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The role of endopeptidase 24.15 in the metabolism of LHRH

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THE ROLE OF ENDOPEPTIDASE 24.15 IN THE METABOLISM OF LHRH

by

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Abstract

THE ROLE OF ENDOPEPTIDASE 24.15 IN THE METABOLISM OF LHRH

by

Abraham M. Lasdun

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Previous work in this laboratory demonstrated that purified preparations of endopeptidase-24.15 (EP 24.15), a zinc-containing metalloendopeptidase, cleaves the Tyr^5-Gly^6 bond of luteinizing hormone releasing-hormone (LHRH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$GlyNH_2$) (Orlowski et al., 1983). This enzyme has both soluble and membrane-associated activities (Acker et al., 1987) and is present in the brain, pituitary and also peripheral tissues (Chu and Orlowski, 1985). Several substrates as well as potent active site-directed inhibitors of this enzyme have been synthesized in this laboratory. We have used these inhibitors to probe the role of this enzyme in both *in vitro* and *in vivo* metabolism of LHRH and its role in regulating the response of pituitary gonadotropes to LHRH. In membrane preparations obtained from the hypothalamus, anterior pituitary, and also in intact AtT 20 cells, EP-24.15 is the primary enzyme responsible for the degradation of LHRH, catalyzing the cleavage of the Tyr^5-Gly^6 bond. The liberated N-terminal pentapeptide ($LHRH^{1-5}$) is then rapidly degraded further to $LHRH^{1-3}$ by angiotensin converting enzyme (ACE). Addition of EP 24.15 inhibitor to incubation mixtures either completely or, to a large degree, inhibited LHRH degradation and completely abolished the formation of both $LHRH^{1-5}$ and $LHRH^{1-3}$ metabolites. Endopeptidase-24.11 (EP 24.11), present in these membrane preparations, also

degraded LHRH, forming the $LHRH^{1-6}$ and $LHRH^{1-4}$ metabolites, although at a much slower rate. The rate-determining reaction in LHRH degradation by brain and pituitary, evidently, is the cleavage of the Tyr^5-Gly^6 bond by EP 24.15. "Superactive" analogs of LHRH in which Gly^6 was replaced by a D-amino acid ("superactive" LHRH analogs with a D-amino acid substitution in position 6 are henceforth referred to as "D-Xaa^6-analogs") are resistant to degradation by EP 24.15; resistance to this primary degrading activity is hypothesized to contribute heavily to their superactive properties.

Inhibitors of EP-24.15 are shown to block the *in vivo* degradation of intracerebroventricularly (ICV) and intravenously (IV) administered LHRH. Concurrent ICV administration of LHRH and N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (cFP-AAF-pAB), a specific inhibitor of EP 24.15, led to a more than 10-fold increase in LHRH recovery above controls treated with LHRH alone. Administration of N-[1-(RS)-carboxy-3-phenylpropyl]-Phe-pAB (cFP-F-pAB) or captopril, inhibitors of "enkephalinase" (EP 24.11) and angiotensin converting enzyme (ACE) respectively, did not significantly increase LHRH recovery. Intravenous administration of LHRH and either cFP-F-pAB or cFP-AAF-pAB but not captopril, led to an increase in the half-life of LHRH from 10 min to 15 and 20 min respectively. Concurrent administration of both inhibitors resulted in a dramatic 8-fold increase in the half-life of LHRH, similar to values reported for D-Xaa^6-analogs. The potentiating effect of cFP-F-pAB resulted from inhibition of the *in vivo* degradation of cFP-AAF-pAB by EP 24.11. It is concluded that EP 24.15 is the dominant factor determining the *in vivo* LHRH degradation both in the CNS and periphery; resistance to this enzyme, therefore, is probably the factor in the prolonged half-life of superactive analogs.

To determine whether EP 24.15 activity regulates the concentration of LHRH reaching pituitary gonadotropes, concentrations of plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured in rats after ICV and IV administration of LHRH alone or in conjunction with inhibitors of EP 24.15. In animals treated with N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate (cFP-AAY-pAB) and cFP-AAF-pAB, two potent EP 24.15 inhibitors, IV and ICV LHRH injections induced a much greater and longer-lasting increase of plasma LH and FSH concentrations than in controls receiving LHRH alone. The magnitude and duration of the increases was similar to those after administration of [D-Trp^{6}]-LHRH or [D-Leu^{6},des Gly-$NH_2{}^{10}$]-LHRH ethylamide, two D-Xaa^{6}-analogs.

It is concluded that: 1) LHRH degradation by EP 24.15 limits the magnitude and duration of the response of the pituitary to LHRH; and 2) the increased *in vivo* activity of the "superactive" LHRH analogs can largely be attributed to their resistance to degradation by EP-24.15.

ICV injection of a potent EP-24.15 inhibitor alone induces, although only mildly, a rise in plasma LH levels in urethane-anesthetized rats, suggesting that the enzyme may have a role in regulating endogenous LHRH-stimulated LH secretion by controlling the concentration of secreted LHRH reaching the portal circulation and pituitary gonadotropes intact.

In *in vitro* experiments measuring the recovery of LHRH following KCl-evoked release, the addition of EP 24.15 inhibitor into the incubation media did not increase LHRH recovery. This indicated, that under the conditions used, no appreciable degradation of endogenous LHRH by hypothalamic (membrane) EP 24.15 took place. These findings are readily understood in light of the low concentration of LHRH in the media and the short (15 minute) period of incubation.

DEDICATION

This thesis is dedicated to:

The one and only Batsheva. This is for you, my dear Batsheva, because you are so generous in giving to me all your love, encouragement and support. You are a shining illustration of how the spirit of our past *Gedolim*, who were giants in *Torah* and *Gemillas Chassodim*, will never leave us, but rather, will pervade our every moment and action, if only we dedicate our lives to this goal. For it is because of you that I have been truly blessed by *Hashem*.

My dear parents. It is impossible to thank you for all that you have done to help me attain this wonderful goal. May Batsheva and I have the privilege to give back to you at least a small fraction of all the good you have done.

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Ms. Charlene Michaud, for guiding me in numerous and difficult technical aspects of my experimental work. She has also taught me to gain a much deeper understanding of the rationale and execution of sundry experimental procedures. Furthermore her amazing organization and efficiency in carrying out her experimental work has educated me in the prerequisites of successful scientific endeavors.

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LIST OF ABBREVIATIONS

ACE:	Angiotensin Converting Enzyme;
AP:	Anterior Pituitary;
cFP-AAF-pAB:	N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB;
cFE-F-pAB:	N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB;
cFP-F-pAB:	N-[1-(R,S)-carboxy-2-phenylpropyl]-Phe-pAB;
CNS:	central nervous system;
CRF:	corticotropin-releasing factor;
D-Leu^6-LHRH-EA:	[D-Leu^6,Des Gly-$NH_2{}^{10}$]-LHRH Ethylamide;
D-Xaa^6-analogs:	"superactive" LHRH analogs with a D-amino acid substitution in position 6;
EP-24.11:	endopeptidase-24.11 (EC 3.4.24.11);
EP-24.15:	endopeptidase-24.15 (EC 3.4.24.15);
FSH:	follicle-stimulating hormone;
HPLC:	high pressure liquid chromatography;
ICV:	intracerebroventricular(ly);
IP:	intraperitoneal;
IV:	intravenous(ly);
K_i:	inhibition constant (competitive)
K_m:	Michaelis constant
LH:	luteinizing hormone;
LHRH:	luteinizing hormone-releasing hormone; pGlu-His-
	Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2;
LHRH-DA:	LHRH-degrading activity;
ME:	median eminence;
OVX:	ovariectomized;
RIA:	radioimmunoassay;
TRH:	thyrotropin-releasing hormone;

INTRODUCTION

The presence of a hypothalamic Luteinizing Hormone-Releasing Hormone (LHRH) was first indicated by the finding that the injection of hypothalamic extracts stimulates the release of Luteinizing Hormone (LH) (McCann et al., 1960; Nikitovitch-Winer, 1962) and Follicle Stimulating Hormone (FSH) in rats (Igarashi and McCann, 1964). The subsequent purification and isolation of LHRH from hypothalamic tissue (Fawcett, 1968) led the way for the determination of its amino acid sequence (Matuso et al., 1971a) and subsequent laboratory synthesis (Matusò et al., 1971b). This work allowed for subsequent studies showing that either purified, natural LHRH or the synthetic peptide, when injected either intravenously, intraarterially, or directly into a hypophysial portal vessel, induces the secretion of LH and FSH in an identical manner (Arimura et al., 1972; Ondo et al., 1973; Rennels et al., 1971; Schally et al., 1971). The ability to study the effects of synthetic LHRH on gonadotropin release opened the door for the massive work geared toward the understanding of LHRH's role in the regulation of pulsatile gonadotropin secretion and gonadal function.

A pulsatile mode of LHRH release from the median eminence (ME) nerve terminals into the pituitary portal circulation has been shown to be the biological signal for the normal pulsatile pituitary secretion of LH (and FSH). Studies, in which concentrations of LH in venous samples were simultaneously measured with LHRH concentrations in portal blood (in sheep) or in third ventricle (of monkeys), have demonstrated a good concordance between LH and LHRH pulses (Clarke and Cummins, 1982; Fink, 1988; Levine et al., 1982; Crowley et al., 1985). Thus, changes in the frequency of plasma LH

concentrations are assumed to reflect similar alterations in the frequency of LHRH stimulation of the pituitary (Reame et al., 1984). Sarkar (1976) and Ching (1982a) described an increase in radioimmunoassayable LHRH concentrations in rat hypophyseal blood shortly preceding the preovulatory LH surge during proestrous. In a similar mode, the changes in LH secretion during the menstrual cycle (in primates and humans), and in particular, the rise in LH pulse frequency during the preovulatory follicular phase, are believed by many researchers to be precipitated by parallel changes in LHRH release (Marshall and Kelch, 1986; Marshall et al., 1988). It is believed that the elevated serum estradiol and progesterone levels prior to the gonadotropin surge mediate these changes in LHRH and LH secretion due to their stimulatory effects at the hypothalamic and pituitary level (Freeman, 1988; Goodman, 1988; Knobil and Hotchkiss, 1988).

In addition, the increase in LH pulse frequency during pubertal development is believed to reflect a maturation in the capability of the brain to generate and release an increased pool of LHRH (Hompes, et. al., 1982; Meijs-Roelofs, 1972). Quite possibly both processes are stimulated (in the female) by the rising titter of estrogen secreted by the developing ovarian follicle (Ojeda et al., 1980; 1983 Andrews, et al., 1978, 1981).

Recent studies have suggested that abnormalities of pulsatile LHRH secretion are responsible for certain forms of reproductive disorders. For example, in a variety of forms of anovulation, pituitary and ovarian function are apparently normal and yet normal cyclical changes in pituitary and ovarian hormones do not occur. These syndromes, which include Kallman's syndrome, amenorrhea associated with weight loss, anorexia nervosa, exercise and emotional stress, have been classified under the general category of

"hypothalamic amenorrhea" since the underlying abnormality, apulsatile LHRH secretion, resides in the hypothalamus (Marshall and Kelch, 1986; Marshall et al., 1988). A similar hypothalamic deficit seems to be the underlying cause in males with hypogonadotropic hypogonadism, a persistence of the prepubertal state associated with gonadotropin deficiency (Santoro et al., 1986). Pulsatile LHRH therapy has been successful in the induction of ovulation and even pregnancies in women with hypothalamic amenorrhea and in the induction of puberty in men with idiopathic hypogonadotropic hypogonadism (Marshall et al., 1988; Hoffman and Crowley, 1982; Fraser, 1984). The fact that long-term pulsatile LHRH administration is now used to promote the maturation of the pituitary-gonadal axis in these and related disorders (Valk et al., 1980; Crowley and McArthur, 1980; Leyendecker and Wildt,1983; Jacobson et al., 1979; Mortimer et al., 1974), is further confirmation that pulsatile LHRH secretion provides the signal for gonadotropin secretion and gonadal function.

Because of the lability and consequent short half life of LHRH *in vivo* (Berger et al., 1988), progress began toward the development of more clinically useful LHRH analogs with increased stability. A series of modifications of the LHRH molecule resulted in an entire generation of so called "superagonists" with an increased magnitude and duration of action (Nestor, 1984). The first important structural modification of LHRH leading to increased potency was substitution at the C-terminus (Fujino et al., 1972). The introduction of a Pro-ethylamide group at this position afforded a 5-fold increase in potency over that of the native molecule (Fujino et al., 1973). More potent analogs resulted from substitution of aromatic and more hydrophobic D-amino acids for glycine in position 6 which tend to further decrease the plasma clearance rates of these compounds (Barron et al., 1982). This modification, combined with the

replacement of the C-terminal glycine amine residue with an ethylamide, had led to the production of analogs with biological activities of up to 100 times that of native LHRH (Fraser, 1984). Since these substitutions conferred to these molecules increased resistance to degradation, it was apparent that their increased potency results, at least in part, to an increased *in vivo* stability and consequent reduced clearance rate (Karten and Rivier, 1986). In addition, a higher binding affinity may play a role in contributing to their biological action (Clayton and Catt, 1980). The relationship between susceptibility to degradation and biological potency underscores the possible importance of peptidases *in vivo* in curtailing the action of the peptide; hence the lower potency of the native molecule than of the superagonists.

Clearly any enzymatic activity responsible for the *in vivo* inactivation and clearance of a peptide hormone would most likely be membrane bound, with the active site facing the extracellular spaces since peptide hormones following systemic administration are believed to be confined to the extracellular fluid volume (Giese et al., 1973). While the information available from the administration of LHRH and its analogs demonstrate the role of peripheral membrane peptidases in inactivating exogenous LHRH, it is also likely that membrane peptidases in the central nervous system (CNS) may play an equally important role in inactivating endogenous LHRH following its secretion from the ME. Thus, the following regulatory process is suggested: the concentration of physiologically released LHRH reaching its site of action (pituitary gonadotropes) may be controlled by the action of peptidases either at the site of release (ME) or at the target site (anterior pituitary).

While this is an attractive hypothesis, an understanding of the mechanism of inactivation of released neuropeptides has been slow to develop. In the case of "classical" neurotransmitters, inactivation at the synapse may

occur by either extracellular metabolism, as in the case of acetylcholine (Whittaker et al., 1972), or by reuptake followed by intracellular metabolism, as in the case of γ-aminobutyrate (Turner and White, 1983). The involvement of uptake mechanisms in neuropeptide metabolism has not been demonstrated, although it has not been rigorously excluded. The consensus of opinion is that the physiological action of peptides is normally terminated by extracellular metabolism (Krieger, 1983; Iversen et al., 1976). This notion, that membrane peptidase-mediated metabolism is the primary mode of peptide hormone inactivation, began to emerge following the initial observation that enkephalins are rapidly hydrolyzed by striatal synaptic membrane preparations (Malfroy et al., 1978; Turner, 1986). These membrane-bound peptidases are generally integral, hydrophilic glycoproteins with a small hydrophobic domain (near the N-terminus) which comprises the transmembrane portion. The active site is located on the hydrophilic domain facing the extracellular space (Kenny and Maroux, 1982). Thus, such enzymes are ideally oriented to catalyze the extracellular hydrolysis of secreted neuropeptides at the synapse.

The aforementioned studies of enkephalin metabolism demonstrated cleavage at the Gly^3-Phe^4 bond catalyzed by an enzyme present in brain membrane preparations, which was later identified as endopeptidase-24.11 (EP 24.11, also referred to as "enkephalinase"). Subsequent evidence has accumulated showing that other neuropeptides such as substance P, may be inactivated by this enzyme in the brain (Matsas et al., 1983). Another cell-surface peptidase, angiotensin converting enzyme (ACE), shown to be present in cerebral tissue (Ganten et al., 1984), may play a role in the inactivation of such neuropeptides as C-terminally extended enkephalin peptides, neurotensin and substance P (Norman et al., 1985; Checler et al., 1984, Cascieri et al., 1984).

While most studies of neuropeptide metabolism has concentrated on their inactivation in the CNS, an identical process has also been shown to occur in the periphery. *In vivo* studies have shown that numerous circulating peptide hormones including the enkephalins, bradykinins, angiotensin I & II and several hypothalamic regulatory factors, are degraded by either peripheral tissue or plasma peptidases (Bennett and McMartin, 1979). Since EP 24.11 and ACE are present in significant concentrations in both the brain and periphery (Llorens and Schwatz, 1981; Kenny and Fulcher, 1983; Kenny, 1986; Erdos and Skidgel, 1985; Correa, et al., 1986; Ryan et al., 1976), it follows that the identical peptidases responsible for the degradation of a neuronally secreted peptide hormone may also be instrumental in the peripheral inactivation of the same peptide, following its systemic administration.

The identification of a physiologically relevant inactivating peptidase for a given peptide substrate is laden with the following difficulties:
(1) Peptidases generally display a broad substrate specificity which depends not only on the amino acids engaged in the scissile amino bond, but also on the adjacent one to three amino acids. Consequently, a given peptide can be hydrolyzed by a variety of enzymes. As a corollary, a given enzyme may hydrolyze several neuropeptides. As a result, observation of cleavage sites within a neuropeptide substrate incubated with tissue preparations affords no information as to the identity of the inactivating enzyme.
(2) *In vitro* specificity studies, as well as the use of specific active site-directed inhibitors, can provide guidance as to the likely physiological substrates for a given peptide. For example, the finding that hydrolysis of tachykinins and enkephalins by synaptic membranes and brain slice preparations is abolished in the presence of EP 24.11 inhibitors, suggested that the physiological

inactivation of these peptides may be catalyzed by EP 24.11 (Matsas et al., 1984; Waksman et al., 1985; Turner et al., 1985). These studies, while suggesting a role of an enzyme in the degradation of a given peptide, cannot determine whether the peptidase is located in the synaptic membrane and with the appropriate orientation for it to have a physiological role in hydrolyzing neuropeptides released at that site. Enzymes instrumental in physiological modes of peptide metabolism, are located *in vivo* in cell membranes in discrete subcellular compartments that may not come in contact with the secreted peptide under physiological conditions (Flouret, 1984). Moreover, *in vivo* rates of enzyme action are influenced by a number of factors such as pH, local concentration of reactants and the presence of unknown cofactors. Thus, studies directed at elucidating the mechanisms for degradation of peptide hormones by a particular organ should include not only *in vitro* preparations but the functional organ *in vivo* as well (Carone et al., 1987).

One subset of neuropeptides, the hypothalamic releasing factors, have been shown to be substrates for several peptidases *in vitro*. Considerable evidence has demonstrated the hydrolysis of TRH, LHRH and CRF by various tissue preparations of brain, pituitary and other organs (Griffiths and Kelly, 1979; Griffiths and McDermott, 1983). Although membrane-bound peptidases form a small percentage of the total neuropeptide-degrading activity in the CNS and pituitary (Joseph-Bravo et al., 1979), they may still play an important part in the inactivation process. This mode of action proposes that inactivation of the peptide occurs at the ME nerve terminal or at the pituitary target cell by extracellular membrane enzymes in the vicinity of the receptor. In addition, regulation of levels of releasing hormone in the general circulation by either plasma or tissue peptidases may be important to prevent pituitary-destined

peptide released from hypothalamus from interacting with extrapituitary sites such as the LHRH receptor in the ovaries (Griffiths and McDermott, 1983).

Since the development of agonist and antagonist analogs of hypothalamic hormones has relied to some extent on the knowledge of exactly how these peptides are enzymatically inactivated at various sites in the body (Morely, 1980; Stewart, 1982), the therapeutic implications of knowledge derived from studying this metabolic process are becoming apparent. One starting point in assessing the physiological and pharmacological significance of peptide-metabolizing enzymes is to identify 1) which bonds in the peptide molecule have to be protected in order to enhance their biological activity, and 2) which enzymes catalyze the cleavage of these bonds. To do this, one evaluates the action of the exogenous substance after protecting it from degradation by either modifying the hydrolyzable bonds or by coadministering a suitable enzyme inhibitor together with the peptide.

As an example, several of the synthetic enkephalin analogues have markedly enhanced opioid activity *in vivo*, an effect attributed not to increased affinity for the receptor, but to resistance to hydrolyzing enzymes (Morely, 1980). Chemical modification at specific sites in the enkephalin molecule confer an increased resistance selectively to either aminopeptidases or EP 24.11. These analogs possess analgesic properties many times greater than those displayed by the native enkephalin molecule (Schwartz et al., 1980). While analog studies can suggest the involvement of peptidases in the curtailment of biological activity, and may even indicate which peptide bonds are cleaved in this process, one cannot derive a positive identification of any specific enzyme involved in this process.

A more direct approach toward identification of inactivating enzymes for a given peptide is by demonstrating that active site-directed inhibitors

increases the recovery of either the exogenous or endogenous peptide in either *in vitro* tissue preparations or *in vivo*. For example, a role for EP 24.11 in the CNS inactivation of both [D-Ala^2,Leu^5]enkephalin and substance P was implicated by demonstrating that hydrolysis of these peptides by synaptic membranes is inhibited by phosphoramidon, a selective EP 24.11 inhibitor (Fulcher et al., 1982; Matsas et al., 1983). The same conclusion was reached by the use of a polyclonal antiserum to EP 24.11 to abolish the hydrolysis of [D-Ala^2,Leu^5]enkephalin by kidney microvillar membranes and synaptic membranes (Matsas et al., 1983). Furthermore, when [Met^5]enkephalin was injected intracerebroventricularly, its half-life is increased by thiorphan, an EP 24.11 inhibitor, demonstrating the importance of EP 24.11 in the *in vivo* metabolism of enkephalins (Schwartz, 1983). The identification of metabolizing enzymes for endogenous peptides is an even more valuable use of these inhibitors. EP 24.11 inhibitors were shown to enhance the recovery of secreted endogenous striatal Met-enekphalin, both *in vitro* (using striatal slices) (Patey et al., 1981; De La Baume et al., 1983), and *in vivo* (following the intracerebroventricular and intraperitoneal administration) (Zhang et al., 1982; and Murthy et al., 1984). In addition, the product of EP 24.11-mediated enkephalin hydrolysis, Tyr-Gly-Gly, is markedly and rapidly depleted from the striatum following ICV thiorphan administration (Llorens et al., 1985). These data support the notion that EP 24.11 is operative in the metabolism of endogenous enkephalins.

More importantly, active site-directed inhibitors can be used to define a possible biological function of a peptidase. The demonstration that inhibitor administration amplifies the biological action of a peptide, implies a role of the inhibited enzyme in modulating the biological activity of the peptide substrate. For example, thiorphan and kelatorphan (a highly potent selective and potent

EP 24.11 inhibitor) amplify the antinociceptive activity (induction of an increased response latency in the hot plate and tail flick test) of intracerebroventricularly administered Met-enkephalin, suggesting that EP 24.11 plays a role in curtailing Met-enkephalin's action at its opioid receptor(s) (Roques et al., 1980; Yaksh and Harty, 1982; Zaluski et al., 1984). In a similar type of study, captopril was shown to enhance the substance P-induced salivation in rats, thus indicating a role for ACE in regulating the biological actions of substance P (Cascieri et al., 1984).

This thesis research project is dedicated to:

1) the identification of membrane peptidases instrumental in the cleavage of LHRH in the CNS and in the periphery; and
2) the determination of a possible role of membrane peptidases in regulating the biological activity of LHRH.

(See below, Introduction, page 18, where a more detailed outline of the thesis goals is delineated).

Therefore, it is appropriate to review the prior studies of LHRH degradation relevant to this research project.

Early workers studying LHRH degradation during incubation with hypothalamic, pituitary and other organ homogenates, measured the disappearance of LHRH as well as the appearance of metabolites through radioimmunoassay (RIA) or HPLC. In both the hypothalamus and pituitary, LHRH-degrading activity (LHRH-DA) was shown to be localized subcellularly in both the soluble and particulate fractions (Loudes et al., 1978; Kelly et al., 1979). In light of the probable importance of membrane peptidases in regulating peptide hormone action, several groups have used several models to study LHRH

metabolism by membrane enzymes. To this end, LHRH degradation was studied following its incubation with either particulate fractions of hypothalamic and pituitary homogenates (McDermott et al., 1982), synaptosomal preparations (McDermott et al., 1983), hypothalamic tissue blocks (Powers and Johnson, 1981; Rotsztejn et al., 1976; Sundberg et al., 1978) or membrane preparations of neuroblastoma cells (Yokosawa et al., 1987). While the preponderance of the LHRH-DA in both the brain and pituitary readily solubilized (Joseph-Bravo et al., 1979; Parker et al., 1979), membrane-bound peptidases may still play an important part in the inactivation process.

The analysis of LHRH metabolites has allowed for examination of the sites of cleavage within the LHRH molecule by the peptidases in these soluble or membrane fractions of tissue homogenates. Several groups have shown that the primary products of LHRH degradation by brain and pituitary include the N-terminal tri-, penta- and hexapeptides, suggesting that the initial cleavage occurs at the Trp^3-Ser^4, Tyr^5-Gly^6 and Gly^6-Leu^7 bonds, respectively (Elkabes et al., 1981; Yosokawa et al., 1987; Krause et al., 1982; Koch et al., 1974; Hazum et al., 1981). Nontheless, because of the multitude of peptide bond-cleaving enzymes in all tissues, two difficulties are inherent in these experimental models:

1) since a given peptide may serve as a substrate for numerous enzymes, determination of the cleavage sites in LHRH affords no definitive identification of the peptidases involved; and
2) the presence of multiple cleavage sites suggests that following primary cleavage of LHRH, the resulting metabolites may be degraded by other enzymes catalyzing "secondary cleavage". Identification of the metabolites appearing following incubation with crude enzymic preparations does not

distinguish the reactions catalyzing primary cleavage from those responsible for formation of secondary cleavage products.

Resolution of these difficulties as well as the determination of a physiological/pharmacological role of membrane peptidases in regulating the biological activity of LHRH can be definitively accomplished by the use of active site-directed inhibitors, i.e if the presence of an inhibitor:

1) retards the disappearance of LHRH, the inhibited enzyme is inferred to be instrumental in its disappearance; and
2) enhances the biological effects of LHRH, the inhibited enzyme is inferred to be instrumental in regulating its action.

