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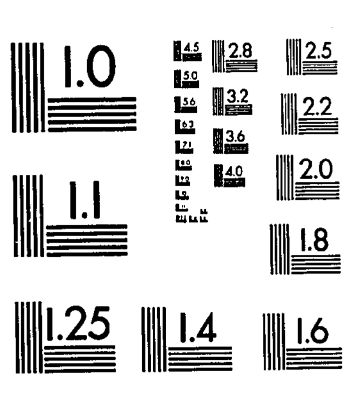

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STUDIES ON THE EFFECTS OF INHIBITORS OF TRH-DEGRADING ENZYMES

by

THEODORE C. FRIEDMAN

A dissertation submitted to the Graduate Faculty in the Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1985

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

STUDIES ON THE EFFECTS OF INHIBITORS OF TRH-DEGRADING ENZYMES

by

Theodore C. Friedman

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The effects of several specific inhibitors of enzymes capable of degrading thyrotropin-releasing hormone (TRH; pyroglutamyl-histidyl-prolyl-amide) was studied. These studies were carried out in rat brain, pituitary and serum and in cell culture (GH3 cells). TRH can be initially degraded by deamidation catalyzed by prolyl endopeptidase (EC 3.4.21.26) or by removal of the pyroglutamyl residue catalyzed either by pyroglutamyl peptide hydrolase (EC 3.4.11.8) or a less well characterized enzyme having the properties of a metalloenzyme. The inhibitors studied were N-benzyloxycarbonyl-prolyl-prolinal (Z-Pro-Prolinal), a specific, potent (Ki = 14 nM), transition-state analog inhibitor of prolyl endopeptidase, 5-oxoprolinal, a newly synthesized transition-state analog inhibitor of pyroglutamyl peptide hydrolase and pyroglutamyl diazomethyl ketone, an inhibitor capable of irreversibly alkylating the active-site cysteine residue of pyroglutamyl peptide hydrolase.

5-Oxoprolinal was synthesized by conversion of pyroglutamate to its methyl ester, reduction of the ester to the alco-

hol (5-oxoprolinol) by sodium borohydride followed by oxidation of the alcohol to the aldehyde. Kinetic experiments showed that 5-oxoprolinal competitively inhibited thiol-dependent pyroglutamyl peptide hydrolase with a Ki of 26 nM. The compound was shown to be a specific active-site directed inhibitor since related aldehydes as well as the corresponding acid and alcohol inhibited the enzyme only weakly or not at all. 5-oxoprolinal also blocked the degradation of luteinizing-hormone releasing hormone, by purified pyroglutamyl peptide hydrolase.

Experiments were conducted in male Swiss Albino mice with the three inhibitors. The activity of prolyl endopeptidase was inhibited by more than 85% in homogenates of all tissues studied 30 minutes after intraperitoneal injection of Z-Pro-Prolinal. The in vivo degradation of a prolyl endopeptidase substrate, N-benzyloxycarbonyl-Gly-Pro-Sulfamethoxazole, was blocked after administration of Z-Pro-Prolinal in a dose- and time-dependent manner, indicating inhibition of the enzyme in vivo. 5-Oxoprolinal, when injected into mice, inhibited pyroglutamyl peptide hydrolase after 10 and 30 min however a relatively high dose (50 mg/kg) was needed to achieve this inhibition. Pyroglutamyl diazomethyl ketone was injected into mice and at a dose of 0.1 mg/kg totally inactivated the enzyme in all tissues studied including brain.

It was expected that the combined use of inhibitors to prolyl endopeptidase and pyroglutamyl peptide hydrolase would

totally protect TRH from degradation in vitro. However, when tissue homogenates or serum was incubated in the presence of inhibiotrs but in the absence of metal chelators, substantial TRH degradation was observed. This indicated the presence of another TRH-degrading enzyme(s) distinct from pyroglutamyl peptide hydrolase. Recent reports in the literature of a third TRH-degrading enzyme which cleaves the pGlu-His bond of TRH led us to develop a coupled assay to measure this activity. The assay uses pGlu-His-Pro-2-Naphthylamide as the substrate and is based on the release of 2-naphthylamine from the product His-Pro-2-naphthylamide by excess diaminopeptidase IV (EC 3.4.14.1). This activity was found to be present in the particulate fraction of rat brain homogenates as well as in rat serum. Distribution studies revealed that the enzyme was primarily localized in the brain membranes and serum. The data suggest that degradation of TRH in brain membranes and serum is catalyzed mainly by an enzyme which cleaves the pGlu-His bond of TRH and is distinct from pyroglutamyl peptide hydrolase.

Z-Pro-Prolinal was injected into rats to see if the inhibition of prolyl endopeptidase would raise endogenous TRH levels. TRH levels were found to be significantly elevated in the pituitary 15 minutes after injection of Z-Pro-Prolinal (5 mg/kg). No significant changes in TRH levels were detected in the pituitary, frontal cortex or the hypothalamus at later times. Prolactin and thyroid stimulating hormone levels in rat serum were not affected by Z-Pro-Prolinal administration.

Experiments were carried out to study the effects of inhibitors in GH3 cells, a cell line cloned from a rat anterior pituitary tumor, which synthesize and secrete prolactin in response to thyrotropin-releasing hormone. A variety of peptide-degrading enzymes were found to be present in homogenates of GH3 cells. The effect of long-term exposure of GH3 cells to 5-oxoprolinal on pyroglutamyl peptide hydrolase activity was studied by incubating cells with inhibitor for 3 days, harvesting, washing to remove inhibitor and assaying for pyroglutamyl peptide hydrolase. In contrast to the expected loss of pyroglutamyl peptide hydrolase activity upon exposure to inhibitor, we found a marked (300-400%) increase in pyroglutamyl peptide hydrolase activity which was not blocked by cycloheximide. This concentration-dependent and time-dependent effect was specific to 5-oxoprolinal, suggesting that the activity of pyroglutamyl peptide hydrolase in GH3 cells is subject to complex regulatory mechanisms.

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Publications resulting from this research

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Friedman, T. C., Orlowski, M. and Wilk, S. (1984) Prolyl endopeptidase: Inhibition in vivo by N-benzyloxycarbonyl-prolyl-prolinal. J. Neurochem. 42, 237-241.

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Abbreviations

BSA	bovine serum albumin
Bz	α-benzoyl
DABA	diaminobenzoic acid dihydrochloride
DAP IV	diaminopeptidase IV
DFP	diisopropylfluorophosphate
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenylhydrazine
DTT	dithiothreitol
h	hour
LHRH	luteinizing hormone-releasing hormone
min	minute
2NA	2-Naphthylamide
NIADDK	National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases
pAB	p-aminobenzoate
PBS	phosphate-buffered saline
PCMB	p-chloromercuribenzoate
PDMK	pyroglutamyl diazomethyl ketone
pGlu	pyroglutamate
pNA	p-Nitroanilide
PPH	pyroglutamyl peptide hydrolase
PTFA	pyridinium trifluoroacetate
RIA	radioimmunoassay
SM	sulfamethoxazole
TCA	trichloroacetic acid
TFA	trifluoroacetate
TLC	thin-layer chromatography
TMS	trimethylsilane
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
Z	N-benzyloxycarbonyl

conventional 3 letter abbreviations were used for other amino acids.

INTRODUCTION

The synthesis of neuropeptides from inactive high molecular weight precursors involves cleavage of selective peptide bonds in a process of limited proteolysis catalyzed by endopeptidases. Endopeptidases also catalyze the degradation of neuropeptides indicating a mechanism for termination of their action. Although neuropeptides are involved in many physiological processes, very little is known about their degradation in vivo. In our laboratory, we have isolated and studied the specificity of several endopeptidases likely to be involved in neuropeptide metabolism. These studies have provided a basis for the design of specific, active site directed inhibitors for these enzymes. The inhibitors are expected to be of value as probes in investigations designed to explore the role of these enzymes in neuropeptide metabolism. The inhibitors are also of pharmacological interest because of the potential to affect those processes that are dependent upon neuropeptide function. My thesis work has been directed toward the exploration of the effects of several specific enzyme inhibitors on the metabolism of neuropeptides, especially thyrotropin-releasing hormone (TRH; pyroglutamyl-histidyl-prolyl-amide).

Prolyl endopeptidase (EC 3.4.21.26), a serine protease thought to be involved in the degradation of TRH, LHRH, bradykinin, angiotensin II, neurotensin and substance P has been

actively investigated in our laboratory (Orlowski et al., 1979; Wilk and Orlowski, 1983a; Wilk, 1983). Prolyl endopeptidase cleaves peptide bonds on the carboxyl side of proline residues within a peptide chain. Z-Pro-Prolinal (Figure 1), a specific and highly potent inhibitor of prolyl endopeptidase has been synthesized in our laboratory (Wilk and Orlowski, 1983a). As an aldehyde analog of the acyl portion of the substrate, the inhibitor interacts with the active site of this serine protease and forms a tetrahedral transition state intermediate analog of the enzyme-substrate complex. It was found to non-competitively inhibit purified rabbit prolyl endopeptidase with a Ki of 14 nM.

