to test whether macrophages, or other immune cell lines, express POMC mRNA. If so, we plan to further evaluate whether immune cells process β-endorphin-1-31 to glycyl-L-glutamine and to identify the factors which regulate its synthesis and secretion. Thus, the overall objective of these studies is to investigate glycyl-L-glutamine synthesis by, and regulation of, T-lymphocytes and other immune cells.

3. β-Endorphin Antinociception: Perhaps our most exciting finding, thus far, is the observation that glycyl-L-glutamine inhibits β-endorphin-1-31 antinociception. In light of prior evidence that β-endorphin-1-27 is also a potent antagonist of β-endorphin-1-31 (Nicolas and Li, 1985), this means that C-terminal cleavage of β-endorphin-1-31 produces two peptides which, when co-released, oppose its antinociceptive action. Our studies were also predicated on previous findings that glycyl-L-glutamine inhibits certain behavioral responses to β-endorphin-1-31, as does β-endorphin-1-27; again, as in our studies, glycyl-L-glutamine produced no effect when administered alone (Hirsch and O'Donohue, 1986). Thus, glycyl-L-glutamine appears to act as a neuromodulator of β-endorphin's behavioral and antinociceptive activities.

We now plan to expand our initial dose-response studies and further test whether glycyl-L-glutamine also modulates the response to icv morphine administration. If so, then future studies will examine whether peripherally administered glycyl-L-glutamine and morphine also interact. This would provide the added opportunity to evaluate glycyl-L-glutamine congeners, including glycyl-D-glutamine and cyclo-glycyl-L-glutamine, which are likely to be resistant to metabolism and, in the case of the cyclic dipeptide, should readily cross the blood-brain barrier (Hoffman et al., 1977). Thus, this line of investigation may be useful toward evaluating whether glycyl-L-glutamine, or its analogs, are effective centrally, when administered peripherally. In the longer term, the conclusive demonstration that glycyl-L-glutamine inhibits morphine antinociception would raise the intriguing possibility

that glycyl-L-glutamine receptor antagonists may be useful adjuncts to opiate analgesia.

B. Analytical Methods: The second broad objective of our research is to develop analytical methods for measuring glycyl-L-glutamine and mapping its distribution in brain. These methods are essential for assessing whether glycyl-L-glutamine is selectively localized in β-endorphin neurons and for studying the regulation of its synthesis and release. Establishing these methods has been more problematic than expected, although we have now made considerable progress toward developing both immunoassay and immunohistochemical methods for detecting glycyl-L-glutamine in brain and pituitary tissue.

The most promising approach to measuring glycyl-L-glutamine now appears to be by ELISA assay, and thus far, we have generated sensitive concentration dependent binding curves for BSA-conjugated glycyl-L-glutamine. As an alternative method, we are now examining the feasibility of using HPLC, combined with orthophthalaldehyde derivitization and fluorescent detection, to measure glycyl-L-glutamine in tissue extracts. Both analytical approaches require preliminary sample extraction, and for this purpose, we have found that small anion exchange columns effectively separate glycyl-L-glutamine from amino acids and other small peptides in crude tissue extracts. Thus we have developed all the necessary components for analyzing endogenous glycyl-L-glutamine and should soon have an assay available.

Immunohistochemical studies have also generated promising results. Thus far, we have succeeded in selectively detecting glycyl-L-glutamine in the intermediate pituitary using a fluorescently labeled second antibody, identifying the necessary perfusion and fixation components in the process, and have established that the antiserum does not recognize β-endorphin or any other anterior pituitary peptide or protein. We have also labeled glycyl-L-glutamine containing neuronal processes in brain tissue although further effort will be required to enhance the sensitivity of our immunohistochemical methods. But more sensitive detection techniques, such as peroxidase/antiperoxidase, are readily available to us and we are now initiating experiments to evaluate their effectiveness. When successful, subsequent studies will map glycyl-L-glutamine's distribution in brain, comparing it to that of β-endorphin, to establish whether the dipeptide is selectively localized in β-endorphin releasing neurons.