In order to chose the inhibitors to be used for these purposes, a prerequisite is the identification of the enzymes for which LHRH serves a substrate in their purified form. To date, LHRH has been shown to serve as a substrate for a number of enzymes which have been purified from brain, pituitary and kidney. For the most part, these enzymes are believed to be localized exclusively in either soluble or particulate fractions of these preparations. Soluble LHRH-DA has been attributed to the actions of several peptidases:

1) neutral endopeptidase which hydrolyzes the Tyr^5-Gly^6 and His^2-Trp^3 bonds which is inhibited by thiol peptidase inhibitors such as p-chloromercuribenzoate (Horsthemke and Bauer, 1980; Horsthemke et al., 1981);
2) a DFP-sensitive, prolyl endopeptidase which cleaves the Pro^9-Gly^{10} bond (Wilk et al., 1979; Krause et al., 1982; Hersh and McKelvy, 1979);
3) a "cation-sensitive multicatalytic proteinase complex" which cleaves the Tyr^5-Gly^6 bond (Wilk and Orlowski, 1980, 1983).

Membrane-bound LHRH-DA has been attributed to:

1) EP 24.11, identical with enkephalinase, which cleaves the Gly^6-Leu^7 and the Ser^4-Tyr^5 bonds (Mastas et al., 1984);
2) ACE, which cleaves the tripeptides from the C- and N-terminus of LHRH (Skidgel and Erdos, 1985).

To date, the use of enzyme inhibitors for characterizing LHRH-DA by brain or pituitary, has been of limited value. Advis et al. (1982a) demonstrated that the Tyr^5-Gly^6 bond in LHRH is cleaved by a metallopeptidase in supernatant fractions of rat hypothalamus by showing that cleavage is blocked by o-phenanthroline. Another group has made similar use of p-chloromercuribenzoate to show that the formation of $LHRH^{1-5}$ by neuroblastoma membranes is catalyzed by a thiol protease (Yokosawa et al., 1987). Yet, these inhibitors, due to their lack of selectivity, provide no information as to the exact identity of the relevant peptidase. In a study of the hydrolysis of exogenous LHRH by synaptic membrane peptidases, the addition of either phosphoramidon (EP 24.11 inhibitor), bestatin (aminopeptidase inhibitor), or an anticatalytic antibody to prolyl endopeptidase had little effect. Thus, still another enzyme besides EP 24.11, aminopeptidase or prolyl endopeptidase appears to play the major role in the hydrolysis of LHRH by synaptosomes (Edwardson and McDermott, 1985). Identification, therefore, of the important (membrane) LHRH-degrading enzymes in the CNS through the use of specific inhibitors, had not yet been accomplished.

In addition to the presence of LHRH-DA in the brain and pituitary, LHRH degradation has been studied in peripheral organs. The appearance of several LHRH metabolites, principally among them $LHRH^{1-5}$ and $LHRH^{1-3}$, were observed following incubation of LHRH with homogenates of liver, lung,

ovaries and testes (Carone et al., 1987). Furthermore, in live animal models, metabolites of radiolabeled LHRH were detected in both blood and urine (Stetler-Stevenson et al., 1983). While the presence of LHRH-DA in the brain and pituitary suggests a role for these enzymes in the inactivation of physiologically released LHRH, LHRH-DA in the periphery is not likely involved in the termination of the action of endogenous LHRH at the gonadotropes. However, peripheral peptidases may heavily influence the disposition of systemically administered LHRH by contributing to its plasma clearance rate and possibly, by causing its sequestration in tissues rich in LHRH-degrading peptidases (Berger et al., 1987).

The biological potency of LHRH has been enhanced tremendously as a result of structural modifications which slow its degradation. For example, replacement of the Gly^6 residue by especially aromatic D-amino acids, such as D-tryptophan, dramatically retards its degradation by soluble and particulate fractions of brain homogenates and increases its biological potency *in vitro* some 30 to 100 times (Barron et al., 1984). Whereas the decline of systemically administered LHRH in plasma is extremely rapid, the modified "superagonists" have a far slower rates of decline, i.e. their half-lives are prolonged (Barron et al., 1982). It is now clear that this precipitous decline of plasma LHRH levels is due, in large part, to degradation by tissue peptidases which inactivate and thus, limit the biological action of LHRH (Berger et al., 1988); the slower decline of the D-Xaa^6-analogs is therefore due to their resistance to these peptidases. Thus, an understanding of the mechanism of inactivation of systemically administered LHRH is relevant in the continued improvement in designing protocols of LHRH and analog administration. As with CNS peptidases, a definitive determination of the important inactivating peripheral enzymes and their biological function can be made only by demonstrating that in the

presence of an active site-directed inhibitor: [1]the disappearance of LHRH from the periphery is retarded; [2]and the effects of systemically administered LHRH are enhanced. While Flouret (1987) has used phosphoramidon, a specific EP 24.11 inhibitor, to demonstrate that LHRH breakdown by renal brush border membranes is catalyzed by EP 24.11, the peripheral peptidases responsible for the disposition of LHRH in the general circulation have not been identified.

In summary, the following conclusions regarding LHRH metabolism and its effects on LHRH action are drawn from the literature:

(1) Exogenous LHRH is cleaved at several bonds by membrane-bound and soluble enzymes present in several *in vitro* preparations of brain, pituitary and peripheral organs.

(2) Exogenous LHRH, injected intravenously, undergoes rapid metabolism *in vivo*, with a consequently rapid clearance rate and short half-life.

(3) The biological half-life and potency of LHRH is increased by structural modifications which increase its resistance to degradation.

On the other hand, several crucial points concerning the biological importance of LHRH metabolism still need clarification:

(1) The identity of the membrane peptidases responsible for catalyzing the cleavage of the various peptide bonds *in vitro*, as well as the order of their importance, has not been deciphered.

(2) Because of the difficulties in extrapolating from (membrane) preparations *in vitro*, to *in vivo* conditions:

a) the identity of the membrane peptidases catalyzing *in vivo* metabolism of exogenous LHRH must still be determined. Furthermore, no definitive

evidence has shown that endogenous LHRH is inactivated by CNS or pituitary membrane enzymes following its release.

b) it must still be definitively demonstrated that CNS metabolism of LHRH curtails its LH- and FSH-releasing activity at the pituitary.

c) it must still be definitively demonstrated that the LH- and FSH-releasing activity of systemically administered LHRH is curtailed by peripheral peptidase activity. In addition, whether the major cause for the increased potency of the LHRH analogs is their resistance to degradation or their increased binding potency, has yet to be determined.

The N- and C-terminus of LHRH is blocked by a pyroglutamyl and an amide group respectively. This confers to the molecule the resistance to degradation by exopeptidases (with the possible exception of pyroglutamyl peptidase, an enzyme with a rather low activity in the pituitary and brain). Enzymatic degradation of LHRH must therefore be initiated by the action of endopeptidases. Previous work in this laboratory has concentrated on two zinc-containing metalloendopeptidases, with LHRH-DA, present in both brain and pituitary. Orlowski and Wilk (1981) have isolated from the pituitary and brain, a membrane-bound metalloendopeptidase that cleaves peptide bonds on the amino side of hydrophobic amino acid residues and subsequently showed its identity with EP 24.11 ("enkephalinase") initially isolated from kidney brush border membranes by Kerr and Kenny (1974), (Almenoff et. al., 1981; Almenoff and Orlowski, 1983). A second metalloendopeptidase distinct from EP 24.11 was discovered and characterized in our laboratory, and assigned by the Nomenclature Committee of the International Union of Biochemistry. the number EC 3.4.24.15, and is therefore referred to as endopeptidase-24.15 (will be abbreviated as: EP 24.15) (Orlowski et. al., 1983). EP 24.15 was initially isolated

from the soluble protein fraction of brain homogenates (ibid). A membrane-bound form of the enzyme, constituting approximately 20 to 25% of the total activity was also identified. This form was found to be associated with brain and pituitary membrane fractions including synaptosomes (Acker et al., 1987). EP 24.15 activity is also found in soluble and membrane fractions prepared from peripheral tissues (Chu and Orlowski, 1985; also: unpublished data from this laboratory). Specificity studies with model synthetic substrates and natural bioactive peptides, showed that the enzyme cleaves preferentially bonds on the carboxyl side of hydrophobic amino acid residues, and that a hydrophobic or bulky residue in the P_3' position greatly enhances the affinity of the substrate toward the active site of the enzyme (Orlowski et al., 1983, Chu and Orlowski 1985). Mapping of the active site of the enzyme provided a rational basis for the design and synthesis of active site directed N-carboxymethyl peptide inhibitors (Chu and Orlowski, 1984), some of them having K_i values in the nmolar range (Orlowski et al., 1988).

Several natural oligopeptides serve as substrates for purified EP 24.15, including bradykinin, neurotensin, dynorphin A^{1-8}, substance P and LHRH. Initial studies have shown that this peptidase cleaves the central Tyr^5-Gly^6 bond in LHRH (Orlowski et al., 1983). The adjacent Gly^6-Leu^7 bond is cleaved by EP 24.11 (Matsas et al., 1984). Since neither of the two enzymes would be expected to cleave a substrate with a D-amino acid in either the P_1 or P'_1 position, it was thought that the relative resistance of the LHRH superagonists with a D-Xaa^6 substitution (P'_1 for EP 24.15 and P_1 for EP 24.11) to tissue degradation suggests that either one or both of these endopeptidases may be responsible for a large portion of native LHRH degradation both *in vivo* and *in vitro*. Moreover, since resistance to enzymatic activity appears to be responsible, in part, for the increased potency of such analogs, a role for one or

both of these enzymes is suggested in regulating LHRH's biological actions. The availability of potent and specific inhibitors of the two enzymes became therefore an important tool in determining the relative importance of the two enzymes in LHRH degradation and in regulating the biological action of LHRH.

EP 24.11, EP 24.15 and ACE all possess membrane-associated peptidase activities. While the activity of EP 24.15 was believed to be concentrated primarily in the brain (Chu and Orlowski, 1985), EP 24.11 and ACE have high activities in numerous peripheral organs including the lung, kidney and intestine (Kenny and Fulcher, 1983; Erdos, 1976), as well as in brain (Almenoff and Orlowski, 1984; Correa et al., 1986). Since all of these three metallopeptidases degrade LHRH, at least in their purified form, it was therefore, our objective to determine their relative importance in both central and peripheral LHRH degradation, and the possible biological ramifications. To carry out this study, the following steps were taken:

1. Active-site directed inhibitors of these three enzymes were used to identify the enzyme(s) instrumental in the *in vitro* degradation of exogenous LHRH by membrane preparations of hypothalamus, anterior pituitary and AtT20 cells. Examination of the products by HPLC and amino acid analysis allowed for determination of the exact bonds cleaved, the enzymes responsible for the cleavage activities, and the order of importance of these cleavages.
2. Active-site directed inhibitors of these three enzymes were administered ICV, in conjunction with LHRH, in order to identify the important activity(ies) involved in the *in vivo* CNS degradation of exogenous LHRH following its ICV administration.

3. Active-site directed inhibitors of these three enzymes were administered IV, in conjunction with LHRH, in order to determine which activity(ies) are responsible for the *in vivo* peripheral degradation of exogenous LHRH following its intravenous administration.
4. Active-site directed inhibitors of EP 24.15 and EP 24.11 were administered in conjunction with LHRH in order to determine if one or both of these enzymes play a role in regulating the LH- and FSH-releasing activity of intravenously and intracerebroventricularly administered LHRH. Furthermore, the LH- and FSH-response to IV "LHRH & inhibitor" treatment was compared to the response to D-Xaa^6-analogs in order to determine whether the increased biological activity of these superagonists can be definitively attributed to their resistance to enzymatic degradation, and to which enzymes in particular.
5. Plasma LH was measured following ICV infusion of inhibitors of EP 24.15, in order to determine if this enzyme may be instrumental in regulating the concentration of bioactive endogenous LHRH reaching the gonadotropes following release from ME nerve terminals.
6. Active-site directed inhibitors of EP 24.15 were used to determine if this enzyme is involved in the metabolism of endogenous LHRH following KCl-evoked release from brain slices.

MATERIALS AND METHODS

MATERIALS

Captopril (K_i, 1.7 nM; Ondetti and Cushman, 1982) was obtained from the Squibb Medical Research Institute (Princeton, NJ). The two EP 24.15 substrates, Bz-Gly-Ala-Ala-Phe-pAB and Boc-Phe-Ala-Ala-Phe-pAB were synthesized as described previously (Orlowski et al, 1983). The EP 24.11 substrate, Glt-Ala-Ala-Phe-2NA, and inhibitors, cFE-F-pAB (K_i, 71 nM) and cFP-F-pAB (K_i, 38 nM) were synthesized as described previously (Orlowski and Wilk, 1981; Almenoff and Orlowski, 1983; Pozsgay et al., 1986). EP 24.15 inhibitors N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP-AAF-pAB; K_i = 27 nM) and N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-pAB (cFP-AAY-pAB; K_i = 16 nM) are more potent analogs of N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (K_i = 1.94 μM) reported previously (Chu and Orlowski, 1984) and were synthesized as described (Orlowski et al., 1988). LHRH, [D-Ala^6]-LHRH, des-Gly^{10}[imidazobenzyl-D-His^6]-LHRH ethylamide, thermolysin and urethane were supplied by Sigma Chemical Company (MO). [D-Trp^6]-LH-RH and [D-Leu^6, des-Gly^{10}]-LHRH ethylamide were supplied by Bachem. Biosci. Inc. (Philadelphia, PA). Aminopeptidase N was prepared from hog kidney according to the method of Pfleiderer (Pfleiderer, 1970) and freed from the contaminating metalloendopeptidase as described previously (Almenoff and Orlowski, 1983). Goat anti rabbit antiserum (Lot 101-5) was provided by Bioreclamation (East Meadow, NY). Horse, rabbit and fetal calf serum as well as Earle's Buffered Salt Solution (EBSS) and HEPES were supplied by Gibco Laboratories (Grand Island, NY).

METHODS

Experiments comprising this thesis project are divided into several phases. In the following section, the general design for each phase, its rationale and aims are described. Each of these descriptions are followed by a more detailed description of experimental methods used in each of these phases.

Phase 1: LHRH degradation by membrane preparations of brain, pituitary and AtT20 cells.

Rationale and general description of experimental procedures:

As described in the *Introduction*, (page 16 & 21), 3 membrane peptidases, with known LHRH-DA (in purified preparations) are present in the CNS. It was our aim to determine which of these peptidases play important roles in the degradation of exogenous LHRH by hypothalamic, anterior pituitary and AtT20 cell membranes (as well as intact AtT20 cells). Measurement of degradation products is a preferred means of studying degradation than by merely measuring disappearance of the reactant. Therefore HPLC followed by amino acid analysis was used to monitor the appearance of products of EP 24.15-, EP 24.11- and ACE-catalyzed LHRH cleavage. In order to determine which activities in these crude enzyme preparations are responsible for each of the identified products, in another experiment homogeneous preparations of EP 24.15 and EP 24.11 were incubated with LHRH and the metabolites were identified by HPLC and amino acid analysis. Subsequently, when identical metabolites were formed with crude enzyme incubations, the enzyme responsible for formation of these products was easily identified; the absence of a metabolite's formation upon addition of enzyme inhibitors confirms the identity of the enzyme responsible for its production. Therefore, the addition of inhibitors of these 3 enzymes to crude enzyme incubation mixtures served 2 purposes: 1) to positively identify

which enzymes in these preparations are responsible for the formation of each of the products; and 2) to determine the importance of each of these enzymes in LHRH hydrolysis by these preparations.

In addition, hydrolysis of LHRH and two D-Xaa^6-analogs by purified EP 24.15 and EP 24.11 preparations were compared. Because of the possible importance of these enzymes in curtailing the biological activity of LHRH (see *Introduction*, page 6 & 25), it was important to determine if the superactive LHRH analogs may attain their increased biological potency due to resistance to these enzymes. Therefore, their susceptibility (or lack thereof) to hydrolysis by these enzymes was determined.

Preparation of particulate fractions from pituitary and hypothalamus.

Male Sprague Dawley rats weighing 250 to 300 g were killed by decapitation. The hypothalami were dissected by the method of Glowinski and Iversen (1966), weighed, and 10% homogenates were prepared in an ice-cold, Tris-HCl buffer (0.05 M; pH 7.6) and then centrifuged at 10,000 g for 20 min. The pellet was washed twice with an equal volume of buffer and then resuspended in the same buffer and stored at -20 C. Particulate fractions of rat anterior pituitary were prepared by the same procedure.

Determination of enzyme activity.

Activity of EP 24.15 was determined with tBOC-Phe-Ala-Ala-Phe-pAB as described previously (Orlowski et al., 1983). The product of the reaction (Ala-Ala-Phe-pAB) was degraded to the constituent amino acids and pAB by an excess of aminopeptidase M added to the incubation mixtures. Free pAB was then determined by a diazotization procedure (Bratton and Marshall, 1939; Goldbarg and Rutenberg, 1958). Activity was also determined in the presence of 20 μM N-

[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP-AAF-pAB), a specific active site directed inhibitor of EP 24.15 (K_i = 27 nM). The difference in activities measured in the two incubation mixtures was taken as a measure of EP 24.15 activity. Activity was always determined in the presence of 20 μM N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB (cFE-F-pAB) (Almenoff and Orlowski, 1983), an inhibitor (K_i = 7.1 x 10^{-8} M) of membrane-bound neutral metalloendopeptidase (EC 3.4.24.11), which cleaves tBOC-Phe-Ala-Ala-Phe-pAB at the Ala-Phe bond and also slowly degrades the inhibitor of the soluble metalloendopeptidase. Enzyme activity is expressed in units, one unit being defined as the amount of enzyme required to release 1 μmol of product/h. Specific activity is expressed in units/mg protein determined (Lowry et al., 1951) with bovine serum albumin as the standard.

The K_m of LHRH with respect to EP 24.15 was measured by incubation of purified enzyme (Orlowski et al., 1983) with LHRH (20 to 200 μM) in a Tris-HCl buffer (0.1 M; pH 7.0) containing 0.4 mM dithiothreitol (DTT). The reaction was terminated by addition of an aliquot of 1.0 M HOAc, and product formation was estimated by HPLC as described below.

In some experiments, LHRH or superactive analogs of LHRH were incubated with purified rat brain EP 24.15 (Orlowski et al., 1983) or rat kidney EP 24.11 (Orlowski and Wilk, 1981; Almenoff and Orlowski, 1983). A total volume of 600 μL contained 0.2 mM LHRH, 0.2 mM DTT, 0.03 M Tris-HCl (pH 7.0) and 1.2 U EP 24.15, or 4.8 U EP 24.11.

Reaction of LHRH with pituitary and hypothalmic particulate fractions.

Activity toward LHRH was determined by following the degradation of the peptide by HPLC. Incubation mixtures contained LHRH (0.1 mM), Tris-HCl buffer (0.1 M; pH 7.0), DTT (0.4 mM) and the membrane fraction (containing

about 2-5 mg protein) in a final volume of 0.8 mL. Incubations were at 37 C. In experiments in which the effect of inhibition of EP 24.15 was tested, 50 μM of cFP-AAF-pAB was included in the incubation mixtures. EP 24.11 and angiotensin converting enzyme were inhibited by cFE-F-pAB (200 μM) and captopril (10 μM), respectively. Aliquots of the reaction mixture (60 μL) were withdrawn at times 0, 1, 2, and 3 hours and treated with 30% trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant was subjected to HPLC as described below.

AtT20 cell culture and reaction of LHRH with cells and particulate fractions.

AtT20 cells were grown in monolayer culture in Ham's F12 medium supplemented with 10 mM sodium bicarbonate, 10 mM HEPES (pH 7.2), 12.5% horse serum, 2.5% fetal calf serum, 1,000 U/mL penicillin, 1,000 μg/mL streptomycin sulfate, and 2.5 μg/mL amphotericin B. Cells were inoculated into 24 cm^2 plastic tissue culture flasks (Falcon, Oxnard, CA) and incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2. The medium was changed twice weekly.

For incubation of cells directly with LHRH in monolayer culture, 25 cm^2 flasks were inoculated with 3.0 mL of a cell suspension, and grown for 1 week as described above. The medium was removed, and cells were washed once with 5.0 mL of fresh medium. 5.0 mL of a sterile-filtered medium containing 0.1 mM LHRH, 110 mM NaCl, 5.3 mM KCl, 1.0 mM $MgCl_2$, 25 mM glucose, 70 mM sucrose, 1.8 mM $CaCl_2$, 0.4 mM dithiothreitol and 2 mM dibasic sodium phosphate (pH of the medium was adjusted to 7.35). Some flasks contained the EP 24.15 inhibitor cFP-AAF-pAB (50 μM) and others contained the angiotensin converting enzyme inhibitor captopril (10 μM). Samples of 100 μL were removed with a sterile pipet

for HPLC analysis as described below. Cells were harvested at the end of the experiment and counted with a hemocytometer.

A particulate fraction of AtT20 cells was prepared and incubated directly with LHRH. Cells from 4 flasks were harvested by addition of 5.0 mL 0.5 mM EDTA, and centrifuged immediately for 5 min at 1000 g. Cells were then washed by resuspending three times in normal medium (see above) to remove EDTA. The final packed cell pellet was homogenized in approximately 10 vol of 0.05 M Tris-HCl (pH 7.0). The suspension was centrifuged for 20 min at 12,000g. The supernatant was removed and the membranes were washed twice in the same buffer. The final pellet was homogenized in 10 vol of the same buffer and frozen in aliquots for later use. Incubation mixtures contained 0.1 mM LHRH, 0.4 mM dithiothreitol, 0.01 M Tris-HCl (pH 7.0) and the particulate fraction described above. EP 24.15 had a specific activity in the incubation mixture of 0.21 μmoles/mg/hr (170 μg protein) using tBOC-Phe-Ala-Ala-Phe-pAB as the substrate.

Separation and identification of the products by HPLC.

High pressure liquid chromatography (HPLC) was carried out on a C_{18} reverse-phase μBondapak column (30 x 0.4 cm; 10 μM; Waters). Products were eluted with a linear gradient established between 0.1% phosphoric acid and acetonitrile. The initial concentration of acetonitrile was increased from 10% to 35% during 30 min at a flow rate of 1 mL/min. The acetonitrile concentration was then maintained at 35% for an additional 20 min. Emerging peaks were monitored at 214 nm.

For isolation of reaction products, the incubation mixtures described above were treated with 2.5 mL of ethanol and concentrated under a stream of nitrogen after removal of protein by centrifugation. The entire incubation

mixture was subjected to HPLC and the emerging peaks were collected for analysis of amino acid composition. Amino acids were determined as previously described (Orlowski and Wilk, 1981) following acid hydrolysis of the peptides in 6 M HCl at 105 C for 24 h.

Phase 2: *In vivo* degradation of exogenous LHRH in the CNS and in the general circulation.

Rationale and general description of experimental procedures:

A. Following the identification of the important CNS LHRH-degrading enzymes *in vitro* through the use of enzyme inhibitors, it was decided to do a similar determination *in vivo*. Enzymes responsible for the degradation of ICV administered LHRH were identified by coadministering inhibitors of EP 24.15, EP 24.11 and ACE with LHRH. The disappearance of LHRH was monitored by radioimmunoassay of LHRH remaining in brain one hour following ICV LHRH infusion (using an antiserum to $LHRH^{1-10}$ that does not crossreact with the products of EP 24.15 and EP 24.11-mediated hydrolysis).

B. In a similar study, we identified the peripheral peptidases responsible for the degradation of IV administered LHRH by the coadministration of inhibitors of EP 24.15, EP 24.11 and ACE. LHRH disappearance was monitored by radioimmunoassay of plasma LHRH concentrations at various time intervals following IV LHRH injection. The effect of the various peptidases on disposition of LHRH in the general circulation was further quantified by determining the plasma half-life of LHRH in the presence and absence of inhibitors of these peptidases. In order to suggest the likely sites of LHRH hydrolysis by EP 24.15 and EP 24.11 in the periphery, the activity of these 2 endopeptidases in several peripheral organs was assayed.

As part of this study, the lability of the EP 24.15 inhibitor, cFP-AAF-pAB, in the general circulation was studied. As will be explained further in the *Results* and *Discussion* sections, it was determined that this inhibitor can serve as a substrate for EP 24.11 and thermolysin. Therefore, the hydrolysis of cFP-AAF-pAB by purified EP 24.11, thermolysin as well as EP 24.11 in crude tissue homogenates, was studied by HPLC and a colorimetric enzyme assay (see below, .

ICV injections of LHRH and inhibitors.

150-175 g male rats were anesthetized by intraperitoneal (IP) administration of urethane (1.3 g/kg), and an incision was made into the scalp along the midline. A hole was drilled through the skull at 1 mm posterior and 1.5 mm lateral to Bregma. A cannula (0.010" O.D.) was lowered 9 mm from the surface of the skull at an angle of 10° from the vertical (traveling from lateral to medial). The guide cannula (0.018" O.D.) was beveled approximately 10° so that the injection could be made directly down into the third ventricle (Walls and Wishart, 1977). A solution containing LHRH with or without inhibitors was injected (1 µl/min) using a syringe pump. Animals were left for 1 hr, then sacrificed by decapitation. Brains were removed, homogenized in 10 volumes of 0.1 M acetic acid, and LHRH was assayed as described below. Third ventricle cannulation was verified by direct observation of the cannula "track" before homogenization.

Intravenous (IV) administration of LHRH and inhibitors.

Male rats (300 - 350 g) were anesthetized with 1.3-1.5 g/kg urethane (IP) and fitted with a right atrial catheter. They subsequently were treated with 733 µl/kg of a 50 mM LHRH solution (36.6 nmol LHRH/ kg) IV through the atrial

catheter. Groups of animals received injections of 3.11 ml/kg of either: 1) 50 mM solution of cFP-AAF-pAB (156 mmol/kg); 2) 50 mM solution of cFP-F-pAB; 3) a mixture of 50 mM cFP-AAF-pAB and 50 mM cFP-F-pAB; 4) 10 mM captopril or 5) 0.9% saline, at 5 minutes prior to the LHRH injection and a second, supplemental dose of 1.1 ml/kg of the same was given at 8 minutes after the LHRH administration. Blood samples of 0.5 ml were drawn, through the atrial catheter at 5, 20, 35, 50, 65 and 80 minutes following LHRH administration. Plasma was separated from red blood cells (RBC) by centrifugation (at 1600g for 15 minutes), and frozen on dry ice for radioimmunoassay of LHRH concentrations as described below. Each RBC pellet was resuspended in heparinized saline (50 I.U./ml) and returned to the animals after the subsequent sampling.

Determination of EP 24.15 & EP 24.11 activity in crude tissue homogenates.

Male rats, 300 g were sacrificed by decapitation. The brain, kidney, spleen, lung, skeletal muscle (gastrocnemius), heart and liver were immediately excised and homogenized in 10 volumes of 0.05 M Tris-HCl, pH 7.6. EP 24.15 activity in homogenates was determined with Boc-Phe-Ala-Ala-Phe-pAB (0.4 mM) as described above (*Results*, phase 1). EP 24.11 activity was determined using Glt-Ala-Ala-Phe-2NA (0.8 mM) as previously described (Orlowski and Wilk, 1981). EP 24.11 activity was measured in the presence and absence of 0.4 mM cFP-F-pAB, a selective active site-directed inhibitor of EP 24.11. The difference in activities measured in the two incubation mixtures was taken as a measure of EP 24.11 activity.