Pyroglutamyl peptide hydrolase (PPH)[1] (EC 3.4.11.8), an enzyme classified as a cysteine protease, cleaves the N-terminal pyroglutamyl residue from pyroglutamyl-containing peptides such as TRH. This enzyme, initially found by Doolittle and Armentrout (1968) in a strain of Pseudomonas fluorescens and later purified from other bacteria (Szewczuk and Mulczyk, 1969), was found to be distributed in animal tissues (Szewczuk and Kwiatkowska, 1970). It has recently been purified from guinea-pig brain (Browne and O'Cuinn, 1983). Pyroglutamyl peptide hydrolase can catalyze the removal of the N-terminal pyroglutamyl residue from proteins such as fibrinogen and human serummucoid and also from fibrinopeptides (Szewczuk and

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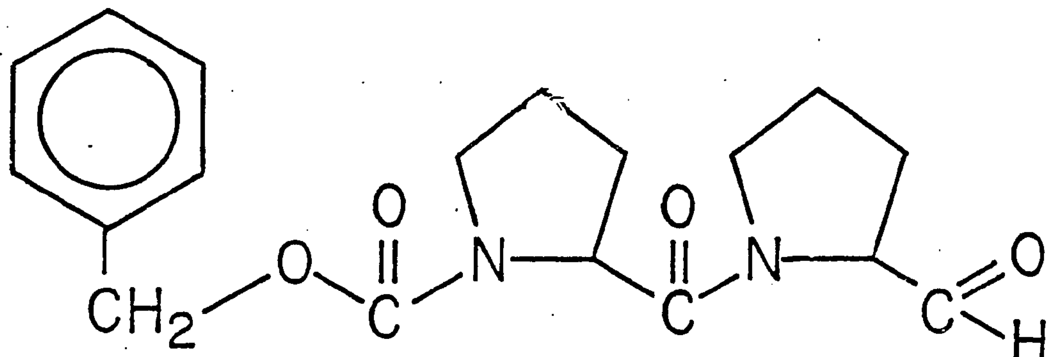
[1]Synonyms of 5-oxoproline are pyrrolidone carboxylic acid, pyroglutamatic acid and pyroglutamate.

Figure 1



Structure of Z-Pro-Prolinal

Mulczyk, 1969; Armentrout and Doolittle, 1969; Orlowski and Meister, 1971). Because of this, initial interest in the enzyme centered on its use as a tool for the removal of the N-terminal pyroglutamyl residue from proteins and peptides in the process of determination of their amino acid sequences.

The physiological role of pyroglutamyl peptide hydrolase is not known. With the finding, however, that many biologically active peptides including TRH, LHRH, neurotensin and bombesin contain N-terminal pyroglutamyl residues, the potential role of the enzyme in the degradation and termination of action of these peptides has attracted interest. A specific and potent inhibitor of pyroglutamyl peptide hydrolase would be a helpful probe in studies investigating the physiological role of this enzyme.

Peptide aldehyde analogs of substrates have been used to inhibit serine and cysteine proteases by the formation of transition-state-like intermediates (Westerik and Wolfenden, 1972; Thompson, 1973). Transition-state theory predicts that the enzyme-substrate binding in the transition-state complex will be stronger than in the initial Michaelis complex between enzyme and substrate (Wolfenden, 1972). An analog which mimics the features of the transition-state complex will also form a very stable complex with the enzyme. Aldehydes react with serine and cysteine proteases to form relatively stable tetrahedral hemiacetals and thiohemiacetals respectively. These complexes resemble the tetrahedral transition-state

intermediates formed between substrate and enzyme. Aldehyde analogs, therefore, have an extremely high affinity for the enzyme and can be used as potent and specific inhibitors of serine and cysteine proteases.

In our laboratory, we have previously synthesized Z-Pro-Prolinal as a specific active-site directed inhibitor of prolyl endopeptidase (Wilk and Orlowski, 1983a) and the design of this inhibitor was used as a model for designing an inhibitor of PPH. Since currently available evidence suggests that pyroglutamyl peptide hydrolase is a cysteine protease, we considered the possibility that 5-oxoprolinal (Figure 2), the aldehyde analog of 5-oxoproline[2], would be a transition-state inhibitor of pyroglutamyl peptide hydrolase. We therefore synthesized 5-oxoprolinal and showed that this compound is a potent and selective inhibitor of pyroglutamyl peptide hydrolase in vitro. However, 5-oxoprolinal was found to be a relatively weak and short lived inhibitor in vivo (see below).

Diazomethyl ketone substrate derivatives have been used as irreversible inhibitors of cysteine proteases (Leary et al., 1977; Green and Shaw, 1981). These inhibitors act by alkylating the active-site cysteine residue. Irreversible inhibitors of bacterial PPH have been synthesized (Fujiwara et al., 1981a; 1981b; 1982). The L-pyroglutamyl chloromethyl ketone inhibitor (Fujiwara et al., 1981a) was found to be an effec-

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[2]Synonyms of pyroglutamyl peptide hydrolase are pyrrolidonyl peptidase and pyroglutamyl aminopeptidase.

Figure 2

Structures of 5-oxoprolinal and the 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal. Hydrogens are labelled for NMR data (see Methods section).

tive inhibitor of the rat liver and kidney PPH, however the in vivo effects of these inhibitors have not been ascertained. In our laboratory, we have synthesized pyroglutamyl diazomethyl ketone (PDMK) (Figure 3) as a potent irreversible inhibitor of PPH for studies in vivo. Preincubation of partially purified PPH with nanomolar concentrations of PDMK rapidly and totally inactivates this enzyme in vitro (Wilk and Friedman, 1985).

Although it has been recognized since the 1950's that the hypothalamus influences the release of pituitary homones, it was not until 1969 that the first hypothalamic releasing factor, TRH, was isolated from ovine and porcine hypothalamic tissue (Boler et al., 1969). TRH was found to be a tripeptide with the chemical formula pGlu-His-Pro-NH2. Although TRH was initially thought to only regulate the release of TSH (Schally and Redding, 1965; Wilber and Utiger, 1967), it was later found to also induce the release of prolactin (Jacobs et al., 1971; Bowers et al., 1971) and growth hormone (Takahara et al., 1974). The availability of purified TRH led to development of specific radioimmunoassays for TRH (Bassiri and Utiger, 1972; Jeffcoate et al., 1973) and to the discovery of TRH in extrahypothalamic brain regions as well as outside the CNS (Jackson, 1983). The localization of TRH in specific areas of the forebrain and spinal cord (Brownstein et al., 1974; Hokfelt et al., 1975) resulted in the consideration of TRH as a neurotransmitter. Other evidence consistent with a neuromodulator or neurotransmitter role of TRH include its

Figure 3

Pyroglutamyl Diazomethyl Ketone

Structure of pyroglutamyl diazomethyl ketone (PDMK)

localization in synaptosomes (Barnea et al., 1978), its Ca^{++}-dependent release from slices and synaptosomes (Charli et al., 1978; Bennett, 1981) and the identification of specific TRH binding sites in various brain regions (Burt and Taylor, 1980). TRH produces certain stimulatory effects and general arousal in animals (Metcalf and Dettmar, 1981), affects thermoregulation (Metcalf, 1974; Horita and Carino, 1975) and respiration (Hedner et al., 1981) and reverses barbiturate and ethanol induced hypnosis (Prange et al., 1974). The clinical uses of TRH have included treating depression (Prange et al., 1972; Kastin et al., 1972), endotoxic and hemorrhagic shock (Holaday et al., 1981) and spinal injury (Faden et al., 1983). Recently, clinical interest in TRH has centered on its use to treat patients with amyotrophic lateral sclerosis (Engel et al., 1983).

The initial breakdown of TRH in various tissues appears to be catalyzed by enzymes which cleave either of two bonds. Earlier studies found that in tissues, the initial degradation proceeded by either deamidation now known to be catalyzed by prolyl endopeptidase (EC 3.4.21.26) or by removal of the N-terminal pyroglutamyl residue catalyzed by pyroglutamyl peptide hydrolase (Prasad and Peterkofsky, 1976; Bauer et al., 1978; Griffiths and McDermott, 1983; Griffiths et al., 1979a; Browne et al., 1981; Hayes et al., 1979; Bauer and Kleinkauf, 1980; Kreider et al., 1981; Griffiths et al., 1980; Busby et al., 1982; Safran et al., 1982; Browne and O'Cuinn, 1983). Estimates of the relative contributions of these two enzymes

to TRH degradation vary widely in the literature, depending on whether a soluble or particulate fraction is used and whether a thiol-reducing agent or metal chelator are present during the assay. Prolyl endopeptidase, an enzyme classified as a serine protease (Yoshimoto et al., 1977), is activated by thiol-reducing agents, such as dithiothreitol (DTT) and is found in the cytosolic fraction of tissues (Dresdner et al., 1982). PPH, classified as a cysteine protease (Mudge and Fellows, 1973), has been reported to occur in both the soluble and particulate fractions (Browne et al., 1981; Kreider et al., 1981; Prasad et al., 1983) of tissues. PPH is activated by thiol-reducing agents and metal chelators and appears to have a broad specificity for pyroglutamyl-containing peptides (Prasad and Peterkofsky, 1976; Busby et al., 1982; Browne and O'Cuinn, 1983). Additionally, a TRH-degrading enzyme which also cleaves the pGlu-His bond of TRH, has been identified in rat serum (Taylor and Dixon, 1978) and was partially purified from porcine serum (Bauer and Nowak, 1979). This enzyme has a molecular weight of 260,000, appears to be relatively specific for TRH and is inhibited by metal chelators (Taylor and Dixon, 1978; Bauer and Nowak, 1979; Bauer et al., 1981b). Recently, a similar TRH-degrading enzyme has been identified in guinea pig brain (Greaney et al., 1980; O'Connor and O'Cuinn, 1984; Garat et al., 1985). This enzyme also has a high molecular weight (230,000), is inhibited by metal chelators such as EDTA and appears to have a narrow specifity for cleaving the pGlu-His bond in TRH-like peptides (O'Connor and O'Cuinn, 1984).