In summary, our results thus far have made considerable progress toward completing our first objective, evaluating glycyl-L-glutamine's pharmacologic activity spectrum in selected experimental paradigms. We have also overcome several impediments toward accomplishing our second objective, establishing the methods necessary to analyze glycyl-L-glutamine's tissue distribution. We plan to continue these efforts, and additionally, to initiate studies of the receptors which mediate glycyl-L-glutamine pharmacological

responses, our third and final research objective. Toward that end, we are now establishing the requisite receptor binding techniques, testing as our initial goal, whether the dipeptide inhibits opiate receptor ligand binding. This, perhaps the most exciting phase of our research, should ultimately resolve whether glycyl-L-glutamine acts through receptors previously identified for other neurotransmitters or, perhaps, through its own, unique receptor.

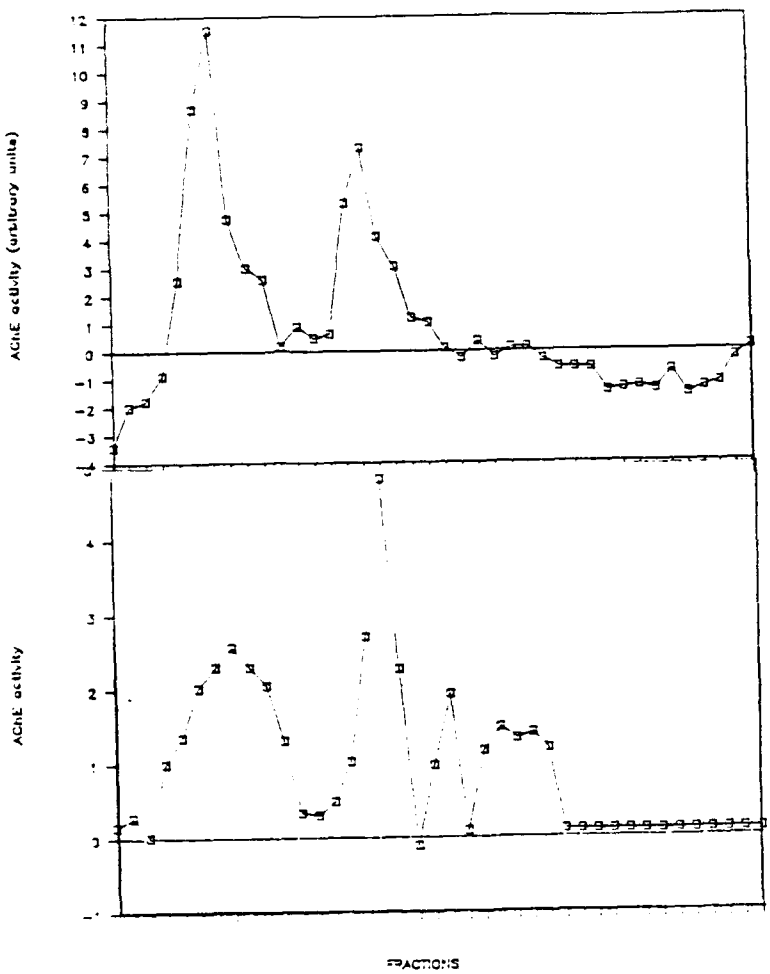

Figure 1. Glycyl-L-glutamine stimulates expression of asymmetric acetylcholinesterase (AChE) forms in cultured fetal myocytes. Dissociated cells from ventricles of neonatal rats were preplated in M199 medium and 0.5% fetal calf serum to remove non-muscle cells, then transferred to laminin-coated culture dishes (1.5×10^5 cells/35 mm dish) in a defined medium and incubated with or without 10^{-6} M glycyl-L-glutamine for 48 h. AChE molecular forms were separated by sucrose density centrifugation and peaks of AChE activity were identified by calibrating the sucrose gradients with standard proteins with known sedimentation coefficients (Nyquist-Battie et al., 1987). The upper panel illustrates the molecular forms of AChE in controls, and the lower panel glycyl-L-glutamine treated myocytes. The peaks of AChE activity correspond, from left to right, with the sedimentation coefficients of (lower panel): G_1 and G_2, G_4, A_8 and A_{12}.