Enzyme activities are expressed in units, one unit being defined as the amount of enzyme required to release 1 µmol of product/h. Specific activity is expressed in units/mg of protein. Protein was determined by the method of Lowry et al., (1951) with bovine serum albumin as the standard.

Degradation of cFP-AAF-pAB and Glt-AAF-2NA by EP 24.11 in kidney, lung, spleen and brain homogenates.

Appropriate dilutions of the crude homogenates of kidney, lung, spleen and brain, prepared as described in previous paragraph, were incubated with cFP-AAF-pAB (final concentration, 0.2 mM) in Tris-HCl buffer (0.05 M, pH 7.6) in the presence of excess aminopeptidase N (10 µg). Incubations were at 37° C in a final volume of 0.25 ml. The release of free pAB was measured as described above for determination of EP 24.15 activity. Activities were measured in the presence and absence of 0.4 mM cFP-F-pAB, a specific active site-directed inhibitor of EP 24.11. The difference in activities measured in the two incubation mixtures was taken as a measure of EP 24.11 activity. EP 24.11 activity in these homogenates also was determined with Glt-Ala-Ala-Phe-2NA (0.8 mM) as the substrate as described above.

Degradation of cFP-AAF-pAB by purified EP 24.11 and thermolysin and identification of the reaction products.

A homogeneous preparations of EP 24.11 (Almenoff and Orlowski, 1983) was incubated with cFP-AAF-pAB (0.25 mM) in a 0.05 M Tris-HCl buffer (pH 7.6) at 37°C, in a final volume of 1.0 ml. After 80 minutes of incubation the reaction was terminated by addition of 50 ml of glacial acetic acid. In reactions with thermolysin the incubation mixtures consisted of 1.0 mM cFP-AAF-pAB, 5 mg of crystalline bacterial thermolysin (Sigma Chemicals), 0.05 M Tris-HCl, pH 7.6 in a final volume of 1.0 ml. Reactions were carried out at 37°C for 30 minutes and terminated by addition of 50 ml of glacial acetic acid. Aliquots of the reaction mixtures were subjected to high pressure liquid chromatography (HPLC) on a C_{18} reverse-phase µBondapak column (30 x 0.4 cm; 10 µm; Waters). Products

were eluted with a linear gradient established between 0.1% phosphoric acid and acetonitrile. The initial concentration of acetonitrile was increased from 10% to 40% during 30 min at a flow rate of 1 ml/min. The acetonitrile concentration was then maintained at 40% for an additional 10 min. Emerging peaks were monitored at 214 nm. For isolation of reaction products, the incubation mixtures described above were subjected to HPLC with a gradient system described above except that phosphoric acid was replaced by trifluoroacetic acid. Emerging peaks were collected and acetonitrile and trifluoroacetic acid were removed (and peptides were concentrated) under a stream of nitrogen for further analysis of the products.

Radioimmunoassay (RIA) for LHRH.

RIA was carried out by the general procedure described previously (Molineaux et al., 1986). 10 µl serum aliquots were incubated with an LHRH specific antiserum (kindly provided by Dr. James Roberts of this institution) and 5000-7000 cpm of [^{125}I]LHRH in a total volume of 300 ml of 150 mM sodium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (w/v) and 0.1% Triton X-100 (v/v). Radioimmunoassays performed on degradation products obtained after incubation of LHRH with EP 24.11, EP 24.15 and aminopeptidase N showed that there is less than 0.015% crossreactivity with equivalent amounts of LHRH. After 18-24 h, the reaction was terminated by addition of 1.0 ml of a charcoal [3% (w/v)]: dextran [0.3% (w/v)] : horse serum [15% (v/v)] mixture, and samples were centrifuged at 5000g for 15 min. Supernatants were counted in a gamma counter (LKB) and immunoreactivity was estimated using a log/logit data reduction from standard curves prepared with each assay.

Statistical Analysis of Data.

For comparison of differences among multiple groups, one-way analysis of variance followed by Scheffe's multiple comparison test (1953), was performed. For comparison of means of only 2 groups, a 2-tailed, unpaired t-test was performed.

Phase 3: Influence of CNS and peripheral EP 24.15-mediated LHRH degradation on the LH- and FSH-releasing activity of exogenous LHRH.

Rationale and general description of experimental procedures:

After determining, in Phase 2, that of the 3 peptidases in question, that EP 24.15 plays a preeminent role in the CNS and peripheral degradation of ICV and IV administered LHRH, respectively, (see *Results*, section 2), it was decided to determine whether EP 24.15 activity has regulatory significance with regard to the LH- and FSH-releasing activity of LHRH. In order to ascertain that CNS EP 24.15-mediated LHRH degradation curtails the (magnitude and duration of the) biological activity of LHRH, plasma LH and FSH were measured following ICV infusion of LHRH in the presence and absence of EP 24.15 inhibitors. LHRH, in the presence and absence of EP 24.15 (and EP 24.11) inhibitors, was also administered IV to determine the possible pharmacological importance of peripheral EP 24.15 (and EP 24.11) in regulating the gonadotropin-releasing activity of systemically administered LHRH. Moreover, it was our wish to know whether the increased potency of the superagonists relative to native LHRH can be attributed to their resistance to the LHRH-DA of EP 24.15 or EP 24.11 (see *Introduction*, page 6 & 25; also see *Results*, phase 1, where the studies of LHRH analog resistance to these 2 endopeptidases are discussed). Therefore, the LH and FSH-releasing activity of 2 superagonists, administered IV, was compared to that of native LHRH in the presence of EP 24.15 and EP 24.11 inhibitors.

ICV injections of LHRH and inhibitors.

Male rats weighing 150-175 g were anesthetized by intraperitoneal (IP) administration of urethane (1.3 g/kg), and fitted with a right atrial catheter for blood sampling. ICV infusion was carried out exactly as described in phase 2 with the exception for the volume and concentration of infused drugs. 0.5 nmol of LHRH (20 µl of a 25 µM solution), dissolved in 0.9% saline was infused alone or in conjunction with 1 µmol of inhibitors (20 µl of a 50 mM solution of either the EP 24.15 inhibitor or both EP 24.15 or EP 24.11) at a rate of 1 µl/min using a syringe pump. The ionic strength of the solution was maintained at 0.3 mosmol/l by adjusting with sodium chloride.

IV administration of LHRH and inhibitors.

Male rats (200 - 250 g) were anesthetized with 1.3-1.5 g/kg urethane (IP) and fitted with a right atrial catheter. 0.2 ml of a 1.6 µM solution (0.32 nmol) of LHRH or LHRH analog (either [D-Trp6]-LH-RH or D-Leu^6-LHRH-EA), per 250 g rat, dissolved in 0.9% saline, was subsequently administered IV through the atrial catheter. 5 minutes prior to LHRH injection, 0.85 ml of either 0.9% saline or a 50 mM solution of either EP 24.15 or EP 24.11 or both inhibitors (42.5 µmol) per 250 g rat was administered IV. Animals receiving LHRH analogs received only saline prior to analog injection.

Blood Sampling Procedure.

In both groups of animals receiving either IV or ICV injections, blood samples of 0.7 ml were drawn at indicated time intervals through the atrial catheter and placed in a tube containing 50 I.U. heparin. Plasma was separated from red blood cells (RBC) by centrifugation at 1600g for 15 minutes, and frozen

for radioimmunoassay of LH or FSH concentrations as described below. Each RBC pellet was resuspended in 0.4 ml heparinized saline (50 I.U./ml) and returned to the animals after the subsequent sampling.

Radioimmunoassay of plasma LH and FSH levels.

LH and FSH concentrations were determined by a double antibody radioimmunoassay procedure using the reagents and protocols generously provided by the National Hormone and Pituitary Program. Briefly, 100 µl serum aliquots were incubated at 4°C with an LH or FSH specific antiserum and 5000-7000 cpm of [^{125}I]LH or [^{125}I]FSH in a total volume of 300 µl of 150 mM sodium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (w/v) and 0.1% Triton X-100 (v/v). After 18-24 h, 50 µl of a 1:40 dilution of rabbit serum and 50 µl of 1:10 dilution of goat anti rabbit antiserum were added to samples and mixtures were incubated for another 18-24 hours at 4°C and then centrifuged at 5000g for 30 min. Supernatants were decanted and pellets were counted in a gamma counter (LKB) and immunoreactivity was estimated using a log/logit data reduction from standard curves prepared with each assay.

Statistical Analysis of Data.

For comparison of differences among multiple groups, one-way analysis of variance followed by Dunnett's test (1955) (for comparison of the mean values in the control group to the mean values of each of the experimental groups), was performed.

Phase 4: Influence of CNS EP 24.15 activity on endogenous LHRH-stimulated LH secretion.

Rationale and general description of experimental procedures:

After determining, in Phase 3, that EP 24.15 plays a preeminent role in the CNS degradation of ICV administered LHRH, (see *Results*, phase 3), it was decided to determine whether EP 24.15 activity has regulatory significance with regard to the LH-releasing activity of endogenous LHRH. This phase of the thesis project is designed to directly probe whether *in vivo* EP 24.15 inhibition through ICV administration of inhibitor, can increase baseline LH secretion. This design is based upon the presupposition that brain EP 24.15-mediated LHRH extracellular metabolism decreases the amount of LHRH, following its secretion from the ME, that reaches the portal circulation intact. Therefore, inhibition of EP 24.15 activity would be expected to allow a higher proportion of the secreted LHRH reaching the portal circulation and the gonadotropes in the intact, bioactive state, thus precipitating a increase in pituitary LH release.

ICV injections of cFP-AAF-pAB, blood sampling, and LH RIA.

Male rats weighing 150-175 g were anesthetized by intraperitoneal (IP) administration of urethane (1.3 g/kg), and fitted with a right atrial catheter for blood sampling. ICV infusion was carried out exactly as described in phase 2 of *Materials and Methods*. 10 µl of a 50 mM cFP-AAF-pAB was infused into the third ventricle at a rate of 1 µl/min using a syringe pump. The ionic strength of the solution was maintained at 0.3 mosmol/l by adjusting with sodium chloride. Blood samples (0.4 ml) were withdrawn through an atrial catheter prior to and at 30 minute intervals after ICV administration of inhibitor. Plasma LH concentration was determined by a

double antibody radioimmunoassay procedure using the protocol described in phase 3 of *Materials and Methods*.

Statistical Analysis of Data.

For comparison of differences among multiple groups, one-way analysis of variance followed by Scheffe's multiple comparison test (1953), was performed.

Phase 5: Influence of CNS EP 24.15 activity on recovery of endogenous LHRH released from hypothalamic slices.

Rationale and general description of experimental procedures:

In another study designed to determine whether EP 24.15 may extracellularly metabolize endogenous LHRH, LHRH release from hypothalamic nerve terminals *in vitro*, was stimulated by high KCl. The recovery of LHRH in the bathing medium, in the presence and absence of the EP 24.15 inhibitor, cFP-AAY-pAB, was measured.

In vitro LHRH release from hypothalamic slices.

250-300 g male Sprague Dawley rats were sacrificed by decapitation. Brains were quickly removed and the hypothalamus was dissected out from chilled brain. The boundaries of the hypothalamic fragment were as follows: anteriorly, 1 mm anterior to the optic chiasm; laterally, the lateral hypothalamic sulci, and transversely, the anterior commisure. 400 µm thick slices from one hypothalamus (approximately 8 slices per hypothalamus) were deposited into a 3 cc syringe (Becton-Dickinson), cut at the bottom, and melted onto a nylon mesh. The syringe was transferred into a vial containing 1.0 ml EBSS (buffered to pH 7.35 with HEPES, final HEPES concentration: 25

mM) and incubated at 37°C for 15 minutes with gentle agitation (in a Dubnoff metabolic shaking incubation chamber) under a 100% O_2 atmosphere. After this incubation period, the syringe was transferred to a second vial for 15 min and this was repeated up to a sixth transfer. LHRH released during the sixth 15 min incubation period was termed "basal release of LHRH". After this, the syringe was transferred into a vial containing EBSS with 60 mM KCl and incubated for 15 min. LHRH released in this vial was termed "stimulated release of LHRH". Immediately after each transfer, 800 µl of media was removed from the vial (from which slices were removed), frozen on dry ice and stored at -80°C.

From each sample, 200 µl of media were taken for LHRH RIA.
For *in vitro* treatment with inhibitor, half of the 60 mM KCl-containing vials also had a final concentration of 50 µM EP 24.15 inhibitor, cFP-AAY-pAB.

Effect of LHRH concentration of LHRH degradation rate by hypothalamic slices

To study the rate of LHRH degradation by (membrane) EP 24.15 activity in hypothalamic slices, brain slices were incubated with 3 different concentrations of exogenously added LHRH: 10 nM, 1 µM and 100 µM, in the presence and absence of 50 µM EP 24.15 inhibitor, cFP-AAY-pAB. Experiments were conducted as follows:
Following the dissection of hypothalami, 10 slices of 400 µm were cut. 5 slices were added to media (with a volume of 300 µl) containing LHRH alone (controls). The other 5 slices were added to media containing LHRH & cFP-AAY-pAB. Aliquots of 30 µl were removed at time 0, and at 30, 60 and 120 min and frozen immediately on dry ice. Samples of aliquoted media were then diluted 10-fold in 0.1 N acetic acid, heated at 90°C for 10 minutes and

centrifuged at 12000 x g for 20 min. 100 µl of the appropriate dilution of the supernatant was added to RIA tubes and the acid was evaporated by lyophylization overnight. The remaining peptide-containing dried pellet was resuspended in RIA buffer (0.15 M Na-Phosphate, pH 7.4) and LHRH RIA was conducted as described above. Brain slices were saved for protein determination (Lowry et al., 1951).

To calculate the rate of EP 24.15-mediated LHRH degradation, the amount of LHRH remaining at 30-min was subtracted from the amount of LHRH measured in 0-time samples for each hypothalamic incubation. This value multiplied by 2, provided "number pmol LHRH degraded/hr". The "number pmol LHRH degraded/hr" in inhibitor-containing incubation was subtracted from that in control incubation. This provided the "number pmol LHRH degraded by EP 24.15/hr". This value was divided by the protein content of the hypothalamic slices to provide the "number pmol LHRH degraded by EP 24.15/mg protein/hr". This calculation was repeated for each of the 3 starting LHRH concentrations used in this experiment.

RESULTS

Phase 1: LHRH degradation by membrane preparations of brain, pituitary and AtT20 cells.

Degradation of LHRH analogs by EP 24.15 and EP 24.11

Purified preparations of EP 24.15 isolated from the soluble fraction of brain homogenates (Orlowski et al., 1983) cleaved LHRH at the Tyr^5-Gly^6 bond (Table 1), and also to a lesser extent at the His^2-Trp^3 bond. LHRH was also shown to inhibit the degradation of synthetic substrates by the enzyme (Chu and Orlowski, 1985), further confirming that the peptide is a substrate for EP 24.15. The ability, however, of the recently identified membrane-bound component of the enzyme (Acker et al., 1987) to degrade LHRH has not been examined. This component, present in synaptosomal membranes, constitutes 20-25% of the total activity in brain homogenates and, like the soluble form, was shown to be capable of converting several proenkephalin and prodynorphin-derived oligopeptides into Met- or Leu-enkephalin (Acker et al., 1987).

Brain and pituitary contain a membrane bound zinc-metalloendopeptidase with a thermolysin-like specificity that cleaves peptide bonds on the amino side of hydrophobic amino acid residues. This enzyme, designated as EP 24.11 (E.C. 3.4.24.11) was first isolated from rabbit kidney (Kerr and Kenny, 1974) and was shown to be identical with a zinc-metalloendopeptidase isolated from membrane fractions of the pituitary (Orlowski and Wilk, 1981) and with brain "enkephalinase" (Almenoff et al., 1981), which cleaves the Gly-Phe bond in Met- and Leu- enkephalin. When purified preparations of EP 24.11 having an activity of 200 units/ml were

incubated with LHRH under conditions similar to those given in Table 1, slow degradation of the peptide (2 to 5% after 1 hour) was observed. Two degradation products were isolated and identified by HPLC.

TABLE 1: Degradation of LHRH and LHRH Analogs by Purified Endopeptidase-24.15.

Compound	Time of Incubation (min)	LHRH Remaining mM (%)
LHRH	0	0.200 (100)
	15	0.152 (76)
	30	0.096 (48)
	60	0.054 (27)
Analog 1	0	0.200 (100)
	30	0.178 (89)
	60	0.185 (92)
Analog 2	0	0.200 (100)
	30	0.197 (99)
	60	0.170 (85)

LHRH and the LHRH analogs (0.2 mM) were incubated for the indicated times at 37 C with 10 µL endopeptidase-24.15, as described in "Materials and Methods". The activity of purified endopeptidase-24.15 using the substrate tBOC-Phe-Ala-Ala-Phe-pAB (0.4 mM) was 40.5 U/ml. Analog 1 was [D-Ala^6]-LHRH. Analog 2 was des-Gly^{10}-[imidazobenzyl-D-His^6]-LHRH.

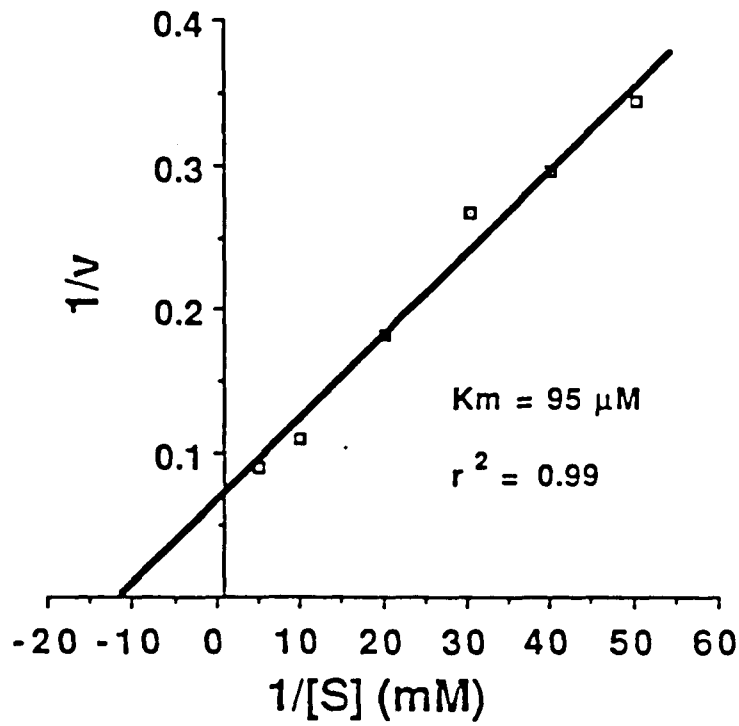
After a short incubation time, a third product was observed which had a retention time identical to pGlu-His-Trp-Ser-Tyr-Gly. This product was not visible after longer incubations, apparently being converted by cleavage of the Ser^4-Tyr^5 bond into pGlu-His-Trp-Ser and Tyr-Gly, the latter being eluted from the HPLC column with the breakthrough buffer peak. These data indicate that EP 24.11 cleaves LHRH at the Gly-Leu bond and that the product of this reaction, pGlu-His-Trp-Ser-Tyr-Gly is further hydrolyzed to yield pGlu-His-Trp-Ser and Tyr-Gly, both cleavages being consistent with the specificity of the enzyme toward bonds on the amino side of hydrophobic residues.

Neither of the two superactive analogs of LHRH, one containing a D-Ala and the other an imidazobenzyl-D-His in place of the Gly^6 residue, were attacked by either EP 24.15 or EP 24.11. This result is consistent with our previous finding that substrates containing a D-amino acid on either side of the scissile bond are resistant to degradation by EP 24.15 (Orlowski et al., 1983), and that D-amino acids at the scissile bond confer resistance against attack by EP 24.11 (unpublished data).

Determination of the Michaelis-Menten constant of LHRH for EP 24.15.

The K_m for LHRH was determined directly by HPLC analysis of reaction products formed in the presence of purified EP 24.15. The rate of formation of pGlu-His-Trp-Ser-Tyr, ($LHRH^{1-5}$), determined by HPLC was plotted as a function of substrate concentration in a double reciprocal plot as shown in figure 1. The K_m as determined by this method was estimated to be 95 μM, which is similar to the K_m values of some of the opioid peptides.

Figure 1: Double reciprocal plot of LHRH degradation by purified endopeptidase-24.15. The formation of pGlu-His-Trp-Ser-Tyr from LHRH was monitored by HPLC analysis of the reaction product. Peak height at A_{214} nm was measured after incubation of enzyme with LHRH for 15 min at 37 °C.


0.4
0.3
0.2
0.1
1/v
Km = 95 µM
r 2 = 0.99
-20 -10 0 10 20 30 40 50 60
1/[S] (mM)

LHRH degradation by hypothalamic and pituitary membranes.

Incubation of LHRH with particulate fractions obtained from homogenates of rat hypothalamus, and analysis of the degradation products by HPLC and amino acid analysis following acid hydrolysis showed that cleavage occurred primarily at the Tyr^5-Gly^6, Gly^6-Leu^7 and Trp^3-Ser^4 bonds. The degradation products which were identified were pGlu-His-Trp-Ser-Tyr, pGlu-His-Trp-Ser-Tyr-Gly and pGlu-His-Trp, (Table 2). Approximately 50-80% of the LHRH added was degraded within 2-3 h. The appearance of pGlu-His-Trp-Ser-Tyr and pGlu-His-Trp was prevented and LHRH degradation was blocked when membranes were incubated with LHRH in the presence of the EP 24.15 inhibitor cFP-AAF-pAB. Only about 30% of the peptide was degraded to several minor products under these conditions. The appearance of pGlu-His-Trp-Ser-Tyr-Gly in the presence of cFP-AAF-pAB indicates that EP 24.11, present in hypothalamic membranes (Acker et al., 1987) cleaves the Gly^6-Leu^7 bond in LHRH (see below).

Incubation of LHRH with a particulate fraction from anterior pituitary resulted primarily in the appearance of pGlu-His-Trp under conditions in which 75% of the LHRH was degraded (Figure 2, Panel A). When N-[1-carboxy-2-phenylethyl]-Phe-pAB, an inhibitor of endopeptidase 24.11 ("enkephalinase") was used, the rate of degradation of LHRH was reduced (Figure 2, panel B; only 55% of the LHRH was degraded). However, when cFP-AAF-pAB was incubated with LHRH under the same conditions, only 30% of the LHRH was degraded to several minor products (panel C). When inhibitors of these two endopeptidases were used together (panel D), only 20% of the peptide was degraded. Thus, a membrane-bound form of EP 24.15 is quantitatively the predominant enzyme responsible for degradation of LHRH in both hypothalamic and pituitary membranes, while EP 24.11 appears to contribute less to LHRH degradation.

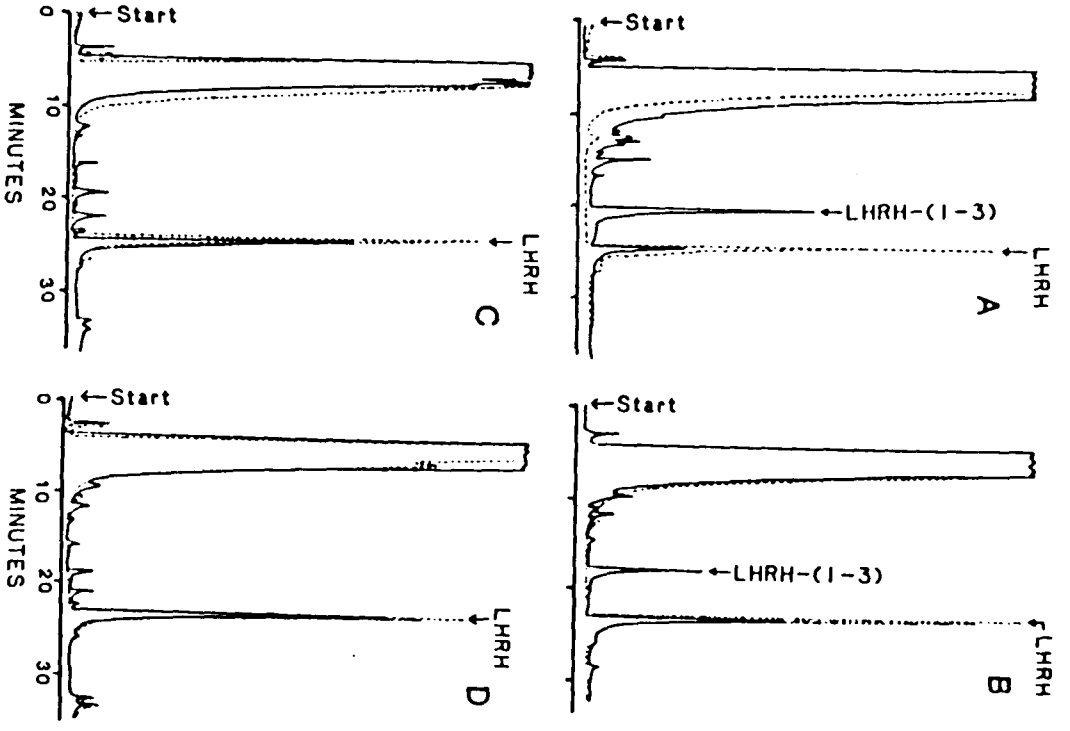
TABLE 2: Degradation of LHRH by Particulate Fractions of the Hypothalamus.

Inhibitor tion	Incuba time (min)	Peak re- tention (min)	Peak Height (cm)	Identification
None	180	22	2.2	pGlu-His-Trp-Ser-Tyr-Gly*
		20	1.6	pGlu-His-Trp-Ser-Tyr
		18	1.2	pGlu-His-Trp
cFP-AAY-pAB	180	22	1.5	pGlu-His-Trp-Ser-Tyr-Gly*

Particulate fractions from the hypothalamus were incubated with LHRH (0.25 mM) in Tris-HCl buffer (0.1 M; pH 7.0) in the presence of DTT (0.4 mM) in a final volume of 0.6 mL. Aliquots of the incubation mixture were withdrawn at various times and the products were separated by HPLC. The reaction was terminated by addition of 50% trichloroacetic acid (50 µL). After removal of the precipitated protein by centrifugation, the products present in the supernatant, were separated by HPLC, and analyzed for amino acid composition after acid hydrolysis.