We initially demonstrated that Z-Pro-Prolinal inhibited prolyl endopeptidase in vivo (discussed below). Z-Pro-Prolinal may therefore increase the levels of those neuropeptides which are degraded by prolyl endopeptidase. Similarly, 5-oxoprolinal and PDMK inhibited PPH in vivo and may therefore increase the level of those neuropeptides degraded by PPH. TRH (pGlu-His-Pro-NH2) was selected as a model peptide for study on the effects of inhibitors on neuropeptide degradation because its initial degradation proceeds through deamidation catalyzed by prolyl endopeptidase as well as removal of the N-terminal pyroglutamyl residue catalyzed by pyroglutamyl peptide hydrolase.

Z-Pro-Prolinal and PDMK were used to assess the roles of prolyl endopeptidase and PPH in the degradation of TRH in vitro. Since PDMK is specific for PPH, this inhibitor could be used to determine if the membrane-bound and serum TRH-degrading enzymes are identical to PPH. The activities of TRH-degrading enzymes in serum and brain fractions were studied. The effect of Z-Pro-Prolinal, PDMK, DTT and metal chelators on these activities was determined. In the particulate fraction of rat brain and in serum we found significant TRH-degrading activity in the presence of the two specific inhibitors using conditions favoring metalloenzyme activity. This suggests that the serum and particulate enzymes are distinct from PPH and contribute to TRH degradation. To facilitate the determination of the particulate and serum enzymes which appear to have a narrow specificity for cleaving the pGlu-His bond in

TRH-like peptides, a coupled assay with pGlu-His-Pro-2NA as the substrate in the presence of excess diaminopeptidase IV (EC 3.4.14.1) (DAP IV) was developed. This assay allowed us to study the distribution of this activity in rat brain regions and rat tissues and compare it to the soluble PPH activity. Additionally, the effect of inhibitors and activators of this particulate enzyme was studied and the Ki of TRH for this enzyme was determined.

The specific inhibitors of these enzymes, Z-Pro-Prolinal and PDMK can be used to determine which pathway predominates physiologically. The use of these two inhibitors, separately or together, should block the metabolism of TRH and elevate its endogenous levels. This was studied by injecting the inhibitor(s) into rats and measuring tissue TRH levels in the hypothalamus, pituitary and frontal cortex by RIA (see below). Additionally, an elevation of TRH should produce an increase in serum levels of TSH and prolactin. These hormones were measured in the serum by RIA.

Preliminary results in our laboratory indicate that prolyl endopeptidase has a high affinity for LHRH (Bier and Wilk, unpublished results). In crude homogenates, the primary sites of cleavage of LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) are the $Pyroglu^{1}$-His^{2} bond, the Tyr^{5}-Gly^{6} bond and the Pro^{9}-$Glycinamide^{10}$ bond (McKelvy et al., 1982, Bauer et al., 1981a). Pyroglutamyl peptide hydrolase (Bauer et al., 1981a), a soluble metalloendopeptidase studied in our laboratory

(Orlowski et al., 1983), and prolyl endopeptidase (Wilk et al., 1979a) could cleave these bonds respectively.

LHRH analogs, modified by replacement of the Glycineamide^{10} residue with Ethylamide and the Gly^{6} residue with a D-amino acid residue, act as long-lasting agonists. These analogs have wide clinical uses (Ziporyn, 1985) including treatment of prostatic cancer (Tolis, et al., 1982), premenstrual syndrome (Muse et al., 1984) and as contraceptive agents (Nillius, et al., 1978; Bergquist et al., 1979). It is possible that the long duration of these modified LHRH compounds is due to their resistance to degradation between the Tyr^{5}-Gly^{6} bond, the Gly^{6}-Leu^{7} bond or the Pro^{9}-Glycineamide^{10} bond. Z-Pro-Prolinal could inhibit the degradation of LHRH at the Pro^{9}-Glycineamide^{10} bond by prolyl endopeptidase and might also prolong the action of LHRH. We, therefore, were interested in examining the effect of Z-Pro-Prolinal on the half-life of LHRH.

Cell cultures offer an interesting and simple system for testing the effect of inhibitors on peptide metabolizing enzymes. GH3 cells are a line of cells cloned from a rat anterior pituitary tumor and propagated since 1965 (Tashjian et al., 1968), which synthesize and secrete growth hormone and prolactin (Tashjian and Bancroft, 1970). Since these cells are derived from a single cell and can be propagated in culture, they offer many advantages to the investigator studying the effects of various compounds on pituitary function.

Since 1971, when increased prolactin synthesis and secretion by GH3 cells in response to nanogram amounts of TRH was noted (Tashjian et al., 1971), GH3 cells have been used extensively to study the mechanism of action of TRH. TRH induces the synthesis of prolactin either by binding to a receptor on the plasma membrane (Martin and Tashjian, 1977) or by entering the cell and binding to a nuclear receptor (Laverriere et al., 1981). Hinkle and Tashjian (1975) showed that internalized TRH is slowly degraded within GH3 cells but made no attempt to investigate which enzymes were responsible for this degradation. The role of intracellular proteolytic enzymes in the regulation of TRH-induced prolactin synthesis remains unexplored. GH3 cells should constitute an ideal system for studies on the role of those enzymes responsible for TRH degradation.

Over the past few years our laboratory group has actively investigated a number of brain and pituitary neuropeptide-degrading enzymes including prolyl endopeptidase (Orlowski et al., 1979) and pyroglutamyl peptide hydrolase. Other neuropeptide-degrading enzymes studied by our laboratory group include a membrane-bound neutral metalloendopeptidase (Orlowski and Wilk, 1981), a soluble metalloendopeptidase (Orlowski et al., 1983) and a multi-catalytic protease complex (Wilk et al., 1979b; Wilk and Orlowski, 1983b). We were initially interested in determining the presence of these enzymes as well as aminopeptidase and lysosomal cathepsin B and D in GH3 cells. We, therefore, compared the activities of these

enzymes in GH3 cell homogenates and in homogenates of rat anterior pituitaries. This information should be of value in studies on the role of proteolytic enzymes in the metabolism of TRH, other neuropeptides and peptide hormones, both in the anterior pituitary and in cell cultures.

With the finding that neuropeptide-degrading enzymes are present in GH3 cells, it was of interest to examine the effects of inhibitors on the enzymes present in these cells. Some of the best documented examples of a compensatory response by cells exposed to an inhibitor include an increase in dihydrofolate reductase produced by methotrexate (Jolivet et al., 1983) and an increase in ornithine decarboxylase by α-methylornithine and α-difluoromethylornithine (Choi and Scheffler, 1981). Initially, Z-Pro-Prolinal and 5-oxoprolinal were tested for their ability to inhibit prolyl endopeptidase and PPH in GH3 cells. Z-Pro-Prolinal effectively inhibited prolyl endopeptidase (see below); 5-oxoprolinal, however, unexpectedly increased PPH activity upon exposure to the inhibitor for 3 days. The concentration-dependent and time-dependent increase was characterized and found to be specific for 5-oxoprolinal suggesting that the activity of PPH in GH3 cells is subject to complex regulatory mechanisms.

The effects of the inhibitors on prolactin response was also studied. TRH was added to the cells in the presence of either Z-Pro-Prolinal, 5-oxoprolinal, or both inhibitors and after 1 hour and 3 days, the medium was removed and assayed

for prolactin by radioimmunoassay. The 1 hour incubation measured newly secreted prolactin and the 3 day incubation measured newly synthesized prolactin. It was expected that since the inhibitors would prevent prolyl endopeptidase and PPH from degrading TRH, the effects of TRH would be more pronounced and the prolactin response would be greater in the presence of the inhibitor(s).

MATERIALS

The following substrates were synthesized in our laboratory as described: Bz-Gly-Ala-Ala-Phe-pAB (Orlowski et al., 1983); Z-Gly-Pro-SM (Orlowski et al., 1979); Z-Leu-Leu-Arg-2NA (Orlowski et al., 1981); Glutaryl-Ala-Ala-Phe-2NA (Orlowski and Wilk, 1981); Z-Gly-Gly-Leu-pNA (Wilk et al., 1979b); Z-Leu-Leu-Glu-2NA (Wilk and Orlowski, 1980); TFA•D-Phe-(O-Benzyl)Ser-Phe-Phe-Ala-Ala-pAB (Orlowski et al., 1984). pGlu-2NA was obtained from United States Biochemical Corporation (Cleveland, OH). pGlu-His-Pro-2NA was obtained from Bachem, Inc (Bubendorf, Switzerland). The following inhibitors were synthesized in our laboratory as described: N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Chu and Orlowski, 1984); Z-Pro-Prolinal (Wilk and Orlowski, 1983a); N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB (Almenoff and Orlowski, 1983); pyroglutamyl diazomethyl ketone (Wilk et al., 1985). TRH, LHRH, L-pyroglutamate, Leu-pNA, leupeptin, dimethyl sulfoxide, DNA (calf thymus, type I), cyclo(His-Pro), chloramine T, cycloheximide, Pro-2NA, t-Boc-His, o-phenanthroline, dithiothreitol and thimerosal were obtained from Sigma Chemical Co. (St. Louis, MO). Thionyl chloride, trifluoroacetate and pyridine were obtained from Fisher Scientific Corp. (Fairlawn, NJ). TRH-OH was obtained from Peninsula Laboratories (San Carlos, CA). Sodium borohydride, N,N'-Dicyclohexylcarbodiimide, 3,5 diaminobenzoic acid dihydrochloride, dinitrophenylhydrazine, N-methylpyrrole-2-carboxaldehyde and

2-thiophenecarboxaldehyde were obtained from Aldrich Chemical Corp. (Milwaukee, WI). Gly-Pro-2NA was obtained from Bachem Chemicals (Torrance, CA). Norit activiated charcoal was obtained from Amend Drug and Chemical Co. (Irvington, NJ). Silica gel (40 um average particle diameter) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Dimethyl sulfoxide was redistilled over NaOH and stored under nitrogen in a flask containing dried 4A molecular sieves. Pyridine was redistilled over KOH and stored under nitrogen in a flask containing dry KOH. LHRH antiserum was obtained from Accurate Chemical Co. (Westbury, NY). I-125-LHRH, I-125-prolactin and I-125-TRH were obtained from New England Nuclear (Boston, MA). Na-I-125 was obtained from Amersham Corp. (Arlington Heights, IL). Trasylol was obtained from Mobay Chemical Corp. (New York, NY). Pentex bovine serum albumin was obtained from Miles Laboratories (Kanakee, IL). Goat anti-rabbit second antibody was obtained from Cappel Co. (Cochranville, PA). Silica-coated plates (Polygram Sil G/UV 254, 40 mm X 80 mm) for thin-layer chromatography were obtained from Brinkmann Instruments (Westbury, NY). Rabbit sera and diisopropylfluorophosphate (DFP) were obtained from Calbiochem-Boehring (La Jolla, CA). All media components were obtained from Gibco Laboratories (Grand Island, NY). Rabbit brains were obtained from Pel Freez (Rogers, AK) and bovine brains from a local abbatoir. TRH antiserum was a generous gift from Dr. Piers Emson of the MRC Neuropharmacology unit, Cambridge, UK.