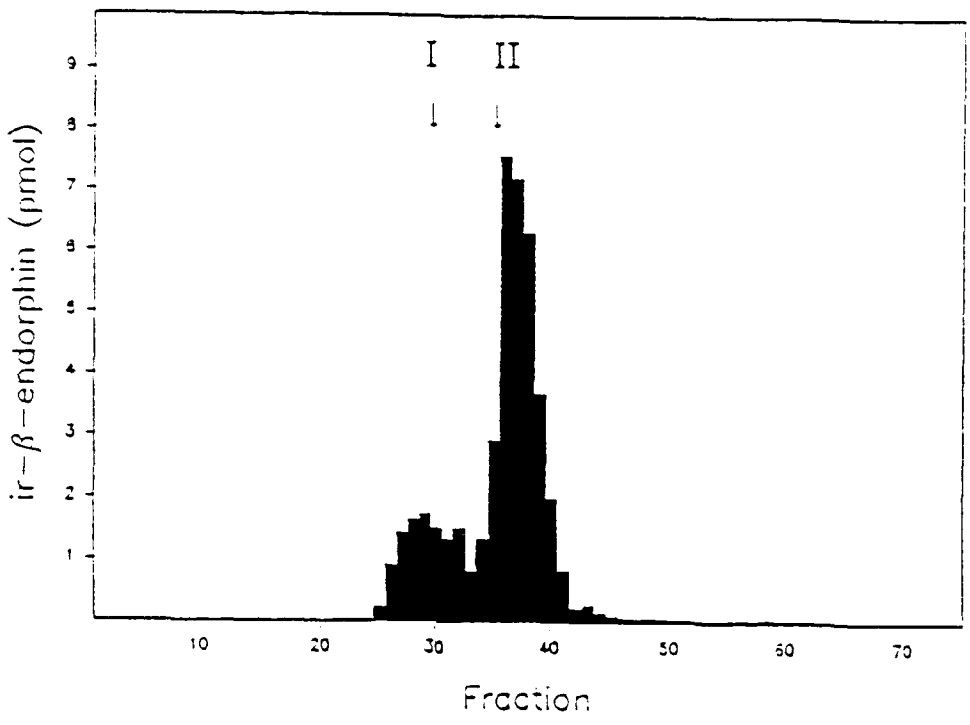

Figure 2. Gel filtration HPLC separation of β-lipotropin and β-endorphin-sized peptides in rat heart. β-Lipotropin and β-endorphin-sized peptides were separated by gel filtration HPLC from pooled extracts of five rat hearts and β-endorphin immunoreactivity was analyzed by RIA. The arrows mark the elution position of porcine β-lipotropin (I) and β-endorphin-1-31 (II).