*Several minor products containing too small amounts of amino acids to be identified were also present. pGlu-His-Trp-Ser-Tyr was the major product of degradation in both preparations.

Figure 2: HPLC separation of the reaction products generated by incubation of a particulate fraction of rat anterior pituitary with LHRH. The identification of the reaction products was derived from amino acid analysis of peaks following acid hydrolysis. The broken and solid lines represent the HPLC traces before and after 120 min incubation, respectively. Panel A represents the HPLC peak profile in the absence of inhibitor; panels B and C were obtained from incubation mixtures containing the endopeptidase-24.11 inhibitor (cFE-F-pAB) and the endopeptidase-24.15 inhibitor (cFP-AAF-pAB), respectively; panel D was obtained from incubation mixtures containing both cFE-F-pAB and cFP-AAF-pAB. The initial concentration of LHRH in the incubation mixtures was 0.25 mM. Final LHRH concentrations were 0.058 mM (23 %), 0.11 mM (46 %) and 0.20 mM (82 %) in panels A, B, C, and D, respectively. Other experimental details are given in Materials and Methods.


Start
LHRH-(1-3)
LHRH
A
Start
LHRH-(1-3)
LHRH
B
Start
LHRH
C
MINUTES
0
10
20
30
Start
LHRH
D
MINUTES
0
10
20
30

The appearance of pGlu-His-Trp in incubation mixtures containing LHRH and either pituitary or hypothalamic membranes (Elkabes et al., 1981; Yokosawa et al., 1987) posed the question of what reaction(s) were involved in its formation. Addition to the incubation mixtures of the angiotensin converting enzyme (ACE) inhibitor captopril blocked the appearance of the pGlu-His-Trp fragment and greatly enhanced the appearance of pGlu-His-Trp-Ser-Tyr (data not shown). This indicated that pGlu-His-Trp is a secondary degradation product formed by cleavage of pGlu-His-Trp-Ser-Tyr, a peptide whose formation is dependent on the presence of EP 24.15 activity. Interestingly, the tetrapeptide pGlu-His-Trp-Ser also accumulated, suggesting that ACE also cleaves the tetrapeptide. The rate of degradation of the LHRH was not affected by addition of captopril, indicating that ACE is not a significant enzyme in the degradation of intact LHRH under the conditions used.

LHRH degradation by AtT20 cells.

EP 24.15 is present as both soluble (75-80%) and membrane-bound (20-25%) forms in rat hypothalamus. The membrane-bound form of the enzyme exists as an integral membrane protein in the synaptosome fraction. However, the exact subcellular localization of the membrane-bound form of EP 24.15 has not been established. We have used a mouse pituitary tumor cell line known as AtT20 for further studies of LHRH degradation. For these experiments, cells were incubated in monolayer culture in the presence of enzyme inhibitors; thus, difficulties in damaging cells during the process of harvesting were avoided, ensuring that the cells were completely intact when used. When AtT20 cells were incubated with LHRH for 12 h, we found again that the only product formed was the tripeptide pGlu-His-Trp (Figure 3). Inclusion of the specific

angiotensin converting enzyme (ACE) inhibitor captopril in the incubation medium prevented the formation of pGlu-His-Trp, and a peak corresponding to the N-terminal pentapeptide pGlu-His-Trp-Ser-Tyr was found. There was a slight decrease in the degradation of LHRH under these conditions. The inhibitor of EP 24.15, cFP-AAF-pAB, on the other hand, completely blocked LHRH degradation. A small peak also was found which had the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro, which might arise from the action of prolyl endopeptidase (post-proline cleaving enzyme) upon LHRH at the Pro^{9}-Gly^{10} bond. This product was not found in the absence of EP 24.15 inhibitor. EP 24.15 appears to have a similar importance in intact AtT20 cells as in pituitary and hypothalamic membrane preparations: it seems to be the primary enzyme which initiates LHRH degradation in this tissue. In order for EP 24.15 to act directly upon LHRH in the incubation medium, the enzyme must be on the external surface of AtT20 cells.

LHRH was degraded by a particulate fraction prepared from AtT20 cells, yielding primarily pGlu-His-Trp and pGlu-His-Trp-Ser-Tyr (Figure 4). A small amount of pGlu-His-Trp-Ser-Tyr-Gly was also formed. When the EP 24.15 inhibitor cFP-AAF-pAB was included in the incubation, LHRH degradation was completely prevented. Thus, like the other preparations discussed above, LHRH degradation is initiated primarily by EP 24.15 acting to hydrolyze the Tyr^{5}-Gly^{6} bond. EP 24.11, which is also present in these cells, may be responsible for the formation of the N-terminal hexapeptide.

Figure 3: HPLC separation of the reaction products generated by incubation of a monolayer culture of unbroken AtT20 cells with LHRH. Plates had a total of 5 mL of media at the beginning of the experiment. The composition of the incubation media, and the separation and identification of products by HPLC is described in "Materials and Methods". Panel A represents an HPLC tracing obtained at incubation time 0; panels B, C, and D were obtained after 12 hours of incubation in the presence of no inhibitor, captopril, or cFP-AAF-pAB, respectively. The elution positions of the reaction products are shown as in Figure 2. The initial concentration of LHRH in the incubation medium was 0.1 mM. After 12 hours, LHRH concentrations were 0.071 mM (71%), 0.089 mM (89%) and 0.10 mM (100%) in panels B, C, and D, respectively. The number of cells in the incubation dish were counted with a hemocytometer at the end of the experiment. Dishes contained 3.12, 2.82 and 3.21 million cells in the experiments shown in panels B, C, and D, respectively.

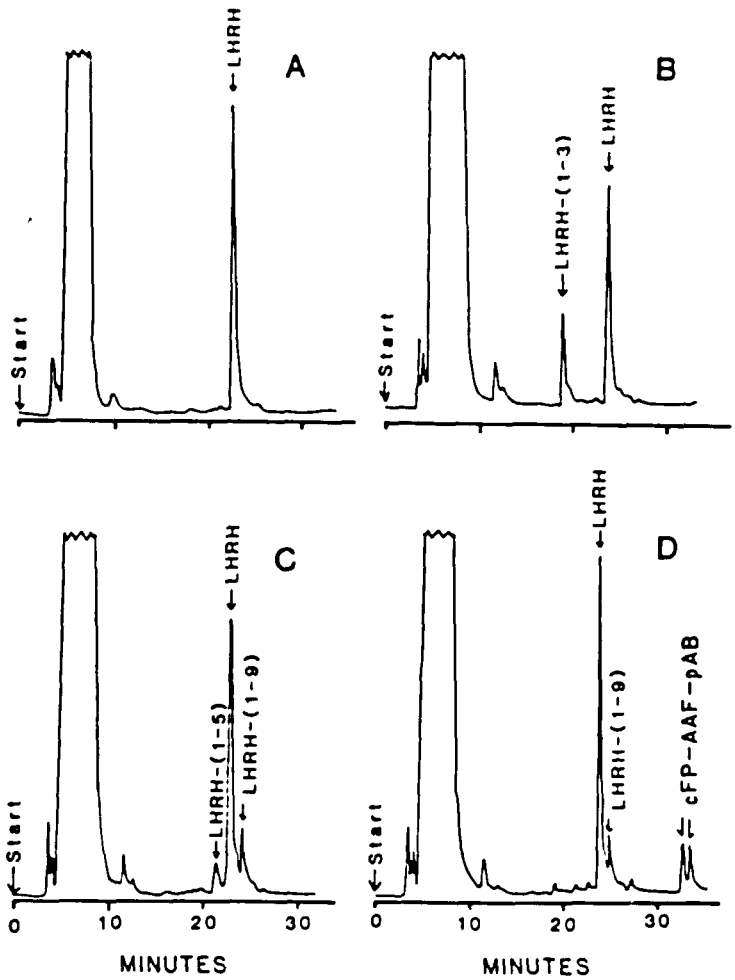
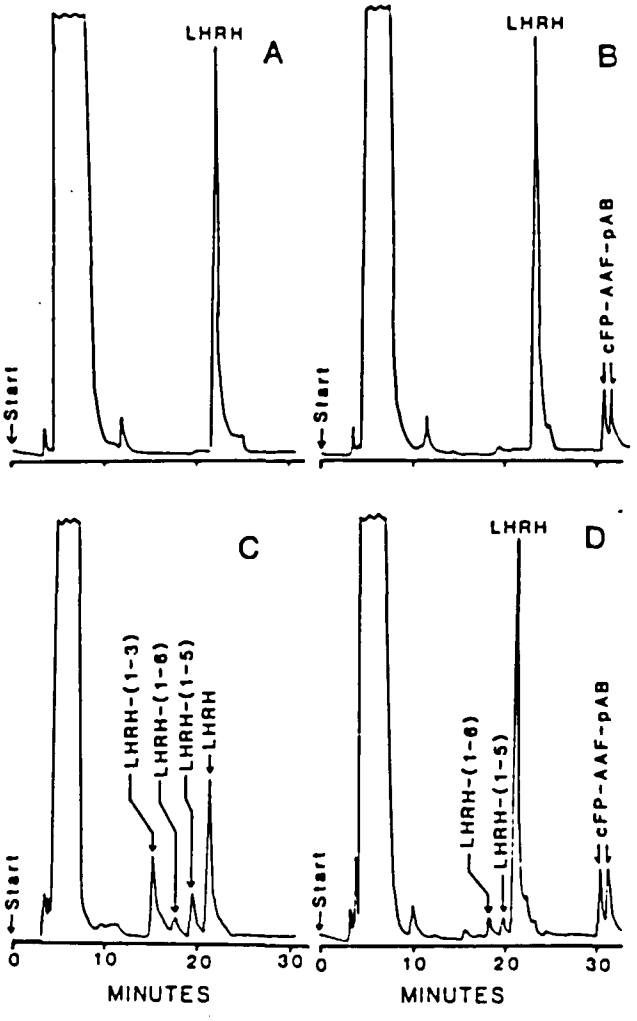

A
B
C
D
Start
LHRH
LHRH-(1-3)
LHRH-(1-5)
LHRH-(1-9)
cFP-AAF-pAB
0
10
20
30
MINUTES

Figure 4: HPLC separation of the reaction products generated by incubation of a particulate fraction of AtT20 cells with LHRH. Panels A and B represent HPLC tracings obtained at incubation time 0 in the absence and presence of endopeptidase-24-15 inhibitor respectively; panels C and D were obtained after 4 hours of incubation. The inhibitor of endopeptidase-24.15, N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (present as 2 diastereomers) was present in D. The initial amount of LHRH in the incubation mixtures was 80 nmol. After 4 hours, the LHRH remaining was 31.6 nmol (40%) and 77.2 nmol (96%) in C and D, respectively.


LHRH
A
LHRH
B
cFP-AAF-pAB
Start
Start
C
LHRH-(1-3)
LHRH-(1-6)
LHRH-(1-5)
LHRH
D
LHRH
LHRH-(1-6)
LHRH-(1-5)
cFP-AAF-pAB
Start
Start
0
10
20
30
MINUTES
0
10
20
30
MINUTES

Phase 2: *In vivo* degradation of exogenous LHRH in the CNS and in the general circulation.

Effect of ICV administration of metallopeptidase inhibitors on LHRH recovery from brain tissue.

One hour after ICV administration of 100 nmol of LHRH, less than 2% of the amount injected could be detected in brain homogenates (Figure 5). The rapid disappearance of the peptide could have resulted from its redistribution in the cerebrospinal fluid, its enzymatic degradation and its exit from the CNS into the peripheral circulation. Inhibition of degradative enzymes would therefore be expected to affect only the fraction of the peptide that undergoes enzymatic degradation. When cFP-AAF-pAB, an inhibitor of EP 24.15, was injected simultaneously with LHRH, significantly higher amounts of LHRH, nearly 20% of the amount injected, were recovered from the brain. No statistically significant increase in LHRH recovery was observed after ICV administration of LHRH together with either the inhibitor of EP 24.11 (cFP-F-pAB) or the inhibitor of ACE (captopril). Nevertheless a tendency to somewhat higher recoveries was observed after each of the last two inhibitors, with captopril having the smaller effect. It is therefore likely that with larger groups of animals the amounts of LHRH recovered after administration of these inhibitors would acquire statistical significance.

The data obtained after simultaneous administration of LHRH and captopril indicate that ACE has only a minor if any effect on the in vivo degradation of LHRH. It was, however, of interest to determine whether concurrent administration of inhibitors of EP 24.15 and EP 24.11 would further increase LHRH recovery beyond that obtained after administration of the EP 24.15 inhibitor alone. The results summarized in Table 3 show that when 10

nmol of LHRH was administered ICV together with cFP-AAF-pAB, the amount of the peptide recovered from brain after one hour was almost 13 times greater

Figure 5: Effect of inhibitors on LHRH recovery after ICV Injection. 10 ml of an LHRH solution (100 nmol) was injected into the third ventricle during 10 minutes. LHRH was co-administered with either 1) 0.9% saline (control), 2) 500 nmol cFP-AAF-pAB, 3) 500 nmol cFP-F-pAB, 3) 100 nmol captopril. The ionic strength of the solution was maintained at 0.3 mosmol/l by adjusting with sodium chloride. At 1 hour following end of infusion, animals were sacrificed and the entire brain was homogenized in 10 volumes of hot acetic acid. Precipitated protein was removed by centrifugation and the samples were frozen for radioimmunoassay analysis. Each group contained five animals. Experimental details are give in Materials and Methods. (*Statistically different from control by Scheffe's test ($p < 0.05$)).

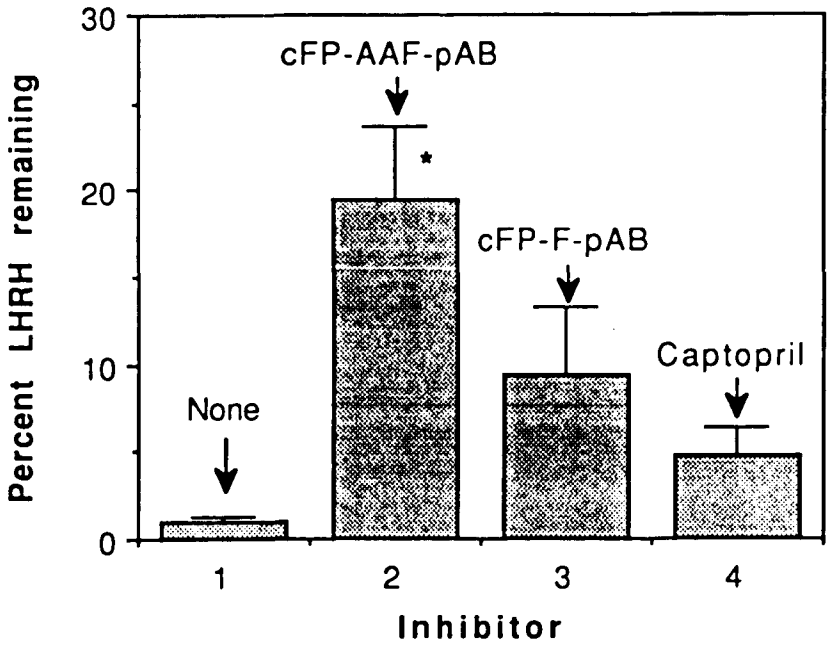

30
20
10
0
Percent LHRH remaining
cFP-AAF-pAB
*
cFP-F-pAB
Captopril
None
1
2
3
4
Inhibitor

TABLE 3: Effect of inhibitors of EP 24.15 and EP 24.11 on recovery of brain LHRH after ICV injection.

	Treatment	Enzyme inhibited	n	nmol LHRH Remaining (mean + S.E.M.)
1.	None	---	7	0.085 ± 0.017
2.	cFP-AAF-pAB	EP 24.15	9	1.092 ± 0.106*
3.	cFP-F-pAB	EP 24.11	4	0.353 ± 0.112
4.	cFP-AAF-pAB + cFP-FpAB	EP 24.15 + EP.24.11	9	1.211 ± 0.163*

Animals received ICV injections of 10 nmol LHRH (10 µl of a 1 mM solution in saline) at a rate of 1 ml/min over 10 minutes (1.). LHRH was co-administered with either 750 nmol cFP-AAF-pAB (2.), 750 nmol cFP-F-pAB (3.) or a solution of both 750 nmol cFP-AAF-pAB and 750 nmol cFP-F-pAB (4.). At 1 hour after the infusion, animals were sacrificed and the entire brain was homogenized in 10 volumes of hot acetic acid. Protein was removed by centrifugation and the supernatant was frozen for radioimmunoassay analysis. N designates the number of animals. *Significantly different from control by Scheffe's test, ($p < 0.01$).

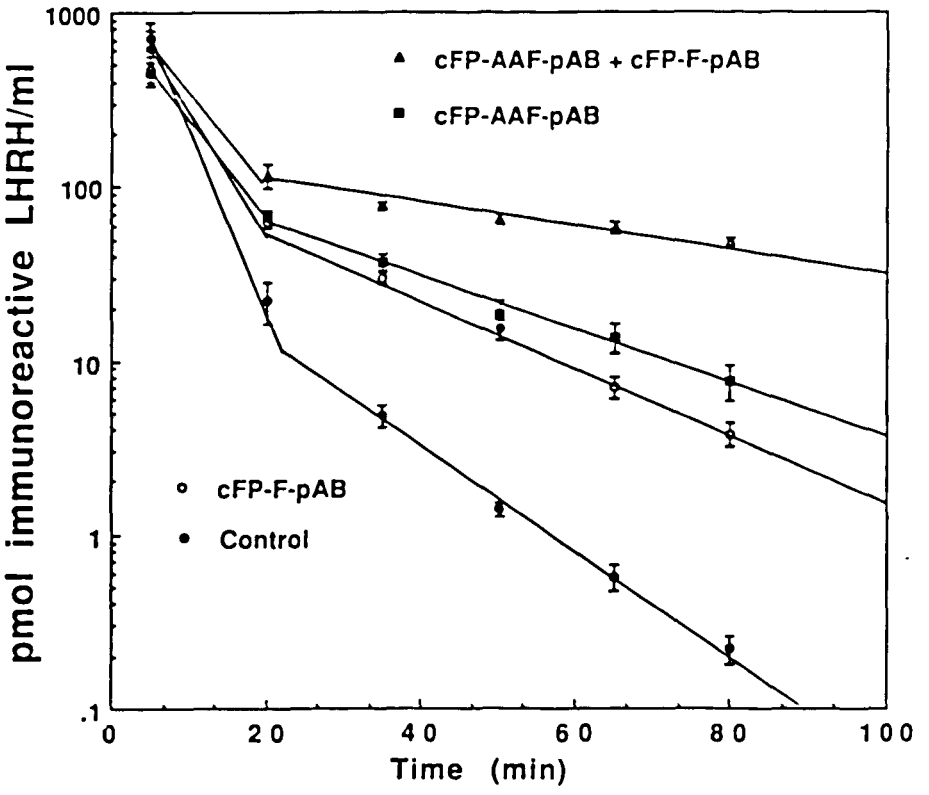
than in controls ($p < 0.01$). Again, administration of the inhibitor of EP 24.11 alone did not significantly increase peptide recovery, although with larger groups of animals the differences might acquire statistical significance. Concurrent administration of both inhibitors did not result, however, in any additional significant enhancement of LHRH recovery above that seen after cFP-AAF-pAB alone.

Effect of IV administration of inhibitors on plasma LHRH concentrations.

The relative contributions of EP 24.15, EP 24.11 and ACE to the *in vivo* degradation of LHRH in peripheral tissues was studied by determining plasma concentrations at various time intervals after IV administration of the peptide alone and in conjunction with enzyme inhibitors. Plasma LHRH concentrations, measured 20, 35, and 50 minutes after intravenous LHRH administration, were significantly higher in animals receiving inhibitors of either EP 24.15 or EP 24.11 than in controls (Figure 6). As early as 20 min after administration of LHRH, there was a clearly discernible protection of LHRH degradation by inhibitors of both EP 24.11 and EP 24.15. At 65 and 80 minutes following LHRH administration, statistically significant elevations of plasma LHRH (above those found in controls) were found only in animals treated with cFP-AAF-pAB. Concurrent administration of both EP 24.15 and EP 24.11 inhibitors produced a much greater elevation of plasma LHRH levels than those found in animals treated with each inhibitor separately. A statistically significant increase in LHRH levels, above those in control animals, was found in this group at all time points after 5 min. Indeed, 65 and 80 minutes after administration of the two inhibitors, plasma LHRH concentrations were as much as 100 to 200 times higher than those in

controls. In animals treated with captopril, plasma LHRH concentrations were not significantly different from those found in controls (data not shown).

Figure 6: Plasma concentrations after intravenous administration of LHRH alone (control) and in conjunction with inhibitors of EP 24.15 and EP 24.11. Data are mean values ± S.E.M. The number of animals were: 11 in the control group, 9 in each group receiving cFP-F-pAB or cFP-AAF-pAB, and 9 in the group receiving both inhibitors. Scheffe's test (1953) was used to determine statistically significant differences between groups. All values for the group receiving both inhibitors were at all time periods significantly different from controls ($p < 0.001$). Corresponding values for the group receiving cFP-AAF-pAB were significantly different from controls ($p < 0.01$ to 0.001). For the group receiving cFP-F-pAB p values were < 0.001 after 20 and 35 min, and < 0.05 after 50 min. For details of experimental procedure see under Materials and Methods.


1000
100
10
1
.1
pmol immunoreactive LHRH/ml
0
20
40
60
80
100
Time (min)
cFP-AAF-pAB + cFP-F-pAB
cFP-AAF-pAB
cFP-F-pAB
Control

The half-life of intravenously injected LHRH was moderately increased from approximately 10 min in the control group to 20 and 15 min in animals receiving the EP 24.15 or EP 24.11 inhibitor respectively (groups 3 and 4, Table 4). Concurrent administration, however, of inhibitors of both enzymes resulted in a dramatic eight-fold increase in the half life of LHRH from about 10 min to approximately 80 min (Table 4). It became evident from the magnitude of this increase, that this could not be attributed to a simple additive effect of the two inhibitors. Since inhibition of EP 24.15 in brain had a much more pronounced effect on LHRH degradation than inhibition of EP 24.11, the possibility was considered that the effectiveness of the EP 24.15 inhibitor in peripheral tissues was limited by its enzymatic degradation catalyzed by EP 24.11. Accordingly, inhibition of EP 24.11 by administration of cFP-F-pAB would have been expected to block the degradation of cFP-AAF-pAB, and consequently result in a more complete inhibition of EP 24.15, which in turn would produce a greater protection of LHRH from degradation. To examine this possibility we proceeded to measure the concentrations of plasma cFP-AAF-pAB in animals after administration of this inhibitor alone and in those receiving concurrently cFP-F-pAB. We also examined the degradation of cFP-AAF-pAB by an isolated preparation of EP 24.11 and thermolysin. Both these enzymes were expected to cleave the Ala-Phe bond in cFP-AAF-pAB in view of their specificity toward bonds on the amino side of hydrophobic amino acid residues.

TABLE 4: Effect of enzyme inhibitors on the half-life of plasma LHRH in rats.

Group	Treatment	Enzyme inhibited	n	LHRH $t_{1/2}$ (min) (± SEM)
1	Control	none	12	10.6 ± 0.72
2	Captopril	ACE	6	11.2 ± 1.37
3	cFP-F-pAB	EP 24.11	9	14.6 ± 0.49
4	cFP-AAF-pAB	EP 24.15	9	19.6± 2.10
5	cFP-AAF-pAB + cFP-F-pAB	EP 24.15 + EP24.11	6	79.6 ± 15.8*

Half-lifes ($t_{1/2}$) were calculated from the LHRH elimination curves in Figure 2. A linear regression program was used to determine the slopes of a best fit line of all time points from 35 to 80 min. The elimination rate constants ($-k_e$) and $t_{1/2}$ ($t_{1/2} = 0.693/k_e$) were calculated.

(*Statistically different from control by Scheffe's test, ($p < 0.01$)).

Plasma concentrations of cFP-AAF-pAB could be conveniently measured by testing the extent of the inhibitory effect of plasma on the degradation of Boc-Phe-Ala-Ala-Phe-pAB by purified EP 24.15 (Orlowski et al., 1983). The concentrations of cFP-AAF-pAB in the 80-minute plasma samples taken from animals receiving this inhibitor alone or in conjunction with cFP-F-pAB (groups 4 and 5, Table 4) were calculated from the following equation:

$$[I] = \frac{i \cdot K_i(1+[S]/K_m)}{1-i}$$

where [I] = concentration of intact inhibitor, i = percent inhibition as a fraction of 1, [S] = concentration of substrate, K_i = inhibition constant of cFP-AAF-pAB, and K_m = Michaelis constant for the substrate (Boc-Phe-Ala-Ala-Phe-pAB). EP 24.15 activity was measured in the presence of plasma from control animals (no inhibitor), and in the presence of plasma from animals treated either with cFP-AAF-pAB alone or treated with both cFP-AAF-pAB and cFP-F-pAB. Percent inhibition (i) was calculated from the equation : $i = 1-(a'/a)$, where a = activity in the presence of serum from control animals and a' = activity in the presence of plasma from the other two groups. The results of this determination showed that the plasma cFP-AAF-pAB concentration measured in the group receiving both inhibitors was more than 10-fold higher (598 ±11 nM) than the concentration in the absence of cFP-F-pAB (52 ± 0.01 nM). The increased cFP-AAF-pAB levels ($p<0.05$, two-tailed unpaired t-test) could have resulted from a decreased enzymatic degradation of the inhibitor or its decreased excretion. The latter possibility was discounted by

the finding that urinary concentrations of free pAB, a measure of the degradation of the parent compound, were nearly 100-fold lower in animals receiving both inhibitors than in those receiving only cFP-AAF-pAB. The low concentrations of free urinary pAB together with the increased plasma cFP-AAF-pAB in animals receiving both inhibitors are a clear indication that cFP-F-pAB protected cFP-AAF-pAB from degradation by EP 24.11, augmenting thereby EP 24.15-inhibition and consequently blocking LHRH degradation by this enzyme.

Hydrolysis of cFP-AAF-pAB by brain, kidney, lung and spleen homogenates and by isolated EP 24.11 and thermolysin.

Indirect evidence cited above indicated that cFP-AAF-pAB is susceptible to degradation by EP 24.11 in peripheral tissues. Confirmation that this degradation is indeed mediated by EP 24.11 would require verification that the tissue distribution of the cFP-AAF-pAB-degrading activity corresponds to the tissue distribution of this enzyme. The degradation of the inhibitor as well as that of Glt-Ala-Ala-Phe-pAB, an EP 24.11 substrate, was therefore studied in crude homogenates prepared from kidney, lung, spleen and brain. Among the four tissues kidney was known to contain the highest and brain the lowest EP 24.11 activity. The data summarized in Table 5 show that the hydrolysis of both the inhibitor and the substrate proceeded at a rate almost 200 times greater in the kidney than in brain. Furthermore, the relative rates of degradation of both the inhibitor and the substrate were the same in all four tissues.