His-Pro-2NA was prepared in our laboratory by coupling t-Boc-His to Pro-2NA in the presence of N,N'-dicyclohexylcarbodiimide. The t-Boc group was removed by treatment with trifluoroacetic acid and the trifluoroacetate salt of His-Pro-2NA was obtained by removal of the trifluoroacetic acid by evaporation followed by precipitation with ether.

DAP IV was purified to apparent homogeneity from rabbit kidney cortex essentially as described by Yoshimoto and Walter (1977). The purified enzyme liberated 500 umol 2NA per mg protein per h from the substrate Gly-Pro-2NA. Calf liver PPH was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Prolyl endopeptidase was purified from rabbit brain to apparent homogeneity as described by Wilk and Orlowski (1983). PPH was partially purified from bovine brain. It co-purifies with prolyl endopeptidase up to the Sephadex G-100 chromatography step (Wilk and Orlowski, 1983a). PPH (MW 24,000-30,000) is then resolved from prolyl endopeptidase (MW 66,000) by the Sephadex G-100 column. At this stage bovine brain PPH which was purified 220 fold from the original supernatant has a specific activity of 66 units/ mg protein (one unit is defined as the amount of enzyme releasing 1 nmol 2NA per h from pGlu-2NA). Aminopeptidase M (EC 3.4.11.2) was purified from hog kidneys according to the procedure of Pfleiderer (1970). To remove contaminating membrane-bound neutral metalloendopeptidase (Almenoff and Orlowski, 1983), the enzyme was chromatographed on phenyl sepharose CL4B.

NMR spectra were performed in $CDCl_3$ on a Varian FT 80-A instrument and are reported in ppm downfield from TMS=0. IR spectra were performed on a Beckman IR8 instrument; only diagnostic peaks are reported.

METHODS

SYNTHESIS OF INHIBITORS

Synthesis of 5-oxoprolinal

5-oxoprolinol (Figure 4, III) (5-hydroxymethyl pyrrolidinone) was synthesized from 5-oxoproline (I) (pyroglutamate) according to the method of Saijo et al. (1980) as described in Figure 4. Thionyl chloride (8.9 g, 75 mmol) was added dropwise to a solution of L-pyroglutamate (I) (12.9 g, 100 mmol) dissolved in 120 ml methanol, placed over a magnetic stirrer and maintained at -20°. After 30 min , the solution was brought to room temperature and stirred for 3 hours. After removal of the solvent, the resulting oil was vacuum distilled (145°-150°, 4 mm Hg) yielding 10.34 g (72.3 mmol) of the methyl ester of pyroglutamate (II).

The methyl ester of pyroglutamate (II) (7.4 g, 51.7 mmol) was dissolved in 72 ml of ethanol. The stirred solution was placed in an ice bath and sodium borohydride (1.96 g, 51.7 mmol) was added over a period of 30 min. The reaction was continued for 2 more hours at room temperature. The mixture was then acidified with concentrated HCl, filtered, and the filtrate was evaporated under reduced pressure to give an oil which was chromatographed on a silica gel column (2 X 25 cm). The column was eluted with ethyl acetate followed by elution with a mixture of ethyl acetate-methanol (90:10). An oil

Figure 4

O= N(H) COOH (I) —$SOCl_2$, CH_3OH→ O= N(H) $COOCH_3$ (II) —$NaBH_4$→ O= N(H) CH_2OH (III) —DCC, DMSO, PTFA→ O= N(H) CHO (IV)

Synthesis scheme for 5-oxoprolinal

(III) was obtained which was crystallized from an ethyl acetate-chloroform mixture. A white solid with a melting point of 72°-74° was obtained, in agreement with the value reported in the literature (Saijo et al., 1980). Thin-layer chromatography in an ether-2-propanol solvent system (85:15), revealed a single spot (Rf of 0.06) when visualized by the chlorine-tolidine method for nitrogen-containing compounds (Krebs et al., 1969).

The alcohol (III) was oxidized to the aldehyde derivative, 5-oxoprolinal (IV), using a modification of the methods of Pfitzner and Moffat (1965) and Jones and Wigfield (1966). A weak acid, pyridinium trifluoroacetate (PTFA), prepared according to Bourne et al. (1954), was the catalyst. N,N'-Dicyclohexylcarbodiimide (2.7 g, 13 mmol) was added to redistilled DMSO (12.5 ml, 175 mmol). After stirring briefly, 5-oxoprolinol (III) (230 mg, 2 mmol) followed by PTFA (240 mg, 1.25 mmol) were added. The pH remained close to neutral, a condition which is necessary because the pyrrolidone ring is labile to acidic and basic conditions. The aldehyde was quantitated by the formation of the 2,4-dinitrophenylhydrazone derivative, using butyraldehyde as standard (Reingold and Orlowski, 1979). After 6 hours, no further increase in aldehyde could be detected. The highest yield obtained from this reaction was about 35%.

Chloroform was added and the reaction mixture was filtered. The filtrate was evaporated under reduced pressure at room

temperature to remove the chloroform and at 55° under vacuum for a short period of time to remove some of the DMSO. The oil was chromatographed on a silica gel column (2.5 X 85 cm) and eluted stepwise with ether:isopropanol (0% to 15% isopropanol). The eluate was monitored for aldehyde by a 2.4-dinitrophenylhydrazine (DNP) spray (0.4% in 2N HCl). The aldehyde emerged with ether-isopropanol (85:15). Removal of the solvent of the pooled aldehyde-containing fractions yielded an oil (IV). TLC analysis in an ether-2-propanol system (85:15) revealed one DNP-positive spot with an Rf value of 0.20. When visualized with the tolidine spray, however, a fast moving spot was also present. To remove this contaminant, the oil was purified using a flash chromatography (Still et al., 1978) silica gel column (1.5 X 60 cm) equilibrated with a mixture of chloroform-ethanol (95:5), eluted under nitrogen pressure with this solvent. The resulting oil, when chromatographed on a TLC plate, afforded only one spot when visualized with the tolidine spray, which coincided with the spot for aldehyde visualized by the DNP spray. This indicated that no other nitrogen-containing compound was present. The amount of aldehyde, however, as quantitated by 2,4-dinitrophenylhydrazone formation with butyraldehyde as a standard was less than the weight of the oil, possibly due to incomplete removal of solvent(s). ^{1}H NMR (CDCl3) δ2.38 m, $H_{\alpha,\alpha}/H_{\beta,\beta}$; δ4.22 m, H_{γ}; δ9.61 br s, CHO. IR vmax (oil, NaCl salt plates): 3400, w, NH; 1680 cm^{-1}, m, CONH overlap CHO.

Synthesis of the 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal

Partially purified 5-oxoprolinal (IV) (75 mg, 0.66 mmol), obtained from the first silica gel chromatography step, was dissolved in 4 ml of 95% ethanol. A DNP solution was prepared by dissolving DNP (0.4 g, 2 mmol) in 2 ml of concentrated H_2SO_4, adding 3 ml of water and 10 ml of 95% ethanol. 3 ml of this solution was added to the aldehyde solution. A solid precipitated immediately which was filtered, dissolved in chloroform, and chromatographed on a silica gel column. The column was washed with chloroform and the DNP-derivative was then eluted with a mixture of chloroform-ethanol (95:5). The pure DNP-derivative as determined by TLC (Rf value of 0.11 using a chloroform-ethanol mixture ; 95:5), eluted as the second component from the column. Removal of the solvent yielded a yellow solid with a melting point of 175°-177°. The NMR of the solid confirmed that the 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal was formed (Figure 1). ^{1}H NMR in CDCL3: δ2.50 br t, J=5.6 Hz, $H_{\alpha,\alpha}/H_{\beta,\beta}$; δ4.55, m, H_γ; δ5.83, br s, exchange with D_2O, lactam NH; δ7.40, d, J=4.9 Hz, CH=N; δ7.90, d, J=9.5 Hz, H_6; δ8.40, dd, J_o=9.5 Hz, J_m=3.0 Hz, H_5; δ9.10, d, J=3.0 Hz, H_3; δ11.10, br s, exchange with D_2O, N-NH; IR vmax (CHCl3 solution, NaCl cell with a 0.2 mm pathlength): 3480, 3460, w, NH; 3350, w, NH; 1690, m, CONH; 1650, 1630, s, C=N; 1550 cm^{-1}, s, NO_2. Anal. calcd. for $C_{11}N_5O_5H_{11}$: H 3.78, C 45.05, N 23.88; Found: H 3.82, C 45.18 N 23.81.