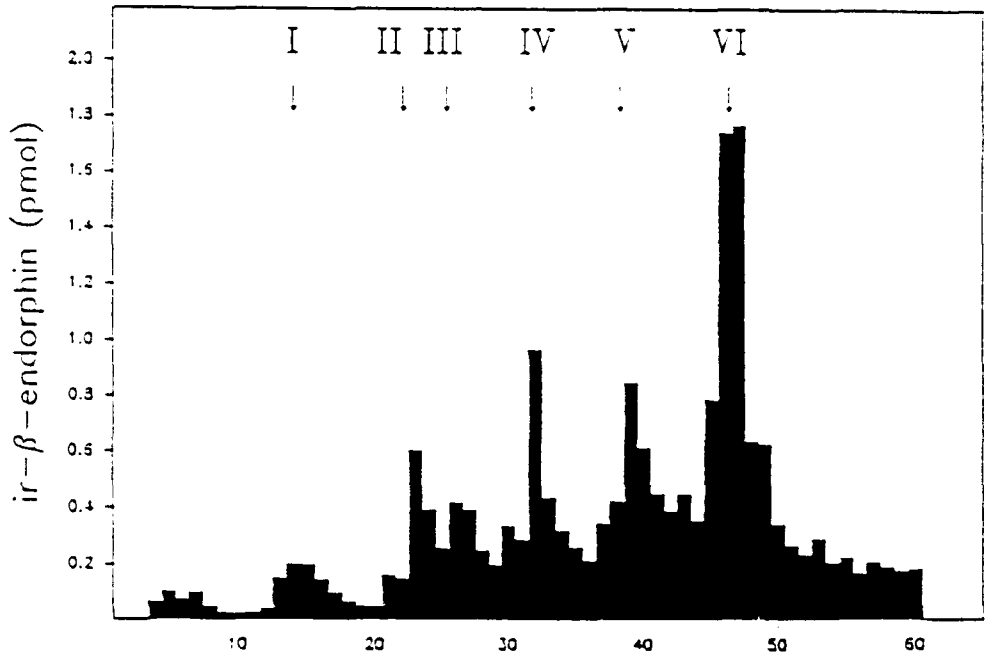

Figure 3. The molecular forms of β-endorphin in rat heart. β-Endorphin peptides were separated by cation exchange HPLC from pooled extracts of five rat hearts previously separated by gel filtration HPLC (Fig. 2). The arrows mark the elution position of N-acetyl-β-endorphin-1-26 (I), N-acetyl-β-endorphin-1-27 (II), β-endorphin-1-26 (III), β-endorphin-1-27 (IV), N-acetyl-β-endorphin-1-31 (V), β-endorphin-1-31 (VI).

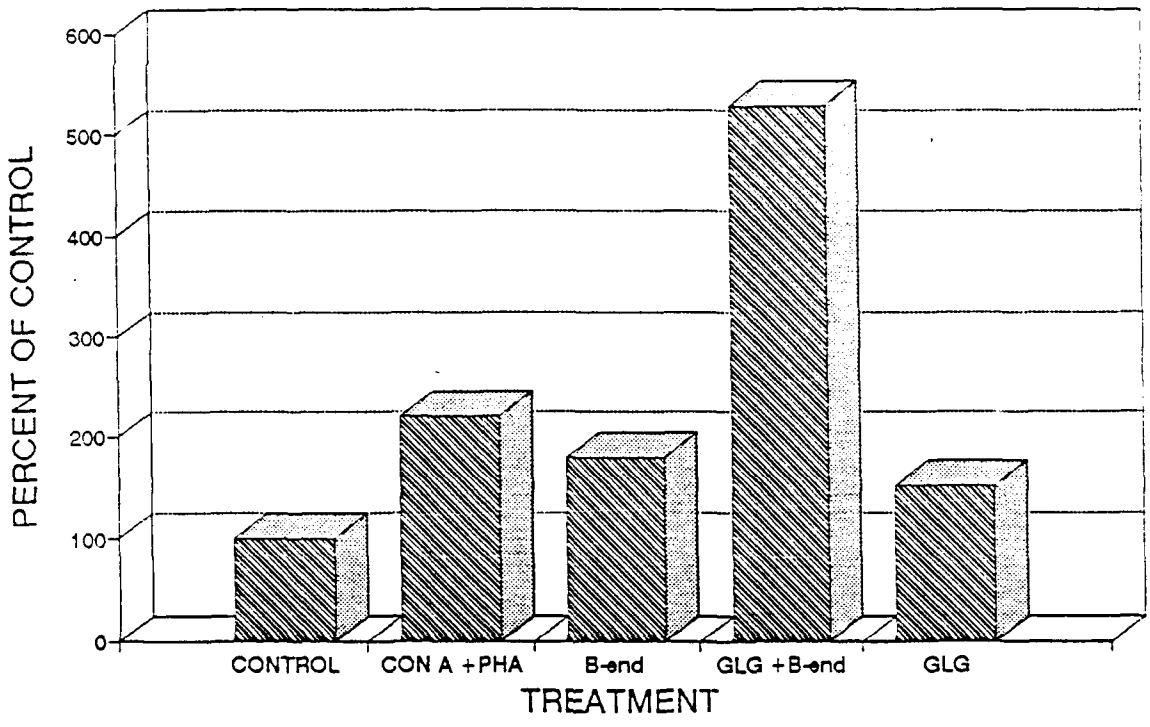

Figure 4. Glycyl-L-glutamine and β-endorphin-1-31 stimulate c-myc oncogene expression in human Jurkat E6-1 T-lymphocyte cells. Jurkat Eg-1 cells were grown in RPMI medium containing 10% fetal calf serum. Mitogenesis was stimulated with concanavalin A (con A) and the cells were incubated for 24 h with glycyl-L-glutamine (GLG; 30 nM) and/or β-endorphin-1-31 (B-end; 30 nM)). C-myc mRNA was isolated by Northern analysis and quantified by densitometric analysis.