EP 24.11 and thermolysin, a bacterial zinc-containing endopeptidase (EC 3.4.24.4), have similar substrate specificities. Purified preparations of both these enzymes were therefore used to study the products of degradation of cFP-AAF-pAB. HPLC separation of the reaction mixtures revealed the formation of

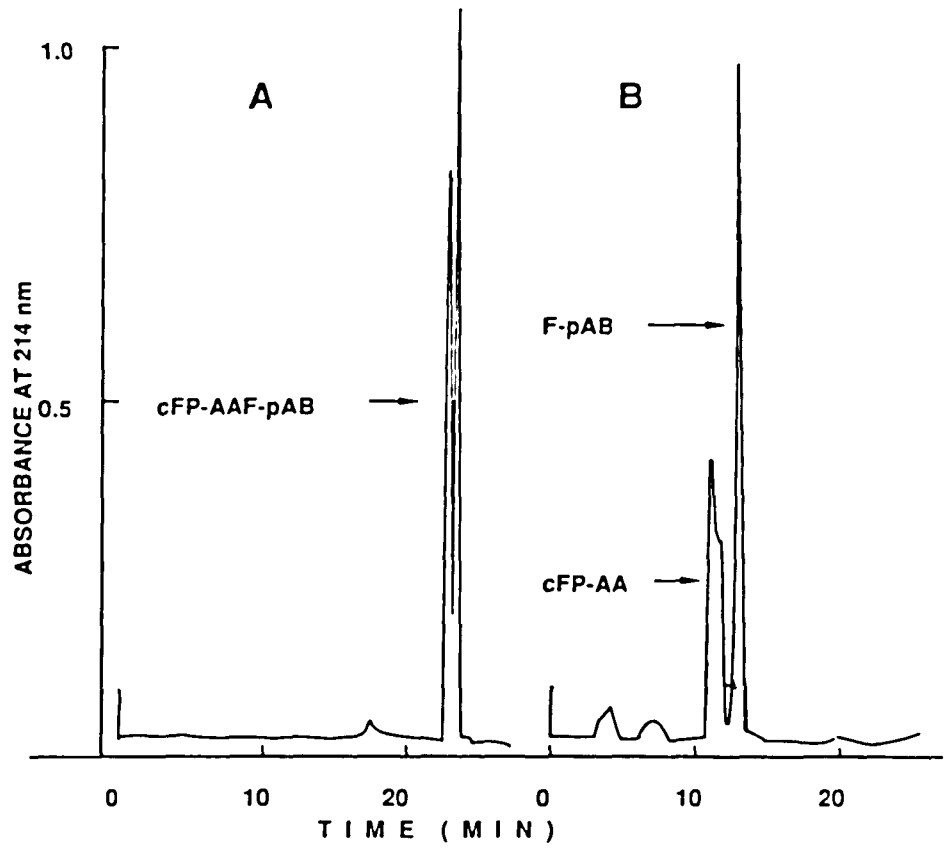
the same 2 products (Figure 7) with each of the enzymes. The slower eluting peak was found to be identical with authentic synthetic Phe-pAB. Accordingly, the early peak was assumed to represent N-[1(RS)-carboxy-3-phenylpropyl]-alanyl-alanine (cFP-AA). It was of importance for the evaluation of the inhibitory effect of cFP-AAF-pAB to determine whether cFP-AA as a

TABLE 5: Degradation of cFP-AAF-pAB and Glt-AAF-2NA by EP 24.11 in kidney, lung, spleen and brain.

	SUBSTRATE			
	cFP-AAF-pAB		Glt-Ala-Ala-Phe-2NA	
TISSUE	Specific activity (U/mg protein)	Relative Activity	Specific activity (U/mg protein)	Relative Activity
Kidney	5.68	189	29.9	199
Lung	0.18	6.0	0.85	5.67
Spleen	0.04	1.33	0.28	1.87
Brain	0.03	1.0	0.15	1.0

Enzyme activities were measured in crude homogenates of kidney, lung, spleen and brain. The concentration of cFP-AAF-pAB and Glt-Ala-Ala-Phe-2NA in the incubation mixtures was 0.2 and 0.8 mM respectively. One unit is defined as the amount of enzyme which releases 1 mmol/product/hr. Relative activities are calculated in relation to the activity in brain arbitrarily set at 1.0. For details of experimental procedures see under Materials and Methods.

Figure 7. A-B: HPLC separation of the reaction products generated by incubation of cFP-AAF-pAB with purified EP 24.11. Panel A. Retention time of cFP-AAF-pAB. The 2 diastereomers of cFP-AAF-pAB are eluted with different retention times. Panel B. Products of complete degradation of cFP-AAF-pAB. The second product peak was identified as Phe-pAB while the first peak is presumed to be cFP-AA. See Materials and Methods for experimental details.


1.0
0.5
ABSORBANCE AT 214 nm
A
B
cFP-AAF-pAB
F-pAB
cFP-AA
0
10
20
0
10
20
TIME (MIN)

degradation product of the inhibitor was inhibitory toward EP 24.15. Small amounts of this product were therefore isolated by HPLC from reaction mixtures in which the inhibitor was completely degraded by incubation with thermolysin. After removal of the solvent and dissolving the isolated cFP-AA in 25 mM Tris-HCl (pH 8.0), its concentration was determined from the HPLC peak heights in a standard curve based on peak heights of known amounts of the same compound appearing in reaction mixtures in which the reaction went to completion. The inhibitory potency of equimolar concentrations (8 mM) of cFP-AA and cFP-AAF-pAB was then measured with purified EP 24.15 using Boc-Phe-Ala-Ala-Phe-pAB as the substrate. The results of this determination showed that cFP-AA was almost completely inactive as inhibitor (1.7 % inhibition), while cFP-AAF-pAB almost completely (97.5%) inhibited the enzyme at the same concentrations.

Several factors could have been responsible for the finding that inhibition of EP 24.15 protects LHRH from *in vivo* degradation. These factors include both the affinity and accessibility of the peptide to the enzyme, and also the magnitude of the activity of EP 24.15 as compared with that of EP 24.11. In order to determine the significance of this last factor we compared the activities of the two enzymes in rat tissue. EP 24.11 and EP 24.15 were determined with Glt-Ala-Ala-Phe-2NA (k_{cat} = 139 sec^{-1}; Pozsgay et al., 1986) and Boc-Phe-Ala-Ala-Phe-pAB (k_{cat} = 86 sec^{-1}; Orlowski et al., 1989) respectively. The results summarized in Table 6 show that with the exception of the kidney and lung EP 24.15 was much more active than EP 24.11 in all other tissues studied. It is important to note that with the exception of the filtered fraction, the unusually high EP 24.11 activity in the kidney would not be expected to contribute greatly to LHRH degradation since this enzyme is primarily localized in microvillar membranes of the brush border of the proximal tubules of the kidney (Kerr and

Kenny, 1974). Accordingly only the fraction of LHRH which enters the glomerular filtrate would be exposed to degradation by EP 24.11. The higher activity of EP 24.15 is even more notable if the turnover rate constants of the two above substrates (k_{cat} values; 139 versus 86 sec^{-1}, respectively) would be considered, since these values would rather tend to underestimate the EP 24. 15 activity.

TABLE 6: Activities of endopeptidase 24.15 and endopeptidase 24.11 in rat tissues.

TISSUE	ACTIVITY EP 24.15 (U/mg)	EP 24.11 (U/mg)
Testis	7.25	0.92
Spleen	1.18	0.258
Liver	0.303	0.005
Kidney	0.621	27.2
Lung	0.661	0.82
Heart	0.331	0.014
Skeletal muscle	0.721	0.165
Brain	0.869	0.148
Plasma (mmol/ml/hr)	0.295	0.014

Activity was determined in crude tissue homogenates obtained from Sprague Dawley rats weighing approximately 350 g, as described in Materials and Methods. Boc-Phe-Ala-Ala-Phe-pAB (0.4 mM) and Glt- Ala-Ala-Phe-2NA (0.8 mM) were used for determination of EP 24.15 and EP 24.11 activity. Data are mean values obtained from two determinations. One unit is defined as the amount of enzyme that degrades 1 µmol of substrate/hr.

Phase 3: Influence of CNS and peripheral EP 24.15-mediated LHRH degradation on LHRH's LH- and FSH-releasing activity

Intravenous administration of LHRH, LHRH agonists and enzyme inhibitors.

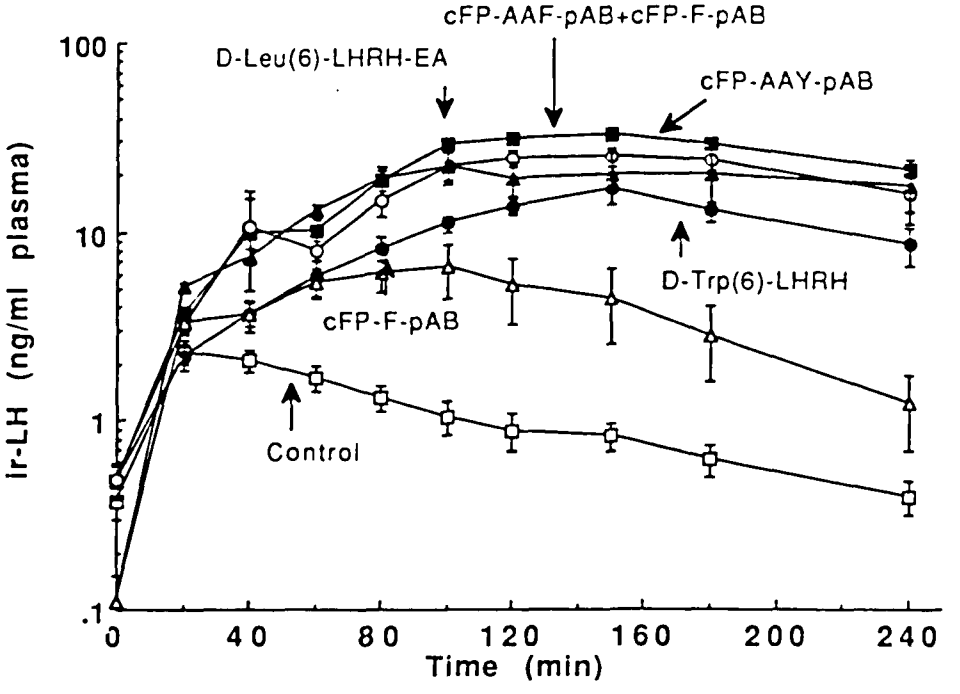
Following the intravenous administration of LHRH alone, plasma LH levels quickly rose to a peak value of 2.4 ng/ml after 20 minutes, and declined thereafter, reaching basal values by 4 hours (Figure 8). Administration of both superactive LHRH analogs, [D-Trp^6]-LH-RH and [D-Leu^6]-LHRH-EA, resulted in a much greater increase of plasma LH levels than after equivalent amounts of LHRH. Indeed, all plasma samples collected (at time beginning at 20 minutes) from animals receiving the analogs, LH concentrations were considerably higher than in plasma samples collected from control animals. Peak values were reached approximately 2 hours after administration of the agonists, and were somewhat higher for [D-Leu^6]-LHRH-EA than for [D-Trp^6]-LH-RH. Increased LH levels after the agonists persisted during duration of the experiment and showed only a small decline after 4 hours. Indeed, after four hours the concentration of LHRH in plasma of animals treated with these analogs was about one order of magnitude higher than in the controls.

As described above (*Results*, phase 2) the effectiveness of inhibitors of EP 24.15 in prolonging the half life of intravenously administered LHRH is limited by the susceptibility of these inhibitors to degradation by EP 24.11. For example cFP-AAF-pAB is degraded by EP 24.11 by cleavage of the Ala-Phe bond, and this reaction can be inhibited by cFP-F-pAB an inhibitor of EP 24.11. Consequently administration of inhibitors of both these enzymes dramatically prolonged the *in vivo* half life of LHRH. Consistent with this finding administration of cFP-AAF-pAB together with cFP-F-pAB, inhibitors of the two enzymes, in

conjunction with LHRH induced an increase in plasma LH concentrations that was nearly identical in both magnitude and duration to that after administration of [D-Leu^6]-LHRH-EA. Thus, plasma LH levels after the two inhibitors reached peak values after 2 hours, and the increased LH concentrations persisted with little change for the duration of the experiments. Also the peak values reached in response to LHRH and inhibitor treatment were similar to those after the superactive analog (30.9 ng/ml versus 32.8 ng/ml) and exceeded by 13-times values in the controls receiving only LHRH. To confirm that the response after the joint administration of inhibitors of the two enzymes can be mainly attributed to the protection of LHRH from EP 24.15-mediated degradation, another experimental group was included in which the EP 24.11 inhibitor was omitted and LHRH was given only in conjunction with cFP-AAY-pAB, an inhibitor of EP 24.15, that is more resistant to EP 24.11-catalyzed degradation than cFP-AAF-pAB. This resulted after 100 minutes in an average peak plasma LH concentration of 22.7 ng/ml, 7.5 times control peak values, that persisted virtually unchanged during duration of the experiments. Indeed, although, the average peak LH values were somewhat lower the pattern of the response to LHRH and cFP-AAY-pAB was similar in both duration and magnitude to the response following the treatment with inhibitors of the two enzymes. These results support the conclusion that the bulk of the LH response after administration of LHRH and equimolar amounts of inhibitors of EP 24.15 and EP 24.11 can be attributed to inhibition of LHRH degradation by EP 24.15 and that cFP-F-pAB, when given together with cFP-AAF-pAB, served mainly to protect the EP 24.15 inhibitor from degradation by EP 24.11. This conclusion is also supported by the finding that in rats treated only with LHRH and cFP-F-pAB an inhibitor of EP 24.11, peak LH values rose after 80 minutes to only 6.65 ng/ml plasma, 2.8 times those in controls and then declined steadily so that

after 4 hours plasma LH concentrations were significantly lower than those in animals receiving inhibitors of both enzymes, and those receiving only cFP-AAY-pAB. These data confirm our previous results (phase 2) which showed that EP 24.15 is the main enzyme responsible for the *in vivo* LHRH degradation.

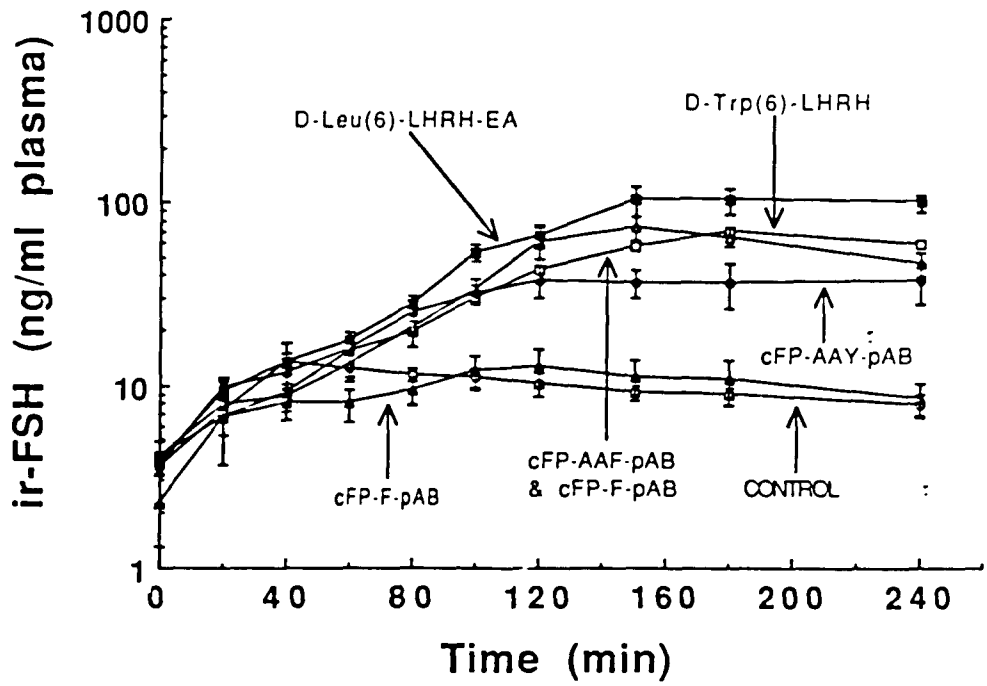
Figure 8: Plasma LH concentrations in response to IV administration of LHRH, LHRH analogs and LHRH together with enzyme inhibitors. Male rats (200-250 g) were treated with IV injections of either 1) LHRH (0.32 nmole); 2) LHRH and cFP-AAY-pAB (425 nmole); 3) LHRH and cFP-AAY-pAB (425 nmole); 4) LHRH and cFP-AAF-pAB (425 nmole) and cFP-F-pAB (425 nmole); 5) [D-Trp^6]-LHRH (0.32 nmole) and 6) D-Leu^6-LHRH-EA (0.32 nmole). Plasma LH concentrations were determined by radioimmunoassy at indicated time intervals as described in Materials and Methods. At 40 and 60 min all experimental groups had significantly ($p < 0.05$ to 0.01) higher levels of LH. At 20, 80, 100, 120, 150 minutes all groups except that treated with cFP-F-pAB had significantly ($p < 0.05$ to 0.01) higher LH levels. At 180 and 240 min groups treated with [D-Leu6]-LHRH EA and LHRH + cFP-AAF-pAB + cFP-F-pAB had significantly ($p < 0.05$ to 0.01) higher LH levels. The number of experiments in each group were : control (LHRH alone) = 7; LHRH + cFP-AAF-pAB + cFP-F-pAB = 7; LHRH + cFP-AAY-pAB = 4; LHRH + cFP-F-pAB = 4; [D-Trp6]-LHRH = 6; D-Leu^6-LHRH-EA = 5.


cFP-AAF-pAB+cFP-F-pAB
D-Leu(6)-LHRH-EA
cFP-AAY-pAB
D-Trp(6)-LHRH
cFP-F-pAB
Control
Ir-LH (ng/ml plasma)
100
10
1
.1
0
40
80
120
160
200
240
Time (min)

Plasma samples obtained from the same experimental groups of animals analyzed for LH were also analyzed for concentrations of FSH. Peak concentrations of FSH (13.24 ng/ml) after LHRH, appeared in this group after 40 minutes (Figure 9), however, the magnitude of the FSH response above baseline values was only about half of that for LH, although the increased levels of FSH persisted much longer. Thus, whereas LH levels had declined to basal values in the control group by the last sampling time (at 240 minutes following LHRH administration), FSH levels were still more than twice those at baseline. The administration of the superactive analogs, [D-Trp^6]-LHRH and [D-Leu^6]-LHRH-EA, produced an elevation of plasma FSH that was far greater in both duration and magnitude than that after LHRH. As with LH, [D-Leu^6]-LHRH-EA produced a greater elevation of plasma FSH than did [D-Trp^6]-LHRH. Administration of LHRH in conjunction with either cFP-AAF-pAB and cFP-F-pAB or with cFP-AAY-pAB alone caused a rise in plasma FSH concentrations that were similar in duration to those induced by treatment with the "superagonists". The pattern of the FSH response after joint administration of LHRH and the two inhibitors resembled most closely the response after [D-Trp^6]-LHRH. Plasma FSH levels 2.5 hours after administration of the two inhibitors rose to a peak of 73.4 ng/ml (5.5 times higher than in controls), a value very similar to that after [D-Trp6]-LHRH (70 ng/ml). In both these groups, the elevated FSH levels persisted throughout the duration of the experiment. A similar response although of somewhat lesser magnitude was seen after administration of LHRH together with cFP-AAY-pAB (a peak value of 37.37 ng/ml was reached at 120 minutes). On the other hand, the response to the co-administration of EP 24.11 inhibitor, cFP-F-pAB and LHRH did not differ from that after LHRH administration alone. The magnitude of the FSH response to treatment with inhibitors and LHRH as well as to treatment with both superagonists was generally lower than that seen with LH.

The analysis of the results obtained for plasma FSH lead to the same conclusion as those derived from measurements of LH levels. In both instances the results indicate that peripheral EP 24.15, but not EP 24.11 activity, plays a prominent

Figure 9: Plasma FSH concentrations in response to IV administration of LHRH, LHRH analogs and LHRH together with enzyme inhibitors. Conditions of the experiments were the same as those in Figure 1. At 80 minutes groups treated with LHRH + cFP-AAF-pAB + cFP-F-pAB, [D-Leu6]-LHRH EA and LHRH + cFP-AAY-pAB had significantly ($p < 0.05$ to 0.01) higher FSH levels than controls. At 100, 120, 180 and 240 minutes all groups except that treated with cFP-F-pAB had significantly ($p < 0.05$ to 0.01) higher FSH levels. At 150 minutes groups treated with LHRH + [D-Leu6]-LHRH EA, LHRH + [D-Trp6]-LHRH, and LHRH + cFP-AAF-pAB + cFP-F-pAB had significantly higher FSH levels than controls. The number of experiments in each group were : control (LHRH alone) = 6; LHRH + cFP-AAF-pAB + cFP-F-pAB = 7; LHRH + cFP-AAY-pAB = 4; LHRH + cFP-F-pAB = 4; [D-Trp6]-LHRH = 6; D-Leu^6-LHRH-EA = 5.


1000
100
10
1
ir-FSH (ng/ml plasma)
0
40
80
120
160
200
240
Time (min)
D-Leu(6)-LHRH-EA
D-Trp(6)-LHRH
cFP-AAY-pAB
cFP-AAF-pAB
& cFP-F-pAB
cFP-F-pAB
CONTROL

role in the metabolism of LHRH and also that after inhibition of EP 24.15 activity, natural LHRH becomes as effective in inducing increases of plasma FSH levels as superactive LHRH agonists having D-amino acid substitutions in the 6-th position.

Effect of intracerebroventricular administration of LHRH and enzyme inhibitors on plasma LH and FSH levels.

Intracerebroventricular administration of LHRH caused an increase in plasma LH concentrations that reached peak values after 90 minutes (4.02 ng/ml), and subsequently, steadily declined (Figure 10). Administration of LHRH together with both cFP-AAF-pAB and cFP-F-pAB or cFP-AAF-pAB alone resulted in in LH increases that at peak values were 7 to 13 times higher than in controls (27.3 ng and 50.4 ng/ml respectively).

In inhibitor treated animals the increased plasma LH concentrations persisted throughout the experimental period, without any appreciable decline. Administration of the EP 24.11 inhibitor, cFP-F-pAB together with the EP 24.15 inhibitor did not lead to any enhancement of plasma LH concentrations over those obtained after administration of cFP-AAF-pAB alone, again indicating that EP 24.15 is the predominant factor in the CNS determining LHRH metabolism, and that EP 24.11 contributes little if at all to the metabolism of this hormone in brain.

Analysis of plasma FSH concentrations in the same group of animals (Figure 11) showed that the increase and subsequent decline of plasma FSH concentrations after ICV LHRH administration was in control animals nearly identical to that of LH. As with the LH response, the magnitude and duration of the FSH response was greatly increased by administration of the EP 24.15 inhibitor together with LHRH, however was not further augmented by

inclusion of the EP 24.11 inhibitor. These results further confirm the primary importance of EP 24.15 in the metabolism of LHRH in the CNS, and point to a role of this enzyme in the modulation of the pituitary response of the pituitary to LHRH.

Figure 10: Plasma LH concentrations in response to ICV administration of LHRH, and LHRH together with enzyme inhibitors. Male rats (150 - 175 g) were treated with ICV injections of either 1) LHRH (0.5 nmole); 2) LHRH and cFP-AAF-pAB (1 μmole); 3) LHRH + cFP-AAF-pAB + cFP-F-pAB (1 μmole of each). Plasma LH concentrations were determined by radioimmunoassy at indicated time intervals as described in Materials and Methods. At all time points both experimental groups had significantly ($p < 0.05$ to 0.01) higher plasma LH levels then the controls. The number of experiments in each group were : control (LHRH alone) = 6; LHRH + cFP-AAF-pAB + cFP-F-pAB = 6; LHRH & cFP-AAF-pAB: n = 5.