A standard curve prepared by reacting known amounts of the solid 2,4-dinitrophenylhydrazone derivative of 5-oxoprolinal with the cellosolve reagent (Reingold and Orlowski, 1979) was found to be similar to the curve with butyraldehyde as standard.

ENZYMATIC ASSAYS

Determination of enzymatic activities

The activities of all enzymes were measured by determining the release of aromatic amines from the appropriate chromogenic substrate using the diazotization procedure of Bratton and Marshall (1939) as modified by Goldbarg and Rutenberg (1958). For samples with low amounts of aromatic amines, the following modification was introduced to increase the sensitivity of the diazotization procedure: 540 ul of mouse tissue homogenate was added to 60 ul of 25% (w/v) trichloroacetic acid or 250 ul of incubation mixture was added to 250 ul of 10% (w/v) trichloroacetic acid. Precipated protein was removed by centrifugation at 4° for 10 min at 3000g. SM concentrations were determined in 375 ul of the supernatant by adding 187 ul 0.2% sodium nitrite followed after 3 min by 187 ul 10% ammonium sulfamate. After an additional 2 min, 250 ul of a 0.16% solution of N-(1-naphthyl)ethylenediamine dihydrochloride was added. The tubes were centrifuged for 10 min and the absorbance of the supernatant was determined at 540 nm. The amount of chromogenic substance released was calculated by a standard curve constructed with known amounts of the chromo-

gen. Under conditions of the assays, enzymatic activity was proportional to incubation time and amount of enzyme protein. Specific activity was expressed as nmoles/ mg protein/h. Protein was measured by the method of Lowry et al. (1951). The significance of changes in the specific activities between the two homogenate preparations was determined using the Student's T-test.

Soluble metalloendopeptidase: Activity was determined using the substrate Bz-Gly-Ala-Ala-Phe-pAB as described by Orlowski et al. (1983). The enzyme cleaves the Gly-Ala bond and the chromogen is released in a coupled assay by incubation with excess aminopeptidase M. Incubation mixtures (final volume 250 ul) contained 10 ul Bz-Gly-Ala-Ala-Phe-pAB (10 mM in 13 mM NaOH). 25 ul of 2.5 mM DTT, Tris-HCl buffer (0.2 M, pH 7.0) and 10 ul of homogenate preparation. Incubation proceeded for 1 hour at 37° and was stopped by placing the tubes in boiling water for two min. Tubes were then placed on ice and 40 ul of distilled water and 10 ul of purified aminopeptidase M was added. The tubes were then incubated for two hours at 37°. The reaction was stopped by addition of 250 ul of a 10% TCA solution. For inhibition studies, 5 ul of 5 mM N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Chu and Orlowski, 1984) (final concentration 10-4 M) or 5 ul of 10 mM o-phenanthroline (final concentration 2 X 10-4 M) was added to the incubation mixture or DTT was not added to the incubation mixture.

Prolyl endopeptidase: Activity was determined using the substrate Z-Gly-Pro-SM as described by Orlowski et al. (1979). The incubation mixture contained 50 ul of Z-Gly-Pro-SM (5 mM in 0.1 M Tris-HCl buffer, pH 8.3), 180 ul of 0.1 M Tris-HCl buffer (pH 8.3), 10 ul of 10 mM DTT, and 10 ul of GH3 cell homogenate or rat anterior pituitary homogenate or 20 ul mouse tissue homogenate. Incubations were for 30 min for GH3 cell homogenates, 1 hour for anterior pituitary homogenates and 10 min for mouse tissue homogenates, at 37°. The reaction was stopped by addition of 250 ul of 10% TCA. For inhibition studies, 10 ul of 2.5 X 10-5 M Z-Pro-Prolinal (Wilk and Orlowski, 1983a) was added to the incubation mixture to give a final inhibitor concentration of 10-6 M.

Cathepsin B: Activity was determined using the substrate Z-Leu-Leu-Arg-2NA as described by Orlowski et al. (1981). The incubation mixture (final volume 250 ul) contained 10 ul of Z-Leu-Leu-Arg-2NA (10 mM in DMSO), 5 ul of 100 mM EDTA, 25 ul of 10 mM DTT, 0.2 M acetate buffer (pH 4.8) and either 10 ul of GH3 cell homogenate or 20 ul of anterior pituitary homogenate. Incubations were carried out at 37° for 1 hour and stopped by the addition of 250 ul of 10% TCA. For inhibition studies, 10 ul of 2.5 X 10-5 M leupeptin (final concentration 10-6 M) was added to the incubation mixture.

Cathepsin D: Activity was determined using the substrate TFA•D-Phe-(O-Benzyl)Ser-Phe-Phe-Ala-Ala-pAB (Orlowski et al., 1984). Cathepsin D cleaves the Phe-Phe bond of the substrate

and the chromogen is released by incubation with excess aminopeptidase M in a coupled assay (Orlowski et al., 1984). The incubation mixture contained 20 ul of the substrate, 420 ul citrate phosphate buffer (0.05 M, pH 3.4), and 10 ul of homogenate preparation and was incubated for 1 hour at 37°. To stop the reaction, the tubes were placed in boiling water for 5 min. The tubes were then placed on ice and 100 ul of 1 M Trizma base and 20 ul of purified aminopeptidase M was added. The tubes were further incubated for 2 hours at 37° and the reaction was stopped by the addition of 230 ul of 30% TCA. The diazotization reaction was modified as follows: to 400 ul of supernatant, 150 ul of 0.34% NaNO2 was added. After 3 min. 200 ul of 1.25% ammonium sulfamate was added. After 2 min. 500 ul of 0.1% N-1-Naphthyl-ethylenediamine diHCl in 95% ethanol was added. The absorbance was determined at 540nm to quantitate the pAB released.

Aminopeptidase: Activity was determined using the substrate Leu-pNA. The incubation mixture (final volume 250 ul) contained 10 ul of 10 mM Leu-pNA (in methanol), 0.05 M Tris-HCl buffer (pH 7.5) and either 25 ul of GH3 cell homogenate or 10 ul of anterior pituitary cell homogenate. Incubations were carried out at 37° for 1 hour and stopped by addition of 250 ul of 10% TCA.

Membrane-bound Neutral Metalloendopeptidase: Activity was determined using the substrate Glutaryl-Ala-Ala-Phe-2NA as described by Orlowski and Wilk (1981). The enzyme cleaves the

Ala-Phe bond and the chromogen is released by incubation with excess aminopeptidase M in a coupled assay. The incubation mixture (final volume 250 ul) contained 10 ul of 10 mM Glutaryl-Ala-Ala-Phe-2NA (in DMSO), 0.05 M Tris-HCl buffer (pH 7.5), 20 ul of purified aminopeptidase M and 40 ul of homogenate. Incubations were carried out for one hour and stopped with 250 ul 10% TCA. For inhibition studies, 10 ul of 1 mM N-[1(R,S)-carboxy- 2-phenylethyl]-Phe-pAB (Almenoff and Orlowski, 1983) (final concentration 4 X 10-5 M) was added to the incubation mixture.

Pyroglutamyl peptide hydrolase: Activity was determined using the substrate pGlu-2NA. The incubation mixture (final volume 250 ul) contained 10 ul of 10 mM pGlu-2NA (in DMSO), 20 ul of 20 mM DTT, 20 ul of 20 mM EDTA (pH 7.5), 50 mM Tris-HCl buffer (pH 7.5) and 50 ul of homogenate. For studies comparing pyroglutamyl peptide hydrolase activity in GH3 cell homogenates with activity in rat anterior pituitary homogenates, 50 ul 10 mM DTT and 5 ul 0.1 M EDTA (pH 7.5) were used instead. Incubations were carried out for 1 hour at 37° and were stopped with 250 ul of 10% TCA.

Multi-catalytic Protease Complex: The activity of the chymotrypsin-like component was assayed using the substrate Z-Gly-Gly-Leu-pNA as described by Wilk and Orlowski (1980). The incubation mixtue (final volume 250 ul) contained 10 ul of 10 mM Z-Gly-Gly-Leu-pNA (in DMSO), 0.01 M Tris-EDTA buffer (pH 8.3) and either 20 ul of anterior pituitary homogenate or 25

ul of GH3 cell homogenate. Incubations were carried out at 37° for 1 hour and stopped with 250 ul of 10% TCA.

The SDS-activated component of the multi-catalytic protease complex was assayed using the substrate Z-Leu-Leu-Glu-2NA as described by Wilk and Orlowski (1980). Incubation mixtures (final volume 250 ul) contained 10 ul of 10 mM Z-Leu-Leu-Glu-2NA, 0.01 M Tris-EDTA buffer (pH 8.3), 10 ul of 1% SDS and either 20 ul of anterior pituitary homogenate or 25 ul of GH3 cell homogenate. Incubations were carried out for 1 hour at 37° and stopped with 250 ul of 10% TCA.

Diaminopeptidase IV: The activity was determined using the substrate Gly-Pro-2NA. The incubation mixture (final vol 250 ul) contained 0.05 M Tris-HCl buffer (pH 7.5), 10 ul purified diaminopeptidase IV (0.084 units) and 10 ul 10 mM Gly-Pro-2NA (in DMSO). Incubations were for 30 min at 37°. The reaction was stopped by the addition of 250 ul 10% TCA and the release of free 2NA was quantitated by diazotization.