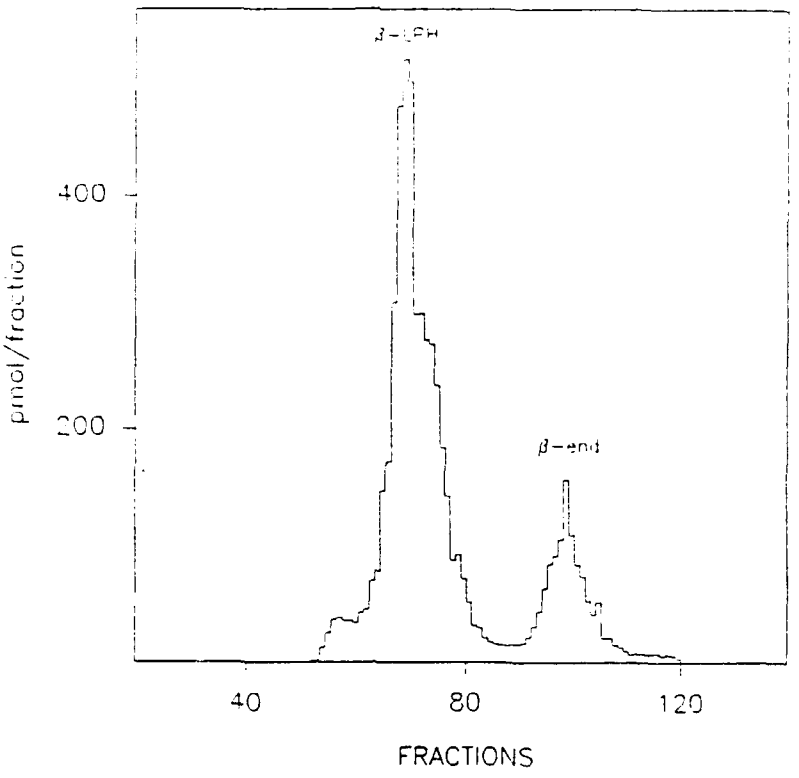

Figure 5. Gel filtration separation of β-lipotropin and β-endorphin-sized peptides in human pituitary. β-Lipotropin (β-LPH) and β-endorphin-sized peptides (β-end) were separated by gel filtration (Sephadex G-50; 2.5 x 100 cm) a single human pituitary and β-endorphin immunoreactivity was analyzed by RIA.

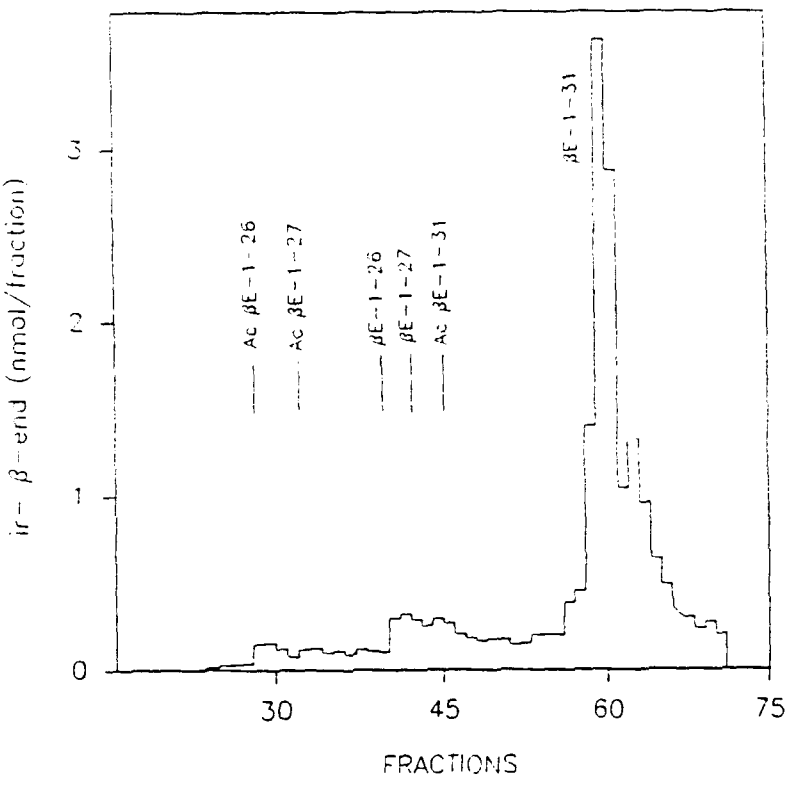

Figure 6. The molecular forms of β-endorphin in the human pituitary. β-Endorphin (β-E) peptides were separated by cation exchange chromatography from a single human pituitary previously analyzed by gel filtration HPLC (Fig. 5) and β-endorphin immunoreactivity was measured by RIA.