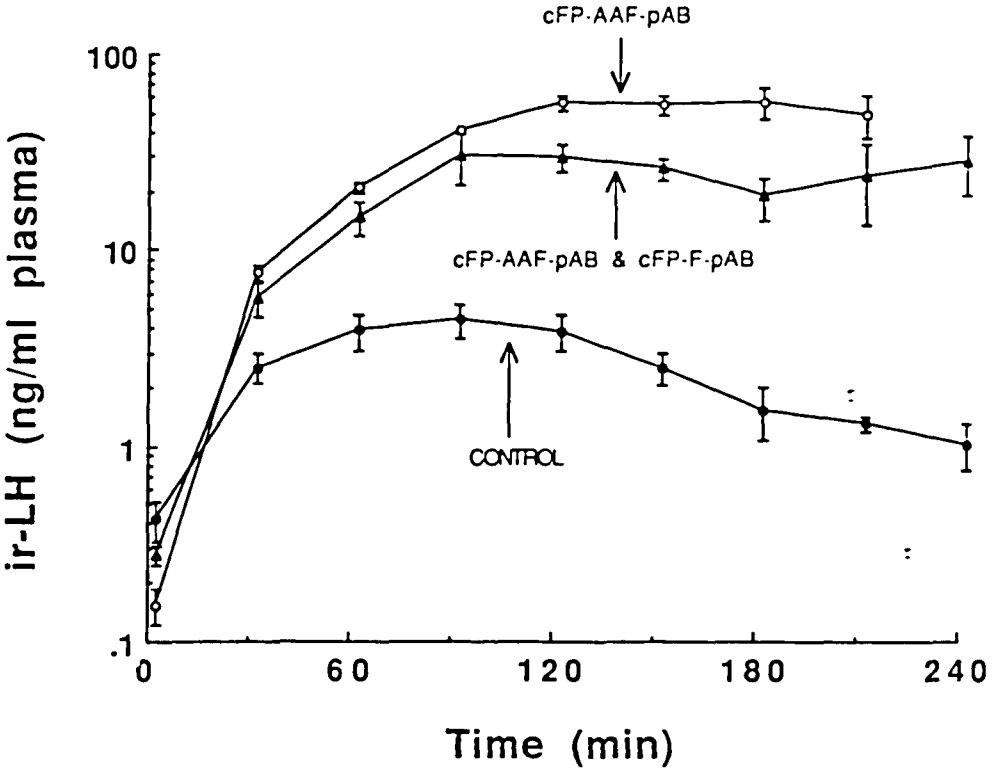
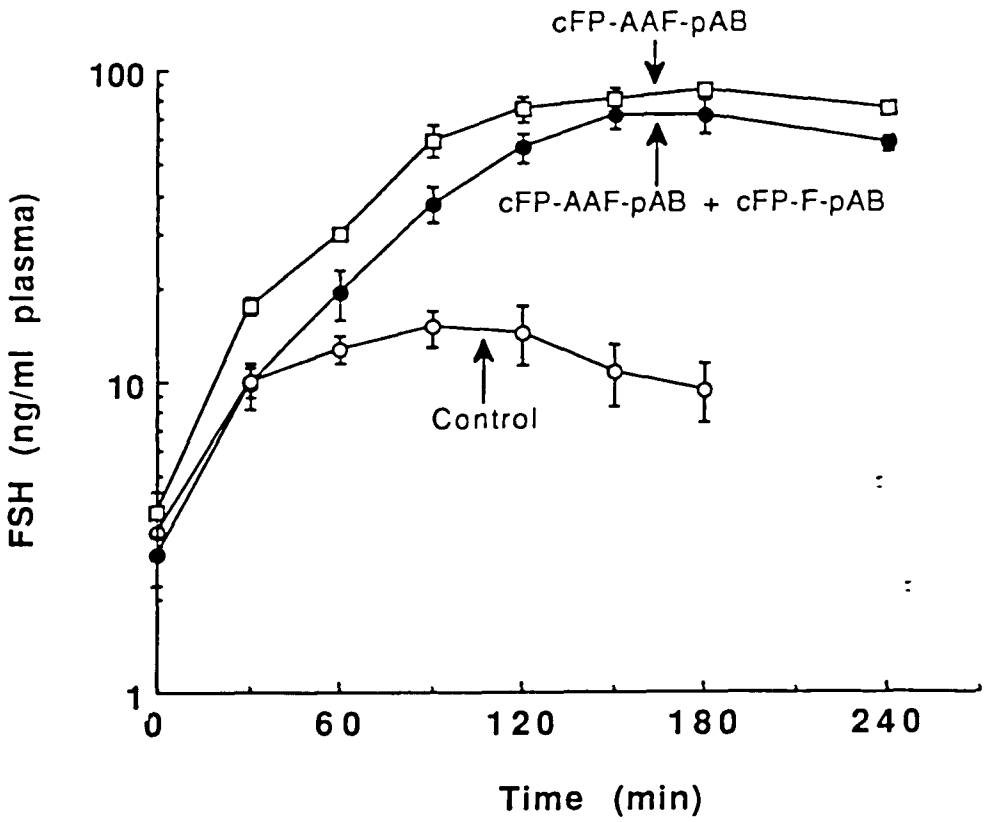

cFP-AAF-pAB
cFP-AAF-pAB & cFP-F-pAB
CONTROL
ir-LH (ng/ml plasma)
100
10
1
.1
0
60
120
180
240
Time (min)

Figure 11: Plasma FSH concentrations in response to ICV administration of LHRH, and LHRH together with enzyme inhibitors. The experimental conditions were the same as those given in Figure 3. At 30 min the group receiving LHRH and cFP-AAF-pAB had significantly ($p < 0.01$) higher plasma FSH levels then the controls. At 60, 90, 120 and 180 minutes both groups receiving LHRH and inhibitors had significantly ($p < 0.05$ to 0.01) higher plasma FSH levels then the controls. The number of experiments in each group were : control (LHRH alone) = 6; LHRH + cFP-AAF-pAB + cFP-F-pAB = 6; LHRH & cFP-AAF-pAB: n = 5.


cFP-AAF-pAB
cFP-AAF-pAB + cFP-F-pAB
Control
100
10
1
FSH (ng/ml plasma)
0
60
120
180
240
Time (min)

Phase 4: Influence of CNS EP 24.15 activity on endogenous LHRH-stimulated LH secretion.

Effect of ICV cFP-AAF-pAB infusion on plasma LH.

Our results show that plasma LH levels are increased following a 10-minute ICV infusion of EP-24.15-inhibitor in urethane-anesthetized rats. At one hour following infusion, LH levels rose to slightly more than twice basal values (table 7). At both 60 and 90 minutes following inhibitor infusion, plasma LH levels were significantly higher ($p < 0.05$) than preinfusion LH levels. By 120-150 minutes after inhibitor infusion, plasma LH levels returned to preinfusion levels. In contrast, in saline-treated controls, plasma LH levels did not rise during the entire sampling period (data not shown).

To obtain statistical significance for LH levels at 60 and 90 minutes (as compared to preinfusion values), it was necessary to infuse inhibitor into large number of animals, (i.e. 10 animals received inhibitor in order to increase the "n" value), since in a few of the inhibitor-treated animals, baseline plasma LH increased very slightly. In addition, when the ICV injection cannula was lowered 1 mm anterior or posterior to the site used in the experiments reported here, no increase in plasma LH was seen. These data, therefore, cannot definitively ascribe an importance of EP 24.15 in the regulation of LHRH and LH-secretion.

TABLE 7: Effect of intracerebroventricular administration of N-[1(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP-AAF-pAB) on the levels of ir-LH in rat plasma.

Time after injection (min)	Plasma luteinizing hormone concentrations (ng/ml)
0	0.339 ± 0.079 (10)
30	0.603 ± 0.077 (9)
60	0.824 ± 0.136 (10)*
90	0.587 ± 0.072 (10)*
120	0.461 ± 0.055 (8)
150	0.081 ± 0.081 (7)

10 ul of inhibitor (50 mM) was infused into the third ventricle through a metal cannula (0.01" O.D.) over 10 minutes (see *Materials and Methods*). Blood samples (0.4 ml) were withdrawn through an atrial catheter prior to and at 30 minute intervals after intraventricular administration of inhibitor. Plama LH concentration was determined by a double antibody radioimmunoassay procedure using the reagents provided by the National Hormone and Pituitary Program.

Data are mean values ± S.E.M. The number of rats is given in parenthesis.

*Statistically significant, compared to preinfusion plasma LH levels, ($p < 0.05$) by one way analysis of variance followed by Scheffe's multiple group comparison test.

Phase 5: Influence of CNS EP 24.15 activity on recovery of endogenous LHRH released from hypothalamic slices.

Effect of EP 24.15-inhibition on the recovery of released LHRH.

The effect of EP 24.15 inhibitors on the recovery of endogenous LHRH released by depolarization of brain slices was evaluated in this study. This model has the advantage of maintaining functional tissue organization. Most importantly, the topographic relationship between neuronal stores of LHRH and their potential synaptic inactivation systems, which exists *in vivo*, is mimicked in this *in vitro* model (Schwartz et al., 1985). Because of the difficulties experienced in demonstrating an role of EP 24.15 in degrading endogenous LHRH *in vivo* (phase 4), we therefore utilized this *in vitro* model to determine whether membrane-bound EP 24.15 associated with hypothalamic slices may inactivate endogenous LHRH following KCl-evoked release. The recovery of endogenous LHRH was therefore measured both in the presence and absence of EP 24.15 inhibitors.

The results show that LHRH release from hypothalamic slices was increased in response to 60 mM KCl stimulation. However, in both control samples and samples with inhibitor, the ratio of stimulated release over basal release was nearly identical. The concentration of LHRH in both inhibitor-containing (high KCl) medium and in control (high KCl) medium was approximately 5 times the concentration in the "basal" media (Table 8). Therefore, it is apparent that in the experimental used here that membrane-bound EP 24.15 does not degrade the LHRH released from the hypothalamus during the 15 min incubation period.

TABLE 8: Effect of EP 24.15 inhibition on recovery of LHRH after KCl-stimulated release from hypothalamic slices.

EXPERIMENTAL CONDITION	BASAL RELEASE (pM LHRH)	STIMULATED RELEASE (pM LHRH)	RATIO OF STIMULATED: BASAL
CONTROL	31.89 ± 3.17 (9)*	171.59 ± 23.90	5.37 ± 0.51
& INHIBITOR	31.66 ± 3.28 (8)*	170.14 ± 19.02	5.54 ± 0.55

LHRH concentration was measured in media which bathed hypothalamic slices during "basal release" and KCl-stimulated release" periods. In all samples, during "basal release" period, no inhibitors were present in the media. KCl-stimulated release of LHRH was measured in either inhibitor-free media (controls), or EP 24.15 inhibitor-containing media (50 µM of cFP-AAY-pAB). LHRH released during the sixth 15 min incubation period was termed "basal release of LHRH". After this, the tissue-containing syringe was transferred into a vial containing EBSS with 60 mM KCl and incubated for 15 min. LHRH released in this vial was termed "stimulated release of LHRH". (Data are mean ± sem). Concentration of LHRH in media was measured by RIA.

* number in parenthesis represents the number of hypothalami per experimental group.

Effect of substrate concentration upon EP 24.15-mediated LHRH degradation

Our inability to demonstrate a role of EP 24.15 in degrading endogenous LHRH following release from brain slices may be explained in light of the low concentration of LHRH in the media bathing the brain slices (i.e. average concentration: < 0.5 nM, (table 8)). The obvious discrepancy, therefore, between data obtained from these experiments and phase 1 experiments where EP 24.15 mediated LHRH degradation by hypothalamic membranes was demonstrated, may be due to the considerably higher LHRH concentration present in the incubation mixtures in phase 1 experiments (0.1 mM). We, therefore, wished to determine if there exists a relationship between the concentration of LHRH in the media and the rate of EP 24.15-mediated LHRH degradation. 3 concentrations of exogenously added LHRH were incubated with brain slices. Data reported in table 9 shows that the rate of EP 24.15-mediated LHRH degradation in brain slice preparations is a concentration-dependent phenomenon. When the starting LHRH concentration is 100 μM, degradation occurs approximately 60 times faster than when the starting LHRH concentration was 1.0 μM and approximately 36,000 times faster than in samples with starting LHRH concentration of 10 nM.

TABLE 9: Substrate-concentration dependence upon EP 24.15-mediated LHRH degradation by hypothalamic slices *in vitro*.

STARTING LHRH CONCENTRATION	RATE OF EP 24.15-MEDIATED LHRH DEGRADATION (in pmol LHRH/mg protein/hr)
100 µM	1620
1.0 µM	27.12
10 nM	0.044

3 concentrations of LHRH, 10 nM, 1 µM and 100 µM, were incubated with hypothalamic slices in the presence and absence of 50 µM EP 24.15 inhibitor, cFP-AAY-pAB, as described in *Materials and Methods*, phase 5. The concentration of LHRH remaining in media was measured by RIA.

The rate of total LHRH degradation, expressed as "pmol LHRH/mg protein/hr" was calculated by measuring the no. pmoles LHRH dissappearing over the 1st 30-min incubation period.
The rate of EP 24.15-mediated LHRH degradation, expressed as "pmol LHRH/mg protein/hr", was calculated by subtracting the rate of LHRH in inhibitor-containing incubation from that in control incubation.

DISCUSSION

LHRH, by virtue of the presence of an N-terminal pyroglutamate residue and a C-terminal amide bond, is resistant to degradation by most exopeptidases such as amino- and carboxypeptidases. The enzymatic degradation of LHRH must therefore be governed by endopeptidases, a class of enzymes capable of hydrolyzing bonds inside a peptide chain. The possible involvement of enzymes in the regulation and termination of action of LHRH has attracted considerable attention in view of the physiological and pharmacological interest in this peptide. The degradation of LHRH by soluble and particulate fractions prepared from rat hypothalamus and pituitary and peripheral organs has been examined by several groups. On the basis of these studies, cleavage of bonds at several sites in the LHRH molecule by a number of peptidases present in these crude enzyme preparations was considered to be of potential importance in the degradation of this peptide, although most of the responsible enzymes were found in cytosolic fractions. In early studies where LHRH was incubated with a 100,000g supernatant of hypothalamus (Koch et al., 1974) or anterior pituitary (Hazum et al., 1981), the major product identified was the hexapeptide pGlu-His-Trp-Ser-Tyr-Gly, indicating that LHRH itself or an N-terminal fragment of LHRH was cleaved at the Gly^{6}-Leu^{7} bond. After incubation of LHRH with particulate fractions of pituitary, Elkabes (1981) found 2 major product peaks, $LHRH^{1-6}$ and $LHRH^{1-3}$ and a minor peak of $LHRH^{1-4}$. McDermott (1982), on the other hand, found that after incubating LHRH with either soluble and particulate fractions of hypothalamus and pituitary, the main peak appearing during early stages of degradation was $LHRH^{1-5}$, while at later stages, the $LHRH^{1-3}$ peak increased concomitantly with a decline of $LHRH^{1-5}$. This led to the conclusion that the important primary cleavage site of LHRH is the Tyr^{5}-Gly^{6} bond and that the product of this reaction,

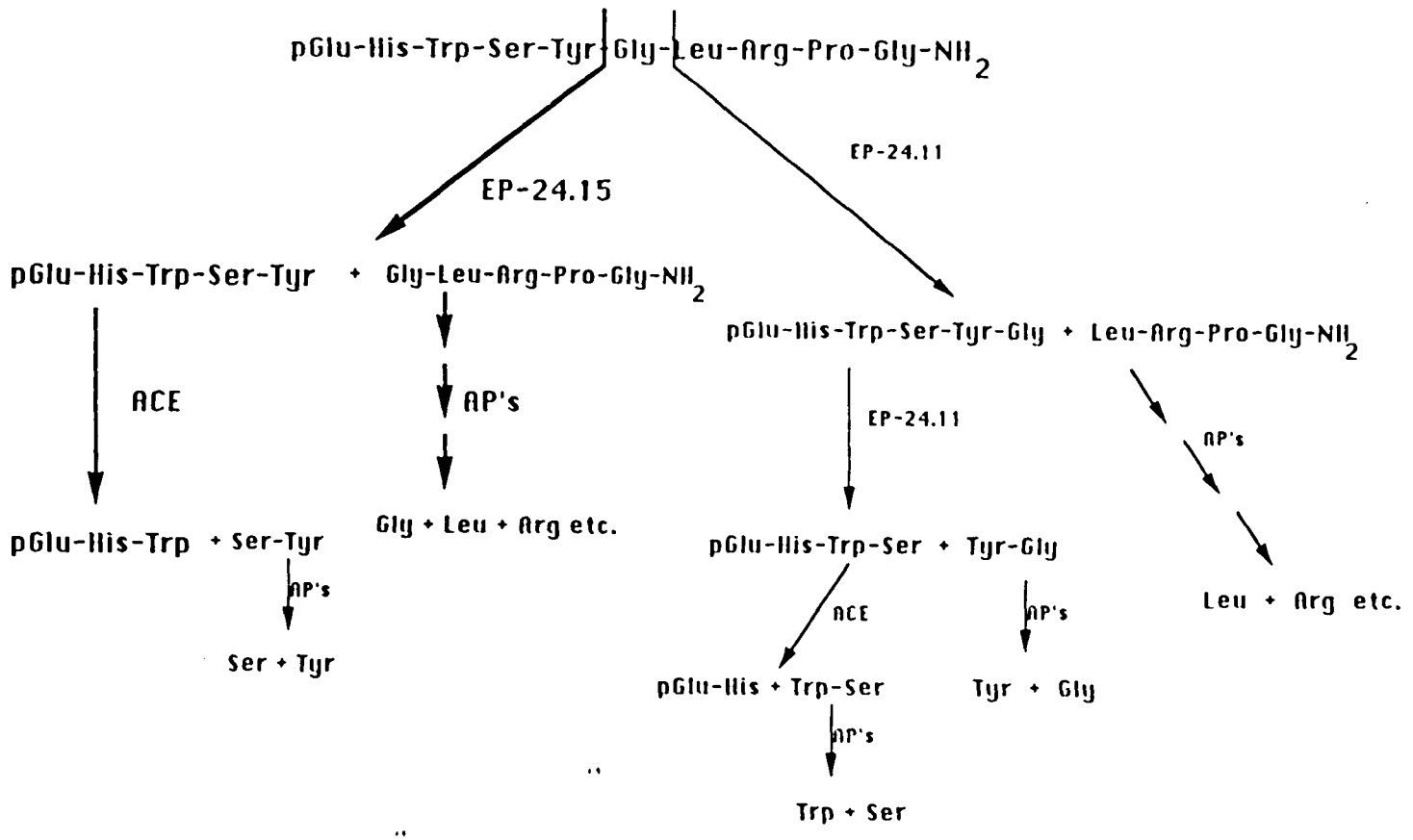
$LHRH^{1-5}$, is further degraded in a subsequent reaction, to $LHRH^{1-3}$. The identical pattern (of product formation versus incubation time) was repeated when LHRH was incubated with membranes prepared from mouse neuroblastoma cells and in crude homogenates of rat brain (Yokosawa et al., 1987; Carone et al., 1987). Thus, these later studies indicate that the Tyr^5-Gly^6 bond is the primary cleavage site of LHRH, catalyzed by a poorly identified enzyme in brain and pituitary membranes, while cleavage at the Trp^3-Ser^4 bond in a secondary reaction is dependent on the initial formation of $LHRH^{1-5}$ by this primary LHRH-cleaving peptidase. It was the goal of our studies (in phase 1) to identify the activity(ies) in both the hypothalamus and anterior pituitary membrane preparations, responsible for primary cleavage of LHRH through the use of active site-directed inhibitors.

It was recently shown in this laboratory that a synaptosomal fraction of rat brain contains a membrane-bound form of EP 24.15 that cleaves the opioid peptides dynorphin A^{1-8}, α-neo-endorphin, β-neo-endorphin and Met-enkephalin-Arg^6-Gly^7-Leu^8 at the 5-6 position to yield the corresponding pentapeptide enkephalins (Acker, et al., 1987). Incubation of rat brain synaptosomal membranes with these peptides in the presence of an inhibitor of EP 24.15 blocked the appearance of the enkephalins. Work presented here strongly indicates that in a particulate fraction prepared from hypothalamic, pituitary and AtT20 cells as well as in intact AtT20 cells, EP 24.15 also functions in the degradation of LHRH by catalyzing the initial hydrolysis of the Tyr^5-Gly^6 bond. EP 24.11, which is also present in these cells, responsible for the formation of the N-terminal hexapeptide by cleaving LHRH at the Gly^6-Leu^7 bond, appears to contribute less to LHRH degradation. A potent, active site directed inhibitor of EP 24.15 recently developed in this laboratory (Orlowski et al., 1988) with a K_i of 27

nM, partially or completely blocked the degradation of LHRH by all of the above preparations. While the rate of LHRH degradation was not significantly affected by the addition of ACE inhibitor, captopril, the appearance of the pGlu-His-Trp fragment was blocked and the appearance of pGlu-His-Trp-Ser-Tyr was greatly enhanced. These data further confirm that the primary degradation of LHRH is catalyzed mainly by EP 24.15 through cleavage at the Tyr^5-Gly^6 bond. Only after liberation of the C-terminal pentapeptide, does the remaining (blocked) N-terminal peptide, pGlu-His-Trp-Ser-Tyr become a substrate for ACE, which forms the tripeptide, pGlu-His-Trp by liberating the C-terminal dipeptide. The EP 24.15 inhibitor blocked the formation of not only $LHRH^{1-5}$, but also $LHRH^{1-3}$ as well, further demonstrating the dependence of the ACE-mediated reactions on the prior EP 24.15-mediated Tyr^5-Gly^6 cleavage, which is the rate-determining reaction. This sequence of LHRH-DA based on the data presented here is schematically presented in Figure 12. LHRH is first degraded mainly at the Tyr^5-Gly^6 bond by a membrane-bound form of EP 24.15, generating a blocked N-terminal pentapeptide which is subsequently cleaved by ACE to yield the tripeptide pGlu-His-Trp. Thus, although the tripeptide, pGlu-His-Trp, rather than pGlu-His-Trp-Ser-Tyr, has been reported as the major end-product by several groups who studied LHRH degradation by crude enzyme preparations from brain and pituitary (Elkabes et al., 1981; Yokosawa et al., 1987), we believe that the scheme postulated here readily reconciles this apparent discrepancy. In both the pituitary and AtT20 preparations, we also have found that pGlu-His-Trp is either the only or primary detectable product in the absence of inhibitors. Only upon the addition of captopril, does the $LHRH^{1-5}$ metabolite tend to accumulate. Thus, the $LHRH^{1-5}$ metabolite appears to be rapidly and nearly completely further metabolized to $LHRH^{1-3}$; this explains the failure of earlier groups to detect the

$LHRH^{1-5}$ metabolite (in significant concentrations) when LHRH was incubated in the absence of inhibitors of ACE. When LHRH is incubated with membranes prepared from mouse neuroblastoma cells, the formation of $LHRH^{1-3}$ and $LHRH^{4-5}$ was observed (Yokosawa et al., 1987). Inclusion of the ACE inhibitor, captopril, causes an increase in the appearance of $LHRH^{1-5}$ and a decrease in $LHRH^{1-3}$ and $LHRH^{4-5}$, suggesting that LHRH is first cleaved at the Tyr^5-Gly^6 bond, and subsequently hydrolyzed at Trp^3-Ser^4 by ACE to yield the di- and tripeptides. The enzyme which cleaved at the Tyr^5-Gly^6 bond was not identified, but these authors considered the likelihood that it was a thiol protease because formation of the $LHRH^{1-5}$ was inhibited by a high concentration (1.0 mM) of p-chloromercuribenzoate. EP 24.15, which is present in high concentration in neuroblastoma cells (Acker and Orlowski, unpublished observations) and is inhibited 33% by 0.2 mM pCMB (Orlowski et al., 1983), was quite possibly the enzyme responsible for the activity seen by these authors. In the present study we have shown that captopril blocks the formation of $LHRH^{1-3}$ by membrane preparations from hypothalamus, pituitary and AtT20 cells as well as in intact AtT20 cells, indicating that LHRH degradation in these preparations proceeds in a similar manner as in neuroblastoma cells. While we have not detected $LHRH^{6-10}$ fragment, the expected metabolite of EP 24.15-mediated LHRH hydrolysis, the amidated C-terminal peptides generated by any cleavage of LHRH are no longer blocked at the N-terminus, and are therefore degraded very rapidly to free amino acids by aminopeptidases, which abound in these tissues.

Figure 12: Proposed scheme for the breakdown of LHRH by hypothalamic and pituitary membranes. Bold arrows are used to indicate the important pathways in LHRH degradation, and large type is used to show the predominant breakdown products seen in the absence of inhibitors. EP = endopeptidase, AP = aminopeptidase, ACE = angiotensin converting enzyme.


pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
EP-24.15
EP-24.11
pGlu-His-Trp-Ser-Tyr + Gly-Leu-Arg-Pro-Gly-NH2
pGlu-His-Trp-Ser-Tyr-Gly + Leu-Arg-Pro-Gly-NH2
ACE
AP's
AP's
EP-24.11
AP's
Gly + Leu + Arg etc.
pGlu-His-Trp + Ser-Tyr
pGlu-His-Trp-Ser + Tyr-Gly
AP's
ACE
AP's
Leu + Arg etc.
Ser + Tyr
pGlu-His + Trp-Ser
Tyr + Gly
AP's
Trp + Ser

When LHRH is incubated with purified EP 24.11 preparations, the peptide is cleaved at the Gly^6-Leu^7 bond. However, this reaction proceeds rather slowly. With longer incubations, EP 24.11 catalyzes cleavage of the Ser^4-Tyr^5 bond (in the $LHRH^{1-6}$ metabolite) into pGlu-His-Trp-Ser and Tyr-Gly. The slow degradation of $LHRH^{1-10}$ (2 to 5% after 1 hour) by purified EP 24.11 would explain the rather poor protection of LHRH provided by EP 24.11 inhibitor, cFE-F-pAB, when incubated with pituitary membranes. pGlu-His-Trp-Ser was also seen when pituitary membranes were incubated with LHRH in the presence of captopril, suggesting that ACE is not involved in formation of the tetrapeptide. The formation of pGlu-His-Trp-Ser can be attributed to cleavage of the Ser-Tyr bond by EP 24.11 acting on the N-terminal hexapeptide of the hormone, as seen with long incubations of purified EP 24.11 preparations with LHRH. Carone (1987) also reported a low recovery of $LHRH^{1-4}$ as compared to the recovery of $LHRH^{1-5}$ following incubation of LHRH with crude homogenates of brain and pituitary; their data are apparently in agreement with our work showing that EP 24.15-mediated hydrolysis of the Tyr^5-Gly^6 bond serves as the primary reaction in LHRH degradation. These data, showing that inclusion of inhibitors of EP 24.15 and not EP 24.11, either completely or at least in large part, inhibit LHRH degradation, are in agreement with data reported by Edwardson and McDermott (1985) showing that inhibitors of EP 24.11, aminopeptidase or prolyl endopeptidase were unable to retard hydrolysis of LHRH by synaptosomes, thus implicating still another enzyme in this process. Perhaps EP 24.15 is that heretofore unidentified primary LHRH-degrading enzyme.

Skidgel and Erdös (1985) have reported that ACE from human kidney purified after solubilization with trypsin, catalyzed an unusual hydrolysis of the Trp^3-Ser^4 and Leu^7-Arg^8 bonds in LHRH with pGlu-His-Trp being the major degradation product. That these bonds were indeed hydrolyzed by ACE was

confirmed by demonstrating that captopril inhibited these reactions. It is therefore of interest that in our experiments no such reactions were observed with brain, pituitary or AtT20 cell membrane preparations, since inhibition of EP 24.15 was sufficient to block the formation of pGlu-His-Trp. The significance of these new ACE-catalyzed reactions in membrane preparations remains to be demonstrated.

Marks and Stern (1974) studying LHRH degradation by crude brain extracts, reported the presence of 2 activities responsible for primary cleavage of LHRH: 1) internal cleavage (at either the Tyr^5-Gly^6 or Gly^6-Leu^7 bond); and 2) a slower C-terminal cleaving enzyme removing the glycineamide from the C-terminus. Evidence for 2 separate primary cleaving activities was provided by studies using structurally modified LHRH analogs. A D-Xaa^6 substitution had a strong inhibitory effect on the internal cleavage activity as demonstrated by fact that internal amino acids were liberated from native LHRH but not from D-Xaa^6-analogs. On the other hand, substitution of the C-terminal glycineamide (such as in [D-Ala^6,desGly^{10}]-LHRH ethylamide) had a lesser, but distinct inhibitory effect which was demonstrated by the fact that the C-terminal ethylamide and adjacent proline was not released from these analogs. Substitutions at both sites provided the most complete inhibition of LHRH hydrolysis In a similar vein, Horsthemke (1981) demonstrated degradation of LHRH by purified preparations of both:

1) a soluble, nonchymotrypsin neutral "LHRH-degrading endopeptidase" which hydrolyzes the internal Tyr^5-Gly^6 bond. This enzyme is inactive towards analogs with a D-amino acid substitution in position 6; and
2) a soluble, post-proline cleaving enzyme which hydrolyses the Pro^9-Gly^{10} bond. This enzyme is less active towards analogs with ethylamide

substitutions in position 10. This enzyme is identical to prolyl endopeptidase described by Wilk (1979).