Membrane-bound pyroglutamyl-peptide hydrolyzing activity The activity was determined with pGlu-His-Pro-2NA in a coupled assay with excess DAP IV in the presence of Z-Pro-Prolinal, a specific inhibitor of prolyl endopeptidase (Wilk and Orlowski, 1983a). The assay is based on the following reaction sequence:

membrane-bound enzyme

(1) pGlu-His-Pro-2NA ----------------------> pGlu + His-Pro-2NA

DAP IV

(2) His-Pro-2NA ----------------------> His-Pro + 2NA

Z-Pro-Prolinal blocks the cleavage of the Pro-2NA bond by prolyl endopeptidase. The incubation mixture (250 ul final volume) contained 10 ul Z-Pro-Prolinal (final concentration 10-5 M), 10 ul DAP IV, 50 ul serum or brain fraction and 50 mM Tris-HCl buffer (pH 7.5). Tubes were preincubated for 10 min at 37° and 10 ul pGlu-His-Pro-2NA (10 mM in DMSO) was then added. The reaction proceeded for 2 h and was stopped by 250 ul 10% TCA. Free 2NA was quantitated as described.

Identification of cleavage products of pGlu-His-Pro-2NA

Rat serum and rat washed brain pellets were prepared as described above. DFP was added to inhibit endogenous DAP IV and prolyl endopeptidase. The incubation mixture (250 ul final volume) contained 10 ul DFP (final concentration 0.66 mM), 10 ul PDMK (final concentration 10-5 M), 50 ul serum or washed brain pellet and 50 mM Tris-HCl buffer (pH 7.5). The tubes were preincubated for 30 min at 37° and 10 ul pGlu-His-Pro-2NA (50 mM in DMSO) was then added. The reaction proceeded for 17 h at which time the incubation mixtures as well as equivalent reaction mixtures which were not incubated were spotted on silica G thin-layer chromatography plates. Authentic His-Pro-2NA as well as His-Pro-2NA generated by incubation

of pGlu-His-Pro-2NA with partially purified bovine brain PPH were also spotted. The plates were developed with both solvent system I (chloroform: methanol: 25% ammonia; 5:3:1) and solvent system II (1-propanol: water: ammonia; 7:3:1) and were visualized under a ultraviolet lamp and by spraying with the Pauly reagent (Krebs et al., 1969).

Kinetic studies of inhibitors

Partially purified PPH (see Methods) and purified prolyl endopeptidase, aminopeptidase M and diaminopeptidase IV were preincubated with inhibitor at 37° for 10 min. The reactions were initiated by addition of substrate. The Ki of 5-oxoprolinal for pyroglutamyl peptide hydrolase was calculated by the methods of Dixon (1953) and Henderson (1972). The Ki of other inhibitors was calculated by the method of Dixon (1953).

Determination of Ki for TRH

The affinity of enzymes toward TRH was studied by determining the inhibition of cleavage of synthetic substrates after addition of several concentrations of TRH. Homogeneous rabbit brain prolyl endopeptidase was assayed as described above. Partially purified bovine brain PPH was assayed as described above in the presence of Z-Pro-Prolinal (10-5 M final concentration) to inhibit contaminating prolyl endopeptidase. The membrane-bound pyroglutamyl-peptide hydrolyzing enzyme was assayed as described above using a washed particulate fraction

of a rat brain homogenate in the presence of Z-Pro-Prolinal and PDMK (10-5 M final concentration of both) to inhibit any contaminating prolyl endopeptidase and PPH respectively. After 10 min preincubation with various concentrations of TRH, substrate was added. The Ki was determined according to the method of Dixon (1953) using two concentrations of substrate.

Determination of TRH degradation

The incubation mixture (final volume 250 ul) contained 10 ul PDMK or Z-Pro-Prolinal (10-5 M) or buffer, 50 ul of rat tissue preparation (serum, 10% brain homogenate or 10% resuspended washed brain pellet) and 50 mM Tris-HCl buffer (pH 7.5). In tubes receiving DTT and EDTA, 20 ul 20 mM DTT and 20 ul 20 mM EDTA (pH 7.2) were added. After a 10 min preincubation, 20 ul TRH (100 ng) was added. Incubations were carried out for 2 h at 37° and were stopped with 250 ul methanol. Control tubes received TRH after the incubation. The tubes were centrifuged at 1000 g for 10 min, 50 ul supernatant was removed and evaporated to dryness under a stream of nitrogen. The samples were reconstituted in 500 ul of RIA buffer (0.1 mM thimerosal, 5% solution of trasylol containing 50,000 Kallikrein Inactivator Units/100 ml buffer and 0.2% bovine serum in PBS) and the samples were frozen at -20° until the RIA was performed.

LHRH degradation

LHRH degradation was studied using purified calf liver

pyroglutamyl peptide hydrolase. Z-Pro-Prolinal was added to prevent degradation by any contaminating prolyl endopeptidase. Incubation mixtures contained 31 ul LHRH (62.5 ug), 10 ul Z-Pro-Prolinal (final concentration 10-5 M), 20 ul 20 mM DTT, 20 ul 20 mM EDTA (pH 7.2), Tris-HCl buffer (pH 7.5) and 50 ul pyroglutamyl peptide hydrolase (3.6 mg lyophilized powder/ml buffer). Incubation tubes contained either 25 ul 5-oxoprolinal (final concentration 10-6 M) or the equivalent amount of buffer. Incubations were carried out at 37° and the reaction was monitored using a Perkin-Elmer series 2 HPLC equipped with a variable-wavelength spectrophotometric detector (LC-55). 20 ul of incubation mixture was chromatographed on a 250 X 4 mm Bio-Sil OD-5S reverse phase column (Bio-Rad Labs, Richmond, CA) as described by Wilk and Orlowski (1982). After 3 h of incubation, most of the LHRH was converted to product in the tube without inhibitor. The incubation mixture in the sample lacking inhibitor was then applied to the column and the product peak (retention time of 9 min) was collected, hydrolyzed and analyzed on a Technion TSM amino acid autoanalyzer as described by Wilk and Orlowski (1982).

RADIOIMMUNOASSAYS

TRH: The RIA procedure for TRH was based on the method of Jeffcoate et. al. (1973). Each assay tube (final volume 300 ul) contained 30 ul sample (20 ul of hypothalamic sample) or 25 ul standard TRH, 100 ul antiserum (1/45,000 final dilution, 69.6 ug of lyophilized powder corresponds to 1 ul of serum).

40 ul [^{125}I]TRH (2.5 pg/assay or about 10,000 cpm/assay) and the above RIA buffer. Incubations were carried out for 18 h at 4°. Then 50 ul of 1/10 diluted rabbit serum and 3 ml of cold 1-propanol was added. The tubes were incubated at 4° for an additional 30 minutes and then centrifuged at 1000 g for 45 minutes. The supernatant was removed and the pellet and supernatant were counted in a LKB 1275 Minigamma counter. Blank tubes without antiserum were carried through the experiments. The (% bound minus blank) was calculated for all samples using the PROPHET computer system which is a national computer resource supported by the Biomedical Research Technology Program, Division of Research Resources, National Institutes of Health. A standard curve was constructed as log [TRH] vs (% bound minus blank) and the amount of TRH in the unknown was determined from the curve. The degradation of TRH in vitro was assayed in duplicate and the RIA for each of the two tubes was run in triplicate. The amount of TRH remaining after incubation was compared to control tubes with no incubation to determine percentage degradation. The antiserum does not cross-react with TRH-OH. Z-Pro-Prolinal and PDMK do not affect the RIA for TRH.

LHRH: The assay for LHRH was similar to that of TRH. To each tube, 30 ul of LHRH antiserum (final dilution of 1/1200), 50 ul of standard LHRH and 170 ul of RIA buffer were added. For half-life experiments, the buffer contained 0.1 mM PCMB and 1 mM o-phenanthroline to prevent degradation of LHRH by serum enzymes. The samples were incubated for 3 days at 4° and then

40 ul of I-125 LHRH (approximately 10,000 cpm/tube) was added. Incubation continued for anotner 3 days at 4°. Precipitation and calculations were performed as described for TRH. The standard curve was linear from about 6.25 pg to 100 pg of LHRH.

The 1-9 LHRH fragment was generated for cross-reactivity studies by incubating 100 ug of LHRH with 50 ul homogeneous prolyl endopeptidase, 50 ul 10 mm DTT and 90 ul 0.1 M Tris-HCl buffer (pH 8.3) for 19 h. An aliquot of the incubation mixture was monitored by HPLC as described above. Authentic LHRH had a retention time of 10 min and 1-9 LHRH had a retention time of 12 min. This fragment was then assayed by RIA for cross-reactivity with LHRH and was found not to displace authentic LHRH even at 2000 pg (20 times greater than the largest amount of LHRH on the standard curve).

Prolactin: The assay for prolactin is based on a procedure established by the NIADDK. The buffer used was the same as for TRH. The antiserum and standard prolactin were supplied by the NIADDK. Iodination of supplied prolactin was unsuccessful.and therefore commercial iodinated prolactin was used. The media from GH3 cells (see below) was diluted 1/6 for the 1 h experiments and diluted 1/300 for the 3 day experiments. To each tube, 100 ul of antiserum (final dilution of 1/2600), 100 ul of standard prolactin (PRL-RP-3) or 40 ul of sample and enough buffer to bring the final volume to 340 ul were added. The tubes were incubated at 4° for 24 hours. 60 ul of I-125

prolactin (approximately 15,000 cpm) was then added to the incubation mixture and the incubation continued for 24 hours at 4° at which time 50 ul of rabbit serum (1/10 dilution) and 50 ul of goat anti-rabbit second antibody was added. After another 24 hours of incubation at 4°, 400 ul of additional RIA buffer was added and the free and bound prolactin were separated as for the TRH RIA. The standard curve was linear between 125 pg and 2000 pg of prolactin.