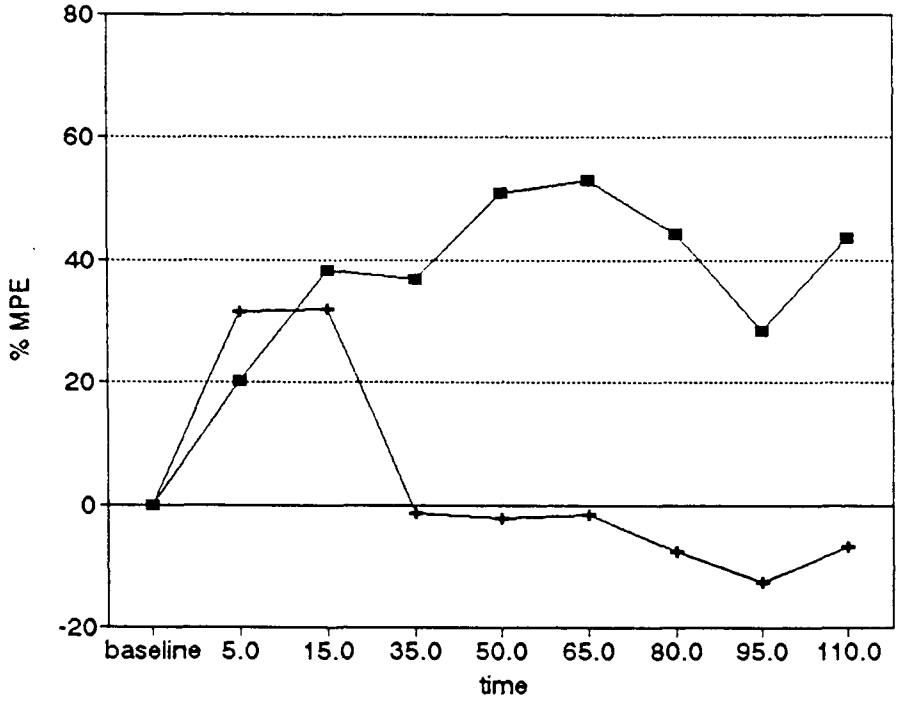

Figure 7. Glycyl-L-glutamine inhibits β-endorphin-1-31 induced antinociception. Groups of 8-10 rats were injected intracerebroventricularly (icv) with either β-endorphin-1-31 alone (5 μg; upper trace) or β-endorphin-1-31 combined with glycyl-L-glutamine (3 μg) and tail flick latencies were recorded at the indicated time points.