We, on the other hand, while also finding major Tyr^5-Gly^6-cleaving activity (by EP 24.15), have found little evidence of any important degrading activity at the Pro^9-Gly^{10} bond in any of the *in vitro* preparations used here. Only upon inhibition of the primary LHRH degrading enzyme, EP 24.15, can the appearance of $LHRH^{1-9}$ be detected (when LHRH was incubated with AtT20 cells). Since prolyl endopeptidase is a cytoplasmic enzyme, its lack of activity towards LHRH in our membrane preparations is readily understood. In fact, Acker et al. (1987) found that only traces of prolyl endopeptidase activity are associated with purified synaptosomal membranes. In a similar vein, Edwardson and McDermott (1985) reported that an anticatalytic antibody to prolyl endopeptidase did not inhibit the degradation of exogenous LHRH by synaptic membranes. While an ethylamide substitution in position 10 is reported to increase the potency of the LHRH molecule, increased potency is not likely provided by any resistance to prolyl endopeptidase since a soluble peptidase is unlikely to be instrumental in the inactivation and clearance of a systemically administered peptide. Some reports have attributed the ethylamide molecule's potency to an increased binding affinity for the LHRH receptor (Perrin et al., 1980).

On the other hand, the increased potency afforded by D-Xaa^6 substitutions may well be attributed to their resistance to membrane endopeptidases cleaving internal peptide bond(s). In the present study we have shown that D-Xaa^6-analogs remained virtually immune to hydrolysis by EP 24.15 as well as by EP 24.11 (table 2). Since both of these enzymes possess membrane-associated LHRH-DA, and are present in high concentrations in periphery (table 6), one or both enzymes may well be instrumental in

terminating the biological activity of circulating LHRH. Accordingly, resistance to degradation by one (or both) of these 2 enzymes might be the main factor in the increased biological activity of D-Xaa^6-analogs. These LHRH analogs evoke an increased pituitary response, i.e. both the magnitude and duration of gonadotropin secretion is dramatically greater than the response to native LHRH (Wass et al. 1979, Soria et al., 1975). The increased activity of these analogs is believed to be due either to diminished rate of degradation and longer half life in the circulation or to greater affinity to LHRH receptors (Barron et al., 1982; Wass et al., 1979, Perrin et al., 1980; Clayton and Catt, 1980). Numerous studies have compared the stability of natural LHRH *in vitro* and *in vivo* with that of the superactive analogs (Barron et al., 1984, Swift and Crighton, 1979). Extensive LHRH degradation has been shown to occur in peripheral tissue preparations (in addition to brain and pituitary preparations) *in vitro* and also in the circulation of live animals (Bienert et al., 1983; Carone et al, 1987; Berger et al., 1987). In contrast, LHRH analogs display resistance to tissue peptidases and also have increased stability in the circulation *in vivo* as demonstrated by their decreased clearance rates and increased plasma half-life (Marks and Stern, 1974; Koch and Baram, 1977; Barron et al., 1982; Chu et al., 1985). These studies as well as others that show a direct correlation between the order of potency among LHRH analogs and extent of resistance to proteolysis (Sandow et al., 1979; Swift AD and Crighton DB, 1979), have strongly implicated the resistance to degradation as the major, if not only factor in their "superactive properties". Since it is the D-Xaa^6-substitution which confers the increased *in vitro* and *in vivo* stability to the LHRH molecule, the biologically important hydrolyzing activity responsible for degradation of native LHRH must occur either at the Tyr^5-Gly^6 or Gly^6-Leu^7 bonds, the sites of EP 24.15 and EP 24.11 activity, respectively (Conn et al., 1984). However, it can not be

deciphered from those studies which of these 2 bonds is the primary site of hydrolysis. Accordingly, the relative importance of EP 24.11 versus EP 24.15 activity in the degradation of LHRH, could not be determined by merely comparing the stability of LHRH versus LHRH analogs. Rather, the use of inhibitors of both enzymes in models for studying LHRH degradation *in vivo* provides the one definitive means of deciphering the relative importance of these 2 enzymes in this process. By employing these inhibitors in our studies, we demonstrated the importance of EP 24.15 in the inactivation of LHRH by hypothalamic and pituitary membranes, and therefore, we were given reason to believe that it is primarily the resistance to this enzyme which affords to these analogs their increased potency. As a corollary, the activity of this enzyme would then be assumed to curtail the biological activity of native LHRH, i.e. EP 24.15 may have a major role in regulating the LH- and FSH-releasing activity of LHRH. Further studies using inhibitors of both EP 24.11 and EP 24.15 in a biological model for demonstrating LHRH's gonadotropin-releasing activity, were necessary to identify the endopeptidase responsible for modulating LHRH's biological activity.

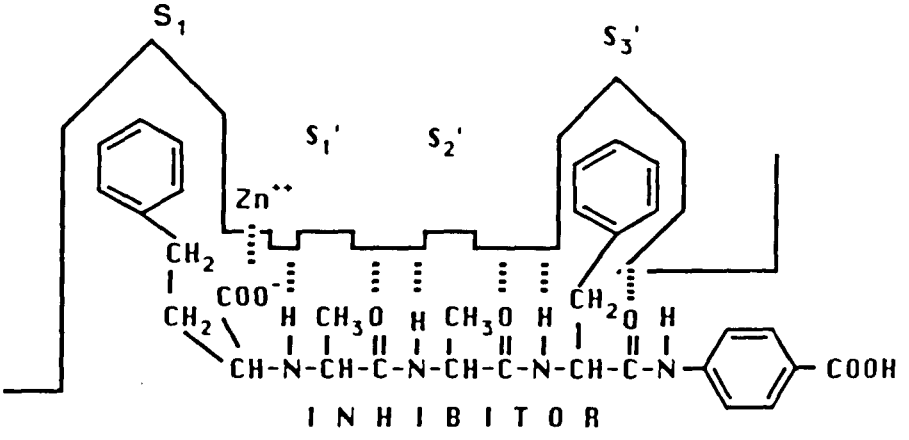
Previous studies in our laboratory have shown that the primary specificity of the enzyme was shown to be directed toward peptides having a hydrophobic amino acid residue in the P_1 and P_2 positions. Secondary enzyme-substrate interactions, however, at sites removed from the immediate vicinity of the scissile bond were also shown to greatly influence the catalytic process (Orlowski et al., 1983). Prominent among those interactions was the enhancing influence of a hydrophobic or bulky residue in the P_3' position on the turnover rate constant. This pattern of specificity was shown to be valid for both synthetic and natural peptides. Consequently, it was not surprising that both the soluble and membrane-bound forms of the enzyme were shown to

hydrolyze the Tyr^5-Gly^6 bond in LHRH (Orlowski, et al., 1983; Molineaux et al., 1988). The synthesis of potent active site-directed inhibitors of the enzyme (Chu and Orlowski, 1984; Orlowski, et al., 1988) provided the necessary tools for the evaluation of the role of this enzyme in the *in vivo* degradation of LHRH. A scheme of interaction of LHRH and the inhibitor, used in the present study, with the active site of EP 24.15 is shown in Figure 13. The scheme underscores the importance of the P_1 and P_3' position for inhibitor and substrate binding. The degradation of LHRH at the Tyr^5-Gly^6 bond by EP 24.15 indicates that Ser^4 and Tyr^5 must bind to the S_2 and S_1 subsites of the enzyme, respectively, with the Arg^8 residue being accommodated at the S_3' subsite. The K_m of 95 mM differs substantially from the K_i value for LHRH previously obtained (811 μM), when the peptide was used as a competing substrate (Chu and Orlowski, 1985). This K_i should be equal to the K_m when the inhibition is purely competitive, and the inhibitor is being used as a competing substrate. The discrepancy found between experiments in which LHRH was used directly as substrate or as a competitive inhibitor suggests that interaction of LHRH with EP 24.15 is more complex than that of a simple substrate or competitive inhibitor (Segal, 1975). Activation of enzyme activity toward synthetic substrates by certain small peptides such as the carboxyl protease inhibitor pepstatin have recently been shown to occur *in vitro* (Orlowski and Michaud, unpublished observations), suggesting that there may be a regulatory site on the enzyme separate from the active site. If LHRH also interacts with such a site, then activation might have masked the inhibition of synthetic substrate cleavage by lower concentrations of the peptide. These questions need further investigation. The measurement of the affinity of LHRH for the membrane-bound form of EP 24.15 must await studies with the purified form of the enzyme. The availability of similar active site directed inhibitors of EP 24.11 (Almenoff and Orlowski 1983) and of ACE

(Cushman et al., 1980) provided the necessary tools for the exploration of the relative significance of LHRH degradation both *in vitro* and *in vivo*.

Figure 13: Scheme of interaction of cFP-AAF-pAB and of LHRH with the active site of endopeptidase 24.15. The Phe and Arg residues of the inhibitor and LHRH respectively are shown to interact with the S_3' subsite of the substrate recognition site of the enzyme. The phenylpropyl group of the inhibitor and the Tyr residue of LHRH bind to the S_1 subsite, while the carboxyl group of the inhibitor and the carbonyl moiety of the substrate coordinate with the active site zinc atom. The arrow indicates the scissile bond in LHRH.

ENDOPEPTIDASE-24.15



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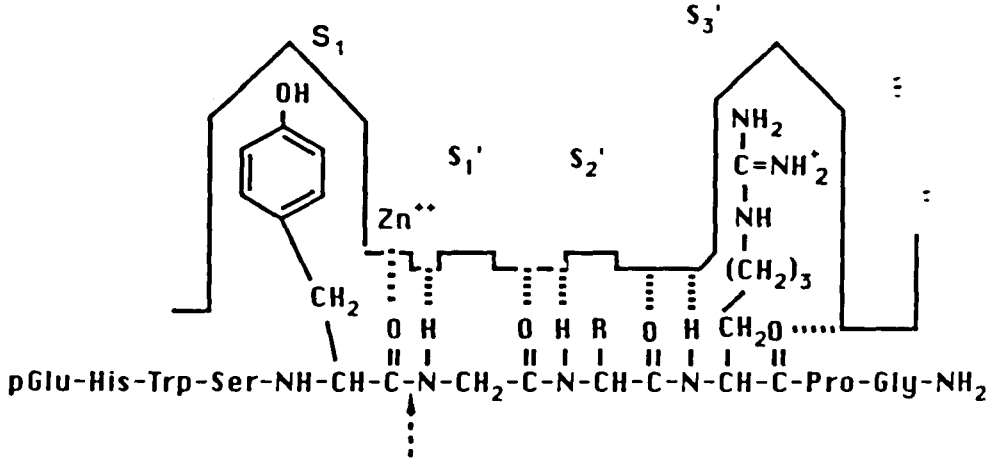

LHRH

The experiments performed in phase 1 with hypothalamic and pituitary membrane preparations demonstrated the use of active site-directed inhibitors of EP 24.15, EP 24.11 and ACE in determining which peptidases are instrumental in LHRH degradation *in vitro*. Since brain membranes possess these 3 activities, the possibility exists that one or more of these enzymes may be active in CNS extracellular metabolism of LHRH *in vivo*. To test the relative importance of these enzymes in this process, we chose as an experimental model in phase 2, the ICV administration of LHRH in the presence and absence of inhibitors of these enzymes. Metabolism of LHRH was monitored by measuring (by RIA) the concentrations of LHRH remaining in brain homogenates at 1 hour following LHRH infusion.

In experiments with ICV administration of LHRH, there was a distinct order of effectiveness among the three inhibitors with regard to enhancement of LHRH recovery (figure 5). Only the EP 24.15 inhibitor gave a statistically significant increase in LHRH recovery. cFP-F-pAB and captopril, on the other hand, did not significantly prevent LHRH degradation in brain. Thus, in the CNS, the order of importance of the 3 enzymes in *in vivo* LHRH metabolism follows the order seen *in vitro*, with EP 24.15 responsible for the greatest proportion of LHRH degradation. EP 24.11 appears to play a role, albeit a lesser one, since the administration of EP 24.11 inhibitor results in a higher recovery of LHRH than that seen in control animals, but still a far lower recovery than that afforded by the use of the EP 24.15 inhibitor. Furthermore, the finding that LHRH recovery from brain increased only very slightly after administration of both EP 24. 15 and EP 24.11 inhibitors (more than the increase afforded by EP 24.15 alone) (table 3), further supported the conclusion that EP 24.11 plays only a minor role in the *in vivo* LHRH degradation. Since captopril affords the least protection of LHRH, it is concluded that ACE is the least important of the 3

enzymes in the CNS metabolism of LHRH *in vivo*. These data are also consistent with those obtained in the *in vitro* experiments with hypothalamic membrane fractions. While the maximal recovery of LHRH from brain in the presence of the EP 24. 15 inhibitor 1 hour after ICV administration was only 20% of the administered dose, the amount recovered must have been affected not only by enzymatic degradation but also by redistribution of the peptide in the cerebrospinal fluid and its exit into the general circulation. Consequently, even upon total inhibition of LHRH-DA, one would not be expected to recover more than a small fraction of the amount infused.

In view of the pharmacological importance of LHRH and its analogs in the treatment of a variety of conditions it was important to identify the enzymes that control LHRH degradation in peripheral tissues. Several groups have observed the degradation of LHRH upon incubation with homogenates of several organs including liver, lung, kidney, gonads and even blood (Bienert et al., 1983; Carone et al., 1987; McDermott et al., 1981). Systemically administered LHRH has been shown to have short half-lives and rapid clearance rates *in vivo* (Chu et al., 1985; Berger et al., 1987; Barron et al., 1982). Moreover, the duration of action of native LHRH as measured by the length of the LH response, is quite short (Coy et al., 1974; Wass et al, 1979). Furthermore, several LHRH metabolites have been detected in the blood and urine of live rats injected with radiolabeled LHRH (Stettler-Stevenson, et al., 1983; Carone et al., 1987), lending further credence to the conclusion that rapid and extensive peripheral LHRH metabolism curtails the pharmacological actions of parenterally administered native LHRH. The susceptibility of LHRH to peptidase activities in peripheral organs may well be a major factor in both the short half life and duration of action of LHRH (Karten and Rivier, 1986). This notion is further supported by the increased half-life as well as duration and magnitude of action of the

superactive LHRH agonists. As indicated above, we had strong reason to believe that the major factor increased *in vivo* survival of these analogs is their resistance to EP 24.15 activity. Accordingly, inhibiting the activity of this endopeptidase by *in vivo* administration of inhibitors should allow native LHRH to mimic the superagonists with regard to their survival time (half-life) and possibly also, biological potency. As in the CNS, the presence of ACE, EP 24.11 and EP 24.15 in peripheral organs has been established. Conceivably any of these activities may hydrolyze and thus be responsible for the short half-life of LHRH in the periphery. We have therefore studied the influence of inhibitors of these enzymes on the plasma half life of intravenously administered LHRH. Among the three inhibitors used in this study, cFP-AAF-pAB caused the largest increment in LHRH half-life, whereas cFP-F-pAB was less effective and captopril had almost no effect (table 4). Thus, the order of effectiveness of the three inhibitors was similar to that found in brain, and therefore the order of importance of the 3 enzymes in peripheral LHRH degradation is identical to that found in the CNS.

Unexpectedly, however, while ICV co-administration of cFP-AAF-pAB and cFP-F-pAB did not significantly increase the LHRH recovery from brain over that seen with the EP-24.15 inhibitor alone, concurrent IV administration of the two inhibitors dramatically increased the half life and concentrations of LHRH in plasma over those seen with either inhibitor given alone. The magnitude of the increase after co-administration of the two inhibitors eliminated the possibility that the increase resulted from a simple additive effect of the two inhibitors. Examination of the structure of cFP-AAF-pAB indicated that this inhibitor may be susceptible to degradation by EP 24.11 and therefore, after intravenous administration of the EP 24.15 inhibitor, it may be inactivated by the large presence of EP 24.11 in peripheral organs. Accordingly, concomitant

administration of cFP-F-pAB, an inhibitor of EP 24.11, would be expected to protect cFP-AAF-pAB from degradation *in vivo*. cFP-AAY-pAB, administered under these conditions, could be expected to provide a far longer and more complete *in vivo* inhibition of EP 24.15 and thus, a more complete protection of LHRH from EP 24.15-mediated degradation than when administered in the absence of EP 24.11 inhibitor. Both EP 24.11 and bacterial thermolysin, having similar specificities, cleave peptide bonds on the amino side of hydrophobic amino acid residues, (Orlowski and Wilk, 1981; Almenoff and Orlowski, 1983 and 1984; Pozsgay et al. 1985 and 1986). It could therefore be anticipated that both enzymes would cleave the Ala-Phe bond in cFP-AAF-pAB. Indeed, experiments reported here showed that purified EP 24.11 and thermolysin degrade cFP-AAF-pAB to F-pAB and cFP-AA according to the following reaction:

$$\text{cFP-Ala-Ala-Phe-pAB} \xrightarrow{\text{EP-24.11 or thermolysin}} \text{cFP-Ala-Ala} + \text{Phe-pAB}$$

This finding together with the demonstration that plasma cFP-AAF-pAB levels after 80 min were more than ten-fold higher in animals treated IV with both inhibitors than in animals treated with cFP-AAF-pAB alone, provided strong evidence for the conclusion that the increased effect seen after both inhibitors could be attributed to inhibition of degradation of the EP 24.15 inhibitor and thereby, the increased protection of LHRH. Further support for this conclusion was provided by the finding that free urinary pAB (a measure of *in vivo* metabolic degradation of cFP-AAF-pAB) was greatly lowered in animals given both inhibitors, and that cFP-AA, a product of cFP-AAF-pAB degradation was ineffective as an EP-24.15 inhibitor. That cFP-AAF-pAB is

degraded by EP 24.11 in peripheral tissues is indeed verified by the finding that the tissue distribution of the cFP-AAF-pAB-degrading activity corresponds to the tissue distribution of this enzyme (table 5).

Our measurements of EP 24.11 activity (tables 5 & 6) showed that the enzyme is about 200 times more active in the kidney than in brain and that most other peripheral tissues also exhibit higher EP 24.11 activities than the brain. As would be expected, the rate of cFP-AAF-pAB degradation by EP 24.11 in different tissues closely followed the rate of degradation of Glt-Ala-Ala-Phe-2NA, an EP 24.11 substrate. The difference in EP 24.11 activity between peripheral organs and brain readily explains the failure of the EP 24.11-inhibitor to substantially increase LHRH recovery after concurrent ICV injections of cFP-AAF-pAB and cFP-F-pAB. Apparently, unlike in peripheral tissues, the low brain EP 24.11 activity is not a significant factor contributing to the degradation of cFP-AAF-pAB.

Collectively, our data strongly indicate that like in brain, EP-24.15 is the primary enzyme responsible for LHRH degradation in peripheral tissues, although EP 24.11 can contribute to this process (Flouret et al., 1987; Stettler-Stevenson et al., 1983). Measurement of EP 24.15 activity in numerous peripheral organs using a synthetic substrate, Boc-Phe-Ala-Ala-Phe-pAB, suggests several anatomical loci for the peripheral degradation of LHRH by EP 24.15. We have found high activity of this enzyme in organs such as spleen, testes, liver, lung and skeletal muscle (table 6). In view of the total mass of these tissues, the observed large contribution of EP 24.15 activity in the periphery towards LHRH degradation can be more readily understood. It is of interest that gonadal tissue, known to have high EP 24.15 activity (Chu and Orlowski, 1985) was also found to have considerable LHRH-DA (Carone et al., 1987), and that the primary degradation product was found to be pGlu-His-Trp-Ser-Tyr ($LHRH^{1-5}$),

a finding consistent with EP 24.15 involvement. Thus the protection of systemically administered LHRH by EP 24.15 inhibition is easily explained. On the other hand, administration of EP 24.11 inhibitor (in the absence of EP 24.15 inhibitor), while it afforded some protection of plasma LHRH, did not significantly prolong the peptide's plasma half-life. Quantitatively, therefore, this enzyme would appear to play a lesser role than EP 24.15 in the inactivation and clearance of systemically administered LHRH. The relative importance of EP 24.15 versus EP 24.11 in LHRH degradation in peripheral tissues is notable in view of the high EP 24.11 activities in such tissues as kidney and lung. A possible explanation is provided by the finding that among 20 different natural peptides one of the slowest rates of degradation by EP 24.11 was observed for LHRH (Matsas et al., 1984). Also, the K_m of LHRH for EP 24.11 (755 μM) was found to be some eight times higher than for EP 24.15 (95 μM; Molineaux et al., 1988). Other factors, however, such as enzyme localization and accessibility of the substrate to the enzyme must also be considered. The unusually high EP 24.11 in the kidney is primarily concentrated in the microvillar brush border membranes of the proximal tubules (Kerr and Kenny, 1974). This localization would tend to limit the action of the enzyme only on that fraction of LHRH that enters the glomerular filtrate. Thus kidney EP 24.11 activity would not likely be of major importance in the inactivation of circulating LHRH since the fraction entering the renal interstitium would not be expected to re-enter the circulation even in the absence of proteolytic degradation. The higher EP 24.15 activity found in most other tissues (with the exception of the lung) may therefore be another contributing factor for the dominant role of this enzyme in LHRH degradation. It is of interest that after intravenously injecting tritiated LHRH into rats, Redding and Schally (1973) observed that the radioactivity was concentrated in the liver and kidney, a finding interpreted to indicate that

these 2 organs are major sites for the inactivation and excretion of LHRH. A comparison of the activities of EP 24.15 and EP 24.11 in crude homogenates of the liver clearly indicates the predominance of EP 24.15 activity, although the relative EP 24.15 activity in this organ compared to other organs such as the testes, brain and spleen is still somewhat low (table 6). However, when one takes the mass of the liver into consideration, the contribution of EP 24.15 activity in this organ to the degradation of circulating LHRH may conceivably be considerable. Inhibition of ACE, on the other hand, provides almost no prolongation of LHRH's half-life and therefore this enzyme is assumed to play the least important role among the 3 peptidases in peripheral LHRH metabolism.

The finding that EP 24.15 is the primary factor that determines the *in vivo* metabolism of LHRH has both theoretical and practical implications. Enzymatic degradation of LHRH has been proposed to be a factor in regulating the amount of LHRH reaching the pituitary gonadotropes and determining thereby the magnitude and duration of the secretory response of the pituitary to this peptide. Advis et al (1982b) examined LHRH-DA in hypothalamic soluble fractions prepared from female rats during the first estrous cycle at puberty. They found that the rate of LHRH degradation decreased on the afternoon of the first proestrus, when the level of LHRH within the hypothalamus is increased, at a time immediately prior to the rise in plasma LH. Furthermore, in ovariectomized rats treated with estrogen and progesterone to induce a proestrous-like LH surge, the same researchers observed a decline in LHRH-DA immediately prior to the LH surge (Advis et al., 1983). These studies point to a functional importance of LHRH degradation with regard to the regulation of pituitary gonadotropin secretion and, especially, the preovulatory LH surge seen on the evening of proestrus, although the enzymes likely to be involved in this process have not been identified. Such regulatory mechanism would

require the presence of LHRH-DA at the a) site of release: where LHRH-DA may serve to reduce the amount of intact LHRH reaching the portal circulation; or at the b) target organ: where LHRH-DA may serve to terminate LHRH's action in a mode analogous to acetylcholinesterase at the neuromuscular junction. Since both the hypothalamus and anterior pituitary are rich in EP 24.15, it is quite possible that one or both of these modes of regulatory enzymatic activity may be physiological functions of this enzyme. A first step in investigating this possibility is to evaluate the role of EP 24.15 in regulating the gonadotropin-releasing activity of exogenous LHRH. In light of the earlier studies, both *in vivo* and *in vitro*, showing the preeminence of EP 24.15 in the CNS metabolism of LHRH extracellularly, we had reason to believe that the CNS EP 24.15 metabolism of LHRH may be instrumental in this process. To test this possibility, in one of the experiments in phase 3 of this project, we monitored the pituitary response, i.e. the magnitude and duration of LH and FSH secretion, following ICV infusion of LHRH in the presence and absence of EP 24.15 inhibitors.

Another aspect associated with our findings is that in view of the therapeutic potential of LHRH, knowledge of the mechanisms of LHRH degradation acquires in addition to its physiological significance, also pharmacological importance. The therapeutic use of LHRH is severely limited by its rapid (extracellular) degradation in peripheral tissues and the very short half life of this hormone in peripheral circulation (8 to 10 min) (Barron et al., 1982; Chu et al., 1985). The notion that rapid and extensive peripheral LHRH metabolism is responsible for its rapid clearance and short half-life is fully supported by our work in which the inhibition of peripheral peptidases, and in particular, EP 24.15, prolongs the half-life of natural LHRH to that of the D-Xaa^{6}-analogs (30 to 150 min; Karten and Rivier, 1986). As mentioned above, this substitution confers resistance to tissue peptidase activity and, in particular, to

EP 24.15 and EP 24.11 activity. Furthermore, while no proof has been forthcoming, it is this resistance, in addition to the increased binding potency of these analogs, which has been thought to be the cause of their increased biological potency (Barron et al., 1982; Clayton and Catt, 1980). The reason for the inability of researchers to definitively determine the cause of these analogs' superactivity is because the following question had never been answered: "Does LHRH-DA, *in vivo*, significantly curtail LHRH's biological (gonadotropin-releasing) activity?" Our previous work showing the role of EP 24.15 in LHRH degradation in crude (membrane) enzyme preparations *in vitro* and in the clearance of LHRH from the CNS and periphery *in vivo*, gave us considerable reason to believe that EP 24.15 activity would curtail the pharmacological actions of LHRH, following either ICV or IV administration. The availability of enzyme inhibitors gave us the means to probe these issues. Plasma LH and FSH levels were measured following ICV and IV administration of LHRH in the presence and absence of EP 24.15 (and also EP 24.11) inhibitors. Because we had a direct means to study the involvement of LHRH-DA in modulating LHRH's biological actions, we were in an ideal situation to also evaluate the cause of the superactive properties of the LHRH analogs. If the increased biological potency of these analogs result from their resistance to LHRH-DA, then responses similar to those seen after administration of these analogs would be expected after natural LHRH administration, if the biologically important LHRH-DA was completely inhibited, i.e. by concurrent administration of inhibitors of the biologically important enzymes. By first determining the biologically important LHRH-degrading enzyme, i.e. the enzyme responsible for curtailing LHRH's biological action, we could then compare the LH and FSH-releasing activity of natural LHRH after inhibition of

this enzyme with that of the D-Xaa^6-analogs. In this way, the contribution of resistance to LHRH-DA to the analog's biological properties could be evaluated.