TSH: The assay for TSH is based on a procedure established by the NIADDK. Antiserum, standard TSH and TSH for iodination were supplied by the NIADDK. The iodination of TSH used the miniiodination method of Ewen and Warren (1983). To a small vial, 2 ug of TSH in 45 ul of 0.05 M Na Phosphate buffer (pH 7.5) was added followed by 100 ul of Na-I-125 (0.1 mC) and 10 ul of chloramine T (2.5 mg/ml in PBS). After 60 seconds at room temperature, 100 ul of Na metabisulfite (2.5 mg/ml in PBS) was added. After another 30 seconds, 200 ul of 1% KI was added. The reaction mixture was applied to a Biogel P 60 column washed with 1% BSA and an initial peak of iodinated TSH was obtained which was separated from the free iodine peak. The iodinated TSH was aliquoted and frozen at -20° until use.

The RIA for TSH used 50 ul of antiserum (1/4500 final dilution), 50 ul of standard TSH (r-TSH-RP-2) or 100 ul of rat serum and RIA buffer so that a final volume of 230 ul was obtained. After incubation at 4° for 2 days, 70 ul of I-125 TSH (about 10,000 cpm /tube) was added. After 3 more days of

incubation at 4°, second antibody precipitation was used as described for prolactin with the modification that 500 ul of buffer was added before centrifugation. The TSH standard curve was linear from 50 pg to 1600 pg of TSH. 100 ul of normal rat serum contains about 50 pg of TSH which is at the lowest limit of sensitivity of the assay.

ANIMAL EXPERIMENTS

Male Swiss Albino mice weighing approximately 35 g and male Sprague-Dawley rats weighing about 250 g were used for all experiments. They were fed a commercial Purina laboratory chow diet.

Effect of Z-Pro-Prolinal on prolyl endopeptidase activity in mouse tissue

Prolyl endopeptidase activity was measured in mouse tissues 30 min after intraperitoneal administration of either Z-Pro-Prolinal (1.25 mg/kg in 10% methanol) or vehicle. The mice were killed by cervical dislocation and brain, heart, skeletal muscle, lung, spleen, duodenum and pancreas, liver and kidney were removed and placed on ice. After addition of 10 volumes of ice cold 0.1 M Tris-HCl buffer (pH 7.0) the tissues were disrupted using a Polytron homogenizer (Brinkmann Instruments) and then homogenized in an ice-cooled homogenizer equipped with a Teflon pestle. Prolyl endopetidase activity was determined with Z-Gly-Pro-SM as a substrate as described above.

Determination of inhibition of prolyl endopeptidase in vivo

Z-Pro-Prolinal was administered by intraperitoneal injection into mice as a solution in 10% methanol, followed at various time intervals by Z-Gly-Pro-SM (145 mg/kg). Control mice received injections of the vehicle. Homogenates were prepared as described above. SM levels were determined using the highly sensitive diazotization described above. A standard curve constructed by carrying known amounts of SM through the procedure was used to calculate the concentration of SM. The concentration of SM in animals receiving inhibitor was compared to the concentration of control animals using the one-tailed Student's T-test.

Effect of 5-oxoprolinal on pyroglutamyl peptide hydrolase activity in mouse tissues

5-oxoprolinal (50 mg/kg in 50% ethanol) or vehicle was administered to mice by intraperitoneal injection. After 10 min or 30 min. mice were killed by cervical dislocation. One mouse received 8 injections of 5-oxoprolinal (50 mg/kg) over a period of 4 days. The tissues were immediately removed and placed on ice. After addition of 5 volumes of ice cold 0.05 M Tris-HCl buffer (pH 7.5), the tissues were disrupted using a Polytron homogenizer (Brinkmann Instruments) and then homogenized in an ice-cooled homogenizer equipped with a teflon pestle. Pyroglutamyl peptide hydrolase activity in the homogenates was determined as described above using 50 ul homogenate. After a 1 h incubation at 37°, the reaction was stopped by

addition of 250 ul 10% TCA. Control tubes in which the enzyme and substrate were omitted separately were also carried through the procedure. Tubes were then centrifuged and 375 ul of supernatant of all organs except liver and kidney were removed. For liver and kidney incubations, 75 ul of supernatant was diluted with 300 ul of 0.05 M Tris-HCl buffer (pH 7.5). 2NA concentrations were determined using the sensitive diazotization method described above. The activity of pyroglutamyl peptide hydrolase in animals receiving 5-oxoprolinal was compared to the activity of control animals using the one-tailed Student's T-test.

Effect of pyroglutamyl diazomethyl ketone on pyroglutamyl peptide hydrolase activity in mouse tissues

Pyroglutamyl diazomethyl ketone (in water) or vehicle was administered to mice by intraperitoneal injection. 5 mice received a dose of 0.1 mg/kg and were killed after 24 h, 3 mice received a dose of 0.1 mg/kg and were killed after 1 h and 3 mice received a dose of 0.01 mg/kg and were killed after 1 h. One mouse received a dose of 0.1 mg/kg and was killed after 5 days. The mice were killed by cervicle dislocation, the tissues were removed and homogenized as described above and PPH was assayed as described above. The activity of pyroglutamyl peptide hydrolase in animals receiving diazomethyl pyroglutamate was compared to the activity of control animals using the one-tailed Student's T-test.

Effect of inhibitors on the particulate pyroglutamyl-peptide hydrolyzing enzyme in rat brain

A solution of Z-Pro-Prolinal (5 mg/kg) and diazomethyl pyroglutamate (1 mg/kg) was injected twice into a rat at time 0 and 10 h and the rat was killed after an additional hour. After addition of 10 volumes of Tris-HCl buffer, the tissue was homogenized, the washed pellet was prepared and the particulate pyroglutamyl-peptide hydrolyzing enzyme was assayed as described above.

Preparation of Anterior Pituitary Homogenates

Pituitaries were removed from male Sprague Dawley rats (225-280g) after decapitation and placed on ice. The anterior pituitaries were removed under a dissecting glass. After addition of 20 volumes of ice cold 0.1M Tris-HCl buffer (pH 7.0), the tissues were homogenized in an ice-cooled homogenizer equipped with a teflon pestle. Homogenates were kept on ice and used on the same day as the preparation to prevent loss of enzymatic activity due to storage.

Preparation of rat organs for distribution studies of pyroglutamyl-cleaving enzymes

Rats were decapitated and the blood was collected and allowed to clot. The blood was centrifuged at 2000 g for 20 min. The serum was removed and assayed immediately for enzyme activity. For organ distribution experiments, the brain, heart, skeletal muscle, lung, spleen, liver and kidney were

removed and placed immediately on ice. After addition of 5 volumes of ice-cold 0.1 M Tris-HCl buffer (pH 7.5), the tissues were disrupted using a Polytron homogenizer (Brinkmann Instruments) and then homogenized in an ice-cooled homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 12,000 g for 20 min. The supernatant was decanted and the pellet was resuspended in the original volume of Tris-HCl buffer, recentrifuged, supernatant decanted and the pellet was once more suspended in the original volume of Tris-HCl buffer. The washed pellet was immediately assayed for membrane-bound enzyme activity.

For experiments on enzyme distribution in brain, the brain was removed and the hypothalamus, cortex, hippocampus, striatum, thalamus, brainstem and cerebellum were rapidly dissected and immediately placed on ice. The pituitary was also removed. After addition of 5 volumes of ice-cold 0.1 M Tris-HCl buffer (pH 7.5), the tissues were homogenized as described above. 300 ul of homogenate was centrifuged in a desk top centrifuge at 9000 g for 10 min. The supernatant was removed and the pellet was resuspended in 300 ul of buffer, recentrifuged, supernatant decanted and the washed pellet once more suspended in 300 ul of buffer. Both soluble and particulate fractions were assayed immediately. Whole brain homogenates, supernatants and washed pellets used for studies on the degradation of TRH and chromogenic substrates were prepared similarly.

Effect of inhibitors on hormone levels in rat tissues

Rats received either Z-Pro-Prolinal (5 mg/kg in 30% ethanol), diazomethyl pyroglutamate (1 mg/kg in water), both inhibitors or vehicle. After 15, 30 or 60 min or 18 h the rat was sacrificed by cervical dislocation and the hypothalamus, pituitary and a section of the frontal cortex was removed and placed on dry ice. The blood was also collected and serum was prepared as described above. The serum was stored at -20° and was assayed for prolactin and TSH as described above. To the tissues, 1 ml of 90% methanol containing 0.1 mM PCMB was added and the tissues were homogenized. The sample was centrifuged at 10,000 g for 15 minutes and the supernatant removed. The supernatants were then evaporated under a stream of nitrogen or by centrifugation under vacuum (Savant Instruments) and were reconstituted in RIA buffer (500 ul for hypothalamic samples, 150 ul for pituitary samples and 75 ul for cortical samples) and stored at -20° until the RIA was performed.

Effects of inhibitors on LHRH half-life in rats

Rats were anesthetized with urethane (1.2 mg/kg) 30 minutes before surgery. The animal's temperature was maintained at 37° by using a heat lamp with a rectal probe. The femoral artery and vein were isolated and cannulas were introduced. The arterial line contained 1% heparin in saline. Z-Pro-Prolinal (1.25 mg/kg) was injected into the femoral vein 2 min before injection of LHRH (210 ug/kg in saline) into the femoral vein. 0, 2, 5, 10, 15, 20, 25, 30, 40 and 60 min after

injection of LHRH, 200 ul of blood was collected from the femoral artery into a plastic tube containing 1 ul of a solution of 100 mM o-phenanthroline and 10 mM p-chloromercuribenzoic acid. This inhibitor solution was added to prevent degradation of LHRH by blood after collection. The blood was centrifuged on a desk top centrifuge (Beckman) for 5 minutes at 9000 g and the plasma was removed. Plasma was frozen at -20° until analysis by RIA. After determination of LHRH concentration in the plasma samples by RIA as described above, the pharmacokinetics of LHRH disappearance was analyzed by the DRUGFUN program of the PROPHET Computer System which simulates a two compartment model with a bolus injection. The duplicate measurements of each RIA determination were averaged and the points were weighted by 1/coefficient of variation of the duplicates.