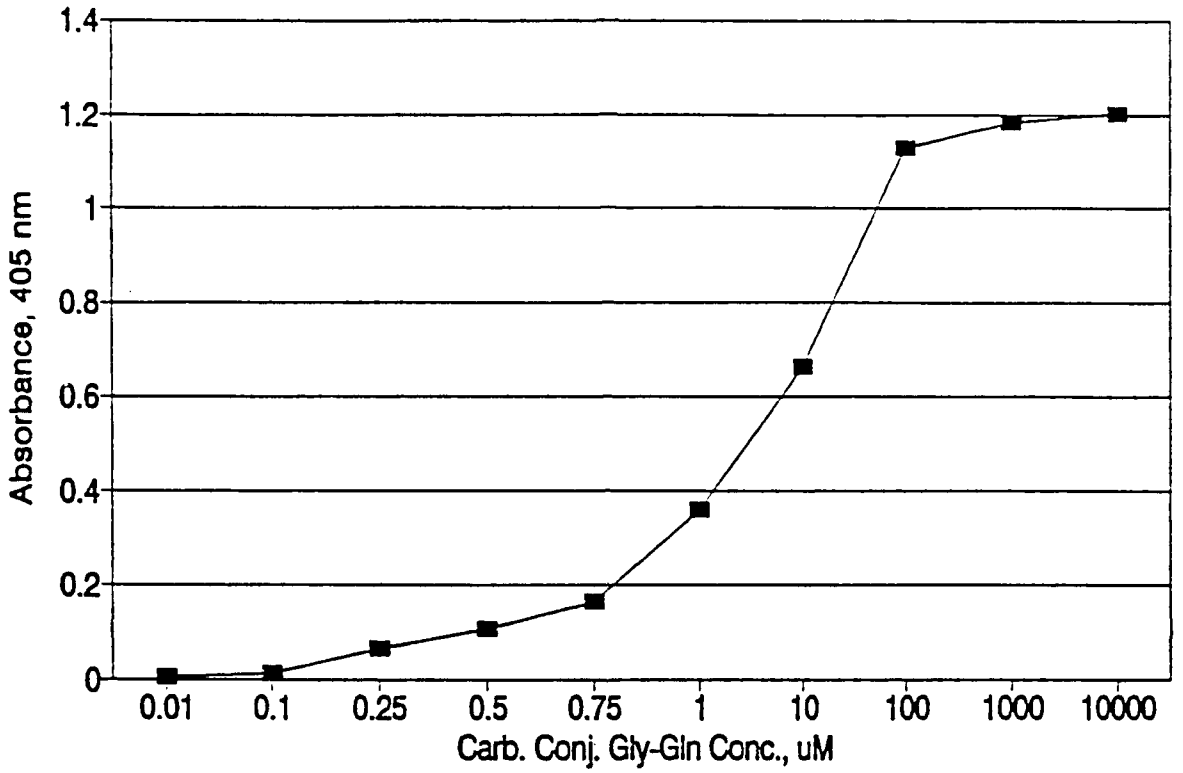

Figure 8. ELISA assay standard curve of BSA-conjugated glycyl-L-glutamine. Glycyl-L-glutamine was covalently linked to BSA by carbodiimide conjugation, dialyzed and plated in 96 well microtiter plates. BSA-conjugated glycyl-L-glutamine was detected using a primary antisera dilution of 1:10,000 followed by goat anti-rabbit IgG coupled to alkaline phosphatase. The carbodiimide conjugated glycyl-L-glutamine concentration (carb. conj. Gly-Gln conc.) was estimated from the glycyl-L-glutamine concentration used in the conjugation reaction. The limit sensitivity of the assay is approximately 1 pmol.

PUBLICATIONS

Five manuscripts were submitted for publication during this project period and two are currently in preparation with submission anticipated in November; in addition, eleven abstracts were submitted and/or presented. Much of this work represents the completion of studies initiated during previous USAMRDC funding (86PP6813) although it was supported, in part, by the current grant.

Manuscripts:

Hirsch, M.D. and Millington, W.R. Endoproteolytic conversion of β-endorphin-1-31 to β-endorphin-1-27 potentiates its central cardioregulatory activity. Brain Res. 550:61-68, 1991.

Millington, W.R. and Smith, D.L. The post-translational processing of β-endorphin in human hypothalamus. J. Neurochem. 57:775-781, 1991.

Millington, W.R., Dybdal, N.O., Mueller, G.P. and Chronwall, B.M. N-acetylation and C-terminal proteolysis of β-endorphin in the anterior lobe of the horse pituitary. Gen. Comp. Endocrinol. (In Press).

Lavigne, G.L., Millington, W.R. and Mueller, G.P. The CCK-A and CCK-B antagonists, devazepide and L-365,260, enhance morphine antinociception only in non-acclimated rats. Pain (Submitted).

Millington, W.R., Mueller, G.P. and Lavigne, G.L. Differential effects of cholecystokinin type A and B receptor antagonists on cholecystokinin stimulated pituitary β-endorphin secretion. J. Pharmacol. Exp. Ther. (Submitted).

Battie, C.N., Hagler, K. and Millington, W.R. Glycyl-L-glutamine regulates the expression of acetylcholinesterase asymmetric forms in cultured fetal cardiac myocytes. (In Preparation).

Evans, V.R., Forman, L.J. and Millington, W.R. Pro-opiomelanocortin-derived peptides in rat heart. (In Preparation).

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