Thus, this became the basis for the design of one of the experiments in the next phase of the thesis project in which the LH- and FSH-releasing activity of IV-administered native LHRH in the presence and absence of several peptidase inhibitors was compared with that of 2 LHRH superagonists, [D-Trp^6]-LHRH and [D-Leu^6,des Gly-NH$_2$^{10}]-LHRH EA. To fully evaluate the biological activity of these peptides, we monitored both the magnitude as well as duration of both the LH and FSH response.

The results presented here, in phase 3, (figures 10-13) show that the LH- and FSH-releasing activity of either IV- or ICV-administered LHRH is dramatically augmented by inhibition of EP-24.15 activity. Thus, both the duration and amplitude of the LH and FSH secretory response to either IV or ICV administered LHRH was increased by EP 24.15 inhibitors many times above values seen in control animals. This substantiates the belief that both CNS and peripheral EP 24.15-mediated hydrolysis of LHRH plays a major role in modulating its gonadotropin-releasing activity. This activity in the CNS is more likely to have physiological importance in that it may catalyze the extracellular metabolism of secreted endogenous LHRH. On the other hand, peripheral EP 24.15 activity is more likely to be important pharmacologically in that it dramatically influences both the pharmacokinetics and pharmacodynamics of systemically administered LHRH.

Furthermore, our data (figures 10 & 11) show that the magnitude and duration of both the LH and FSH response to natural LHRH given IV in conjunction with inhibitors of EP 24.15 & EP 24.11, was comparable to those seen after IV administration of equivalent amounts of 2 LHRH D-Xaa^6-analogs. However, since the dramatically increased response to native LHRH was seen

after joint administration of both EP 24.15 & EP 24.11 inhibitors, a direct quantitative evaluation of EP 24.15's role in regulating the gonadotropin-releasing activity of LHRH, could not be made.

In response to this problem, in phase 2 of this study, we proved that the increased plasma half-life of LHRH following joint administration of inhibitors of the two enzymes can be mainly attributed to the protection of LHRH from EP 24.15-mediated degradation, while cFP-F-pAB, an inhibitor of EP 24.11, when given together with cFP-AAF-pAB, served mainly to protect the EP 24.15 inhibitor from degradation by EP 24.11. As a logical extension, the increased gonadotropin-releasing activity exhibited by LHRH jointly administered with both inhibitors can be almost entirely directly be attributed to the protection of LHRH from EP 24.15-mediated hydrolysis. This conclusion is further supported by the finding that cFP-AAY-pAB, another EP 24.15 inhibitor which is more resistant to EP-24.11-degradation than cFP-AAF-pAB, is nearly as effective in augmenting the response to LHRH as the joint administration of both EP 24.15 and EP 24.11 inhibitors. Furthermore, when cFP-F-pAB alone was co-administered with LHRH, the LH and FSH response resembled more closely the response seen in controls (treated with no inhibitors) than the response to either natural LHRH & EP 24.15 inhibitor or LHRH analogs. These data support our hypothesis that EP 24.15 activity $^{1)}$is the main activity responsible for the *in vivo* peripheral LHRH degradation, and $^{2)}$curtails the gonadotropin-releasing activity of LHRH. Therefore, since the response to natural LHRH given in conjunction with inhibitors of EP 24.15 & EP 24.11, or EP 24.15 inhibitors alone, was comparable to the response to the LHRH analogs, we can strongly argue that the biological potency of the D-Xaa^6-modified superactive analogs can be attributed, in large part, to their resistance to EP 24.15-mediated hydrolysis of the Tyr^5-Gly^6 bond.

Experimental data presented in the literature which support the notion that the decreased susceptibility to enzymatic degradation contributes to the biological potency of LHRH superagonists has been reviewed above. The increased secretory response to superactive analogs of LHRH might be also derived from their higher affinity to pituitary LHRH receptors. The degree, however, of the contribution to the increased potency of these analogs by their higher affinity to LHRH receptor remains nevertheless to be fully evaluated (Clayton and Catt, 1980; Clayton et al., 1979; Perrin et al., 1980). That the ratio of the biological potency of the superagonists to that of LHRH is many times higher than the ratio of their binding affinities argues for the involvement of factors beyond the binding affinities in the ultimate biological effectiveness of these analogs (Clayton and Catt, 1980; Wagner et al., 1979). Our findings showing that administration of LHRH together with inhibitors of EP 24.15 elicits a biological response similar in magnitude and duration to that obtained after superactive LHRH analogs, strongly argues for the view that the *in vivo* resistance to enzymatic degradation, and in particular, EP 24.15-mediated degradation, is the primary if not the only factor responsible for the increased biological activity of these analogs. Indeed, data have been presented which suggest that the increased affinity of the analogs might result, in large part, from their resistance to degradation by membrane peptidases present in the incubation mixtures used in binding assays (Clayton and Catt, 1980).

In both intravenous and intracerebroventricular experiments, the amplitude of the LH response was higher than that of the FSH response, in all experimental groups (control, inhibitor-treated and "superagonist"-treated). This observation is consistent with earlier work demonstrating that a higher threshold of stimulation (by exogenous LHRH) is required for FSH release than for LH release (Ondo et al., 1973; Arimura et al., 1972). Also, a single

(submaximal) dose of LHRH elicits a secretion of LH higher in magnitude than the secretion of FSH (Ondo et al., 1973; Rennels et al., 1971). The finding that the duration of the FSH response was longer than that of LH in the control group may be an expression of a slower metabolic clearance rate of FSH than of LH, a condition likely to be related to the uniqueness of the beta chains of the two hormones. (Emmanouel et al.,1984; Kamberi et al., 1971).

Following the characterization and isolation of LHRH in the early 1970's, it was anticipated that synthetic LHRH and its analogs would prove to be valuable in the treatment of certain forms of female infertility. Superactive agonists acutely increase gonadotropin secretion; however, after the initial stimulatory phase, lasting 1 to 2 weeks, a chronic state of decreased pituitary responsiveness ensues, probably as a result of down-regulation of LHRH receptors and desensitization of gonadotropes (Conn et al., 1984; Fraser, 1984; Filicori and Flamingo, 1988). Accordingly, long term administration of LHRH agonists reduce LH and FSH secretion and gonadotropin-dependent gonadal function. As a result, profound suppression of plasma testosterone and estradiol secretion occurs. These pharmacological properties, however, can be utilized therapeutically to manage disorders that are closely linked to gonadal steroid secretion, where suppression of gonadal function would be advantageous. These include female contraception, precocious puberty, endometriosis, hormonally-dependent malignancies such as prostatic and breast cancer, uterine leiomyomas, and the polycystic ovarian syndrome (Filicori and Flamingo, 1988; Marshall et al., 1988; Vickery, 1986). In patients with prostatic or breast cancer, suppression of gonadotropin and gonadal steroid secretion by LHRH agonist treatment has led to prostatic and breast tumor regression (Santen et al., 1984; Harvey et al., 1984; Fabrie et al., 1986). Endometrial atrophy has also occurred in patients with endometriosis (Filicori and Flamingo, 1988). In children with

precocious puberty, analog administration decreased growth velocity and particularly, skeletal maturation, to normal prepubertal rates. In addition, this therapy precipitated a regression of secondary sexual development (Boepple et al., 1986; Comite et al., 1984). Since the therapeutic value of LHRH agonists is based, in part, to their resistance to degradation, the therapeutic potential of inhibitors of LHRH degrading enzymes deserves consideration. *In vivo* inhibition of EP 24.15 both prolongs the half-life of LHRH and increases the biological response to LHRH to that seen with certain LHRH agonists. The possibility therefore exists, that the administration of native LHRH, in conjunction with EP 24.15 inhibitors, could potentially substitute for the the pharmacological efficacy of the superactive analogs.

As mentioned in the *Introduction*, a wide spectrum of reproductive disorders exist in both men and women which have as their common denominator, a hypothalamic deficit, which results in apulsatile LHRH secretion. Consequently, the currently accepted mode for treating both women with "hypothalamic amenorrhea" and men with idiopathic hypogonadotropic hypogonadism is pulsatile LHRH therapy (Marshall et al., 1988; Fraser, 1984). Since the biological activity of centrally administered LHRH is highly amplified by inhibition of CNS EP 24.15 activity, a therapeutic role of CNS-accessible EP 24.15 inhibitors may perhaps be considered in situations where endogenous LHRH secretion is suppressed but not completely absent. The synthesis of a broader spectrum of EP 24.15 inhibitors and detailed studies on the effect of EP 24.15 inhibition on the secretion and gonadotropin-releasing activity of endogenous LHRH will be needed to evaluate these possibilities.

As an extension of the work in phases 2 and 3 where the metabolism and LH-releasing activity of ICV-administered LHRH was shown to be determined by CNS EP 24.15, it was our goal to determine whether this enzyme may degrade endogenous LHRH following its secretion from the hypothalamus. This LHRH-DA would thereby decrease the concentration of intact LHRH reaching the portal circulation and eventually, at the gonadotropes. Thus, indirectly, EP 24.15-mediated LHRH-DA may be instrumental in determining the level of pituitary LH secretion. If this were the case, then inhibition of CNS EP 24.15 activity would precipitate a rise in basal LH secretion. We, therefore measured plasma LH concentrations prior to and following the ICV administration of EP 24.15 inhibitor, cFP-AAF-pAB. Our results show that baseline plasma LH values were only mildly raised in these experiments (table 7). Furthermore, this rise was very transient. In addition, it was disconcerting that, in order to obtain statistical significance, it was necessary to utilize many animals since in a few of the animals receiving inhibitor, baseline plasma LH increased very slightly. Furthermore, the veracity of our results can be questioned in light of the fact that no increase in plasma LH was seen when the inhibitor was infused into an anatomical site only slightly removed from the cranial coordinates which were used in studies reported here. Therefore, since these data are far from convincing, we cannot as of yet, ascribe a major contribution by EP 24.15 to the regulation of endogenous LHRH-stimulated LH-secretion. However, we believe that we can attribute the somewhat disappointing results to the fact that the animals were under the effects of general anesthesia and surgical stress, both of which are known to dramatically suppress LHRH and LH secretion (Ching, 1982a, 1982b; Euker et al., 1975; Stoebel and Moberg, 1982). Perhaps, some of these problems can be bypassed by changing the experimental protocol and administering the inhibitor to freely moving rats with chronic cannulae

implanted several days prior to experimental procedure. The absence of the inhibitory influences of anesthesia and surgical stress on LHRH release should provide for a higher baseline of LHRH and LH secretion which may enable one to show more convincingly, that inhibition of EP-24.15 gives rise to increased LH secretion. In addition, measurement of LHRH in portal plasma following inhibitor administration would still provide a more direct assessment of the effect of peptidase activity upon the concentration of bioactive hypothalamic peptides reaching portal plasma. These approaches will allow one to determine whether LHRH metabolism is a primary regulatory mechanism in the regulation of *in vivo* LHRH and LH secretion.

In vitro brain slice-release studies have been employed in studying catabolism of several peptides in brain extracellular fluid. Extracellular metabolism of substance P by rat substantia nigra slices is believed to be catalyzed by EP 24.11 and calpain since recovery of endogenous substance P following KCl-evoked release from substantia nigra slices, was increased by inhibitors of these 2 enzymes (Littlewood, et al., 1988; Mauborgne et al., 1987). Similar paradigms have been used to demonstrate the involvement of the membrane-bound pyroglutamyl peptidase II in the degradation of thyrotropin releasing hormone (Charli et al., 1989) and EP 24.11 in the degradation of [Met^5]enkephalin (Patey et al., 1981) following evoked release from brain slices. In all of these experiments, media assayed for measurement of "stimulated release" were allowed to incubate with tissue slices for 4-10 minutes. Thus, in this relatively short period of time, significant degradation of the released peptide took place as evidenced by the fact that, in the presence of selective peptidase inhibitors, significantly higher quantities of the peptide were recovered. In our experimental design, samples containing "stimulated release of LHRH" were incubated with tissue for 15 minutes. Based on our data,

during this period of time, no EP 24.15-mediated degradation of released LHRH occurred as indicated by the fact that the presence of EP 24.15 inhibitor, cFP-AAY-pAB, did not increase recovery of LHRH (table 8). Our failure to demonstrate degradation of LHRH by EP 24.15, in light of the substance P degradation by EP 24.11 in brain slices, is difficult to explain. One possible difference is that the specificity constant (k_{cat}/K_m) of EP 24.11 for substance P, 2.64×10^6 $S^{-1} \cdot M^{-1}$, (Littlewood, et al., 1988) is some 20 times higher than the specificity constant of EP 24.15 for LHRH (1.26×10^5 $S^{-1} \cdot M^{-1}$) (Orlowski et al., 1989). While the degradation of endogenous LHRH released from medial basal hypothalami blocks was demonstrated by Powers and Johnson (1981) as evidenced by the fact that 1 mM bacitracin increased recovery from the bathing medium, an incubation period of 4 hours was necessary to show a significant difference in LHRH recovery between bacitracin-containing and control samples. In addition, these same workers observed significant leakage of intracellular enzymes into the bathing medium during the 4 hour incubation period. Most of the LHRH degrading enzymes in the brain are subcellularly localized in soluble fractions (Parker et al., 1979). Therefore, the LHRH degradation seen by this group is probably predominantly catalyzed by soluble enzymes which escaped into the medium rather than by membrane peptidases, and thus, cannot be taken as a demonstration of true extracellular peptide metabolism.

Our data measuring exogenous LHRH metabolism by brain slices, demonstrate a substrate concentration dependent effect upon EP 24.15-mediated LHRH degradation, with the rate drastically falling off below the starting LHRH concentration of 1.0 μM (table 9). The K_m of EP 24.15 for LHRH has been shown above to be 95 μM (figure 1). Therefore, at 10 nM, some 9500 times lower than the K_m, the rate of degradation would be expected to be negligible, and, indeed,

data obtained in this experiment, substantiated this prediction. Since the average concentration of endogenous LHRH in the "KCl-evoked release" samples is below 0.5 nM (table 8), it becomes obvious why, during the short 15 min incubation period, no EP 24.15-mediated degradation of endogenous LHRH was seen in the brain slice experiments. Thus, an explanation is readily provided for the discrepancy between our failure to observe EP 24.15 mediated LHRH degradation following KCl-evoked release and our observation (in phase 1) that EP 24.15 activity in hypothalamic membrane preparations degrades LHRH significantly. The 0.1 mM LHRH concentration used in phase 1 is some 200,000 times greater than the 0.5 nM concentration found in phase 5; thus the rate of LHRH degradation is negligible in the latter case, while in the former case, the rate is quite rapid.

On the other hand, while the concentration of endogenous LHRH in the bathing medium is quite low, the local LHRH concentration in the interstitial spaces of the hypothalamic slices (following LHRH release from the nerve terminals) may be many times higher, a phenomenon that may also occur *in vivo* following LHRH release. If this were so, one would expect to observe a measurable degradation of LHRH by EP 24.15 as the higher local (substrate) concentration would increase the rate of the reaction. The failure of EP 24.15 inhibitor to increase the recovery of endogenous LHRH, must then be explained in an alternate manner. Perhaps some time (i.e. a few minutes) was needed for the inhibitor molecules (present in the bathing medium) to equilibrate with the enzyme molecules on the surface of the brain slices, and until such equilibration was attained, the enzyme was largely uninhibited. Therefore, during this time period, a significant portion of the LHRH molecules released (by the high KCl in the medium) was degraded by the uninhibited enzyme despite the presence of inhibitor in the bathing medium. This would explain

our failure to observe any difference in recovery of endogenous LHRH between samples incubated in the presence or absence of inhibitor.

The endeavor of establishing a functional role of an individual enzyme entails biochemical, pharmacological and physiological investigations. Biochemical investigations of the functionality of an enzyme should include inhibitor as well immunohistochemical studies (Turner et al., 1986). For example, 2 lines of biochemical evidence have suggested a role for EP 24.11 in the degradation of substance P. Immunohistochemical work has demonstrated a colocalization of the peptidase, EP 24.11 and peptide substrate, substance P. Areas of the brain rich in substance P, especially in the striato-nigral pathway, show also an abundance of EP 24.11 activity, thus implicating a role for this enzyme in the CNS metabolism of substance P (Matsas et al., 1986). Support for this putative functional importance of EP 24.11 was provided by *in vitro* inhibitor studies where metabolism of substance P by striatal synaptic membranes was abolished by phosphoramidon (Fulcher et al., 1982; Matsas et al., 1983, 1985). In similar studies, the formation of [^{3}H]Tyr-Gly-Gly by cleavage of the Gly^3-Phe^4 bond in tritiated [Met^5]enkephalin by slice and particulate preparations from rat striatum was inhibited by thiorphan, thus demonstrating the role of EP 24.11 in the extracellular metabolism of Met-enkephalin *in vitro* (De La Baume et al., 1983). A similar course of study was employed in evaluating the role of EP 24.11 in the metabolism of atrial natriuretic factor (ANF). Following the demonstration that ANF is cleaved by EP 24.11 (Stephenson and Kenny, 1987), inhibitors of EP 24.11 were shown by Olins et al. (1989) and Sybertz et al. (1989) to provide almost complete protection of ANF from inactivation by renal brush border membrane preparations *in vitro*, indicating that EP 24.11 represents a major activity responsible for ANF degradation in

these preparations. In *in vivo* biochemical studies, the disappearance of a peptide is monitored following its co-administration with an inhibitor of the enzyme in question. For example, the half-life of intracerebroventricularly injected [Met^5]enkephalin is significantly increased following thiorphan co-administration, thus implicating a role for EP 24.11 in the *in vivo* inactivation of [Met^5]enkephalin (Schwartz, 1983). In work by Sybertz et al. (1989) and Olins et al. (1989), the IV injection of EP 24.11 inhibitors significantly delayed the disappearance of ANF from plasma after an IV infusion of the peptide; thus, a role for peripheral EP 24.11 in the clearance of ANF was implicated. In pharmacological investigations of enzyme functionality, one attempts to demonstrate that the biological activity of an exogenous peptide can be augmented following the administration of inhibitors; thereby, the enzyme(s) instrumental in its inactivation are established. For example, inhibitor treatment in live rats has served to indicate a role for EP 24.11 and ACE, not only in the degradation of enkephalin, substance P and ANF *in vivo*, but more importantly, in the modulation of the biological activity of these peptides. In several studies, thiorphan and kelatorphan were shown to augment the antinociceptive activity of ICV administered [Met^5]enkephalin, by inhibiting EP 24.11-mediated enkephalin degradation, thus accumulating Met-enkephalin at the CNS opioid receptors, leading to increased antinociception (Roques et al., 1980; Yaksh and Harty, 1982; Zaluski et al., 1984). An inhibitor of EP 24.11, SCH 39370, was shown to augment the hypotensive response to injection of ANF in spontaneously hypertensive rats, thus suggesting a role for this enzyme in regulating ANF's biological actions (Sybertz et al., 1989). In a similar type of study, the substance P-induced salivation in rats was enhanced upon the administration of captopril due to inhibition of ACE-mediated metabolism of substance P, thus indicating a role for ACE in curtailing the biological activities

of this peptide (Cascieri et al., 1984). EP 24.11 may also modulate substance P's biological action since both phosphoramidon and thiorphan enhanced the bronchoconstrictor effect of this peptide *in vitro* (Stimler-Geraro, 1987). Studies with a direct physiological focus, investigating the involvement of an individual enzyme in the inactivation of an endogenous peptide, have generally utilized the *in vitro* brain slice-release paradigms. Such studies have employed enzyme inhibitors in an attempt to increase the recovery of neuropeptides following stimulated release, thus implicating the inhibited enzyme in the extracellular metabolism of the peptide in question. Examples of such studies were delineated above and have demonstrated the importance of EP 24.11 in the metabolism of endogenous substance P and enkephalins (Patey et al., 1981; Littlewood et al., 1988). *In vivo* physiological studies directed at identifying the functionality of a peptidase, include the use of inhibitors to increase the recovery of endogenous peptides. For example, the importance of EP 24.11 in endogenous enkephalin metabolism was suggested when ICV administration of thiorphan enhanced the recovery of endogenous striatal Met-enekphalin (Zhang et al., 1982). The *in vivo* physiological importance of peptidase activity is even more directly demonstrated when the biological activity of an endogenous peptide substrate is increased following inhibitor treatment. De La Baume et al. (1983) and Murthy et al. (1984) have demonstrated that the administration of potent EP 24.11 inhibitors prolonged the latency time of responses during the hot plate and tail flick tests (tests used to demonstrate the nociceptive reflexes to noxious stimuli). Thus the physiological modulation of endogenous enkephalin's antinociceptive activity by EP 24.11 is shown by inhibitor studies.

These three modes of research, i.e. biochemical, pharmacological and physiological studies, directed at identifying functionality of EP 24.15, have been employed during the course of this thesis project. The biochemical studies of this thesis project were carried out during phases 1 and 2. In phase 1, inhibitors of 3 different membrane metallopeptidases inhibitors enabled us establish the predominance of EP 24.15 in the degradation of LHRH by brain and pituitary membrane preparations *in vitro*. In addition, the exact cleavage sites within LHRH, the relative importance of these cleavage activities and the identity of the enzymes responsible for these activities, were established by the use of these inhibitors. In phase 2, similar studies used EP 24.15 inhibitors to demonstrate this enzyme's involvement in the metabolism of exogenous LHRH in the brain and general circulation, *in vivo*. Phase 3 comprised the pharmacological studies of this project where EP 24.15 inhibitors augmented the gonadotropin-releasing activity of exogenous LHRH, demonstrating the importance of EP 24.15 in the CNS and periphery in modulating LHRH's biological action. In phases 4 and 5, we attempted to establish a physiological role of EP 24.15 in inactivating endogenous LHRH. Plasma LH was monitored following infusion of EP 24.15 inhibitor, in order to validate our hypothesis that EP 24.15 activity maintains the concentration of LHRH in portal circulation in check. If this were true, inhibition of this enzyme would allow an increase in the concentration of LHRH reaching the gonadotropes, thus precipitating a rise in LH release. Lastly, in phase 5, in another attempt to ascertain a functional role for EP 24.15 in metabolism of endogenous LHRH, the recovery of endogenous LHRH released from brain slices *in vitro*, was measured in the presence of inhibitors of this enzyme. Results obtained during phase 5 work were negative and phase 4 results, albeit somewhat positive, were

unconvincing. In summary, while the use of active site-directed inhibitors in phases 1 through 3 allowed us to definitively establish the 1)*in vitro* and *in vivo* preeminence of EP 24.15 in the extracellular metabolism of exogenous LHRH in the CNS and periphery, and 2)the importance of such enzymatic activity in modulating the gonadotropin releasing activity of exogenous LHRH, a similar functional role for this enzyme in endogenous LHRH metabolism could not be established by the inhibitor studies performed in phases 4 and 5.

The notion that LHRH metabolism may be initiated by cleavage LHRH of the Tyr^5-Gly^6 bond, has been supported by observations of many other groups. The work of McDermott et al. (1982, 1983) indicating that this activity constitutes the primary LHRH-DA in particulate and soluble preparations of rat hypothalamus and pituitary, has been reviewed above. Similar findings were reported with regard to LHRH-DA in soluble fractions of sheep hypothalamus (Advis et al. 1985). A soluble LHRH-DA from rat hypothalamus has been shown by Advis (1982) et al. to decline prior to the proestrous-associated LH surge (discussed above). In subsequent work, Krause et al.(1982) has shown that this hydrolysis of LHRH, which is subject to fluctuations during the reproductive cycle, is due to cleavage of the Tyr^5-Gly^6 bond. This rate limiting step is responsible for at least 80% of the total LHRH-DA, and is inhibited by o-phenanthroline, indicating that it is catalyzed by a metalloendopeptidase (Advis et al, 1982a). Quite possibly, the activity studied by this group was, in reality, EP 24.15, since this activity is responsible for the Tyr^5-Gly^6 cleavage and since EP 24.15, a metalloendopeptidase, is inhibited by o-phenanthroline (Orlowski et al., 1983). In work from this laboratory (manuscript in preparation), administration of estrogen plus progesterone, a treatment that induces an LH surge, to ovariectomized rats caused a precipitous decline in soluble EP 24.15 activity in the preoptic area of the hypothalamus. Moreover, the activity of this

enzyme was shown to decline in the anterior pituitary as the female rat matures towards the first preovulatory proestrous. Thus a possible functional importance of this enzyme appears to exist with regard to the induction of the LH surge. The possible operative mechanism for this phenomenon would be that the activity of this enzyme normally inactivates LHRH en route to, or at the pituitary gonadotropes, and that at times of increased LH secretion, i.e. the evening of proestrous, this LHRH-DA declines, allowing a higher concentration of LHRH to arrive intact at the pituitary and to be active at that site for a longer period of time. This phenomenon would be in concert with the data reported here where the activity of this enzyme is shown to dramatically curtail the LH and FSH releasing activity of exogenous LHRH. While we demonstrated a pharmacological importance for EP 24.15 with regard to exogenous LHRH, we could not rigorously show, in phases 4 and 5, a similar physiological function, i.e regulation of the LH-releasing activity of endogenous LHRH, for this enzyme. However, the lack of more positive results in the phase 4 ICV experiments can possibly be attributed to the depressed state of LHRH- and LH-secretory system due to anesthesia and surgical stress. Furthermore, while no endogenous LHRH metabolism by EP 24.15 was observed in slice preparations (phase 5), the likelihood of this enzyme being operative in endogenous LHRH degradation *in vivo* is far greater since the local concentration of endogenous LHRH at the enzyme's active site either in ME interstitial spaces or at the gonadotropes, may be orders of magnitude higher than the concentration present in the media bathing the hypothalamic slices. Consequently, the fact that: 1)EP 24.15 activity, both in the brain and in the periphery modulates the magnitude and duration of the gonadotropin-releasing activity of exogenous LHRH; and 2) that the increased biological actions of LHRH superagonists could

be shown to be almost entirely, due to their resistance to this enzyme, allows us to suggest that:

1) EP 24.15 in the CNS may still have an important physiological role in modulating the gonadotropin-releasing activity of secreted endogenous LHRH; and
2) EP 24.15 may have important pharmacological ramifications in that it decidedly determines the disposition (i.e. plasma half-life) as well as the biological action of systemically administered LHRH *in vivo*.

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