GH3 CELL STUDIES

A line of GH3 cells was obtained from Dr. Marvin Gershengorn of Cornell Medical Center and grown in the laboratory of Dr. Terry Davies. Cells were cultured in 75 cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) containing 12 ml of Hams F10 medium supplemented with 15% horse serum, 2.5% fetal calf serum (Tashjian et al., 1968) and 1% penicillin-streptomycin-fungizone. The media was filtered through a Millipore 0.45 um filter unit before use. All additions were sterilized by filtering through a Millipore 0.22 um filter unit. The media was changed twice a week.

Preparation of GH3 cells for determination of enzymatic activities

Two weeks after plating, the GH3 cells were harvested by addition of 0.02% EDTA. The cells were centrifuged at 300g for 10 min and washed with 5 ml of 0.1M Tris-HCl buffer (pH 7.0). After centrifugation at 300g for 10 min, the cells were placed on ice and the above buffer was added to the cells to give a protein concentration of about 2 mg/ml. Cells were homogenized in an ice-cooled homogenizer equipped with a Teflon pestle. Cell homogenates were used for assaying within 2 days of homogenization to prevent loss of enzymatic activity. For experiments in which the inhibitor was added to the cells, the cells were transferred from 75 cm^2 flasks to 25 cm^2 flasks and received 5 ml of the above media. Two weeks after replating, the cells received inhibitor for the indicated period of time. Cells were harvested by addition of 0.02% EDTA, centrifuged at 300g for 10 min and washed with 5 ml phosphate-buffered saline. After centrifugation at 300g for 10 min, the cells were placed on ice and resuspended in 180 ul 0.1 M Tris-HCl buffer (pH 7.0) and homogenized as described above.

Effect of inhibitors on enzymatic activity in GH3 cells

Initially the effect of Z-Pro-Prolinal on prolyl endopeptidase activity in GH3 cells was studied. Cells were plated in 35 mm diameter sterile wells in plates of 6 wells/plate (Costar, Cambridge, MA) to a density of about 0.1 mg protein/well.

3 ml of media were added to each well. For one hour studies, sterile Z-Pro-Prolinal was added to a final concentration of 10-6 M. After 1 hour, the media was removed and the GH3 cells were harvested by the addition of 0.02% EDTA. For 3 day experiments, Z-Pro-Prolinal was added to the cells so that a final concentration of 10-5 M was obtained and the cells were harvested after 3 days. The cells were washed, centrifuged and homogenized and prolyl endopeptidase was assayed as described above. Soluble metalloendopeptidase activity was also assayed as a control.

The effect of 5-oxoprolinal on pyroglutamyl peptide hydrolase activity was then studied. Sterile 5-oxoprolinal was added to GH3 cells in 25 cm^2 flasks to a final concentration of 10-5 M. Initially the cells were incubated for 3 days. The cells were harvested, washed and homogenized and assayed for pyroglutamyl peptide hydrolase activity as described above. Upon finding that pyroglutamyl peptide hydrolase activity increased when GH3 cells were exposed to 5-oxoprolinal, concentration-response and time-course studies were conducted to study this increase. For concentration-response studies, 5-oxoprolinal was added to the cells so that final concentrations ranging from 10-4 M to 10-8 M were obtained. The cells were incubated for 3 days. For time-course studies, 5-oxoprolinal (10-5 M) was added to the cells and the cells were incubated from 9 h to 6 days.

Specificity of the increase in pyroglutamyl peptide hydrolase activity

5-Oxoprolinal (10-5 M) was added to the GH3 cells for 3 days. The cells were harvested, washed and homogenized and prolyl endopeptidase activity was determined as described above. Similarly, Z-Pro-Prolinal (10-5 M) was added to the cells and pyroglutamyl peptide hydrolase activity was determined as described above. TRH (10-6 M) and the TRH metabolites, pyroglutamate (10-4 M) and cyclo(His-Pro) (10-4 M) were also added to GH3 cells for 3 days and pyroglutamyl peptide hydrolase and prolyl endopeptidase activity were assayed as described above.

Effect of 5-oxoprolinal in vitro on pyroglutamyl peptide hydrolase

GH3 cells were exposed to 5-oxoprolinal (10-5 M) for 3 days. The cells were harvested, washed and homogenized as described above. Pyroglutamyl peptide hydrolase was assayed as described above in the presence and absence of 5-oxoprolinal (10-5 M) in the incubation mixture.

Exposure studies of 5-oxoprolinal in GH3 cells

Flasks of cells received 5-oxoprolinal (10-5 M) and then after 1 h the media was replaced. In other experiments, 5-oxoprolinal (10-5) was added on days 0, 1 and 2. On day 3, the cells were harvested, washed, homogenized and assayed for pyroglutamyl peptide hydrolase as described above. Control

flasks and flasks receiving 5-oxoprolinal (10-5 M) for 3 days were carried through the procedure in parallel.

Km determination of pyroglutamyl peptide hydrolase

Cells receiving 5-oxoprolinal (10-5 M) for 3 days were grown in parallel with control cells in 75 cm^2 flasks. Cells were harvested, washed and homogenized as described above. Substrate concentrations between 0.066 mM and 2 mM were used. The reaction proceeded for 1 h and was stopped by placing in boiling water for 2 min. 250 ul of water was added and free 2NA was determined by fluorometry as described above. Lineweaver-Burk plots obtained by a linear regression program were used to calculate the Km.

Subcellular distibution of pyroglutamyl peptide hydrolase in GH3 cells

GH3 cells exposed to 5-oxoprolinal (10-5 M) for 3 days and parallel control cells were harvested, washed and homogenized as described above. The homogenate was centrifuged in a desk top centrifuge at 9000g for 10 min. The supernatant was removed and the pellet was washed with 0.1 M Tris-HCl buffer (pH 7.5), recentrifuged and resuspended in buffer. 10 ul of the supernatant and 10 ul of the washed pellet were assayed for pyroglutamyl peptide hydrolase activity as described above. After 1 h, the reaction mixture was placed on ice and diluted with 250 ul water. The release of free 2NA was determined by fluorometry using an excitation wavelength of 355 nm

and an emission wavelength of 440 nm. A blank tube which received substrate at the end of the incubation was carried through the experiment. The amount of free 2NA was calculated from a standard curve constructed with known amounts of 2NA.

Cycloheximide experiments in GH3 cells

GH3 cells were exposed to cycloheximide at a concentration of 0.5 ug/ml for 24 h or at a concentration of 2 ug/ml for 10 h. No cytotoxic changes were apparent microscopically during either condition. For each experiment, 3 flasks were used as controls. 3 flasks received 5-oxoprolinal (10-5 M), 3 flasks received cycloheximide and 3 flasks received cycloheximide plus 5-oxoprolinal (10-5 M). The cells were harvested, washed, homogenized and assayed for pyroglutamyl peptide hydrolase and prolyl endopeptidase activity as described above.

Studies on the degradation of pGlu-2NA by GH3 cells

1 ml of media was added to the GH3 cells followed by addition of 10 ul of 40 mM pGlu-2NA in DMSO (final concentration 0.4 mM). The cells were incubated for 1 h and 3 days in the presence and absence of 5-oxoprolinal (10-5 M). The media was removed and combined with the harvested cells. After homogenization, free 2NA was determined in the media plus cell mixture by diazotization.

Effect of inhibitors and substrates on TRH binding in GH3 cells

The TRH binding experiment was performed with the help of Dr. Marvin Gershengorn. 10 ul of Z-Pro-Prolinal, 5-oxoprolinal, pGlu-2NA or Z-Gly-Pro-SM was added to 900 ul GH3 cell suspension (2 million cells/ tube) so that the final concentration of each compound was 100 nM. Unlabelled TRH (1 uM) was added to measure nonspecific binding. This was followed by addition of 100 ul 3H-TRH (10 nM final concentration). The tubes were incubated at 37° for 1 h, the cells were then washed twice with GH3 media and 400 ul 0.4 N NaOH was added. The cells were frozen and thawed to disrupt the pellet and 100 ul of incubation was placed in a scintillation vial and counted in a scintillation counter.

Effect of inhibitors on prolactin response in GH3 cells

The effects of inhibitors on prolactin response to TRH in GH3 cells used the methods of Dannies and Tashjian (1976). The effect of inhibitors on prolactin response was measured in two types of experiments. In the first type of experiment, new media was added to the cells along with the inhibitor in either the presence or absence of TRH (10 nM final concentration). Either 5-oxoprolinal (10-5 M), Z-Pro-Prolinal (10-5 M) or both inhibitors were added to the cells and were compared with control cells and cells receiving only TRH (10 nM). Incubation for 1 h measured prolactin release and for 3 days measured prolactin synthesis. The media was removed and assayed for prolactin as described above.

In a second type of experiment, 5-oxoprolinal was added to GH3 cells for 3 days and compared to a parallel set of control cells. The cells were washed with 3 ml of media and 3 ml of new media was added. At this time half of the cells received TRH (10 nM) and the other cells were used as controls. The cells were incubated further and the media was removed after 1 h and 3 days and assayed for prolactin as described above.

Assay of DNA in GH3 cells

DNA in GH3 cells was assayed according to the method of Hinegardner (1971). The cells were harvested with 3 ml 0.02% EDTA, centrifuged at 300g for 10 min, washed with 5 ml PBS and recentrifuged. The cells were resuspended in 150 ul 0.1% SDS. 50 ul of the cell suspension was added to 100 ul of a diaminobenzoic acid dihydrochloride (DABA) solution (0.4 g in 5 ml water plus 0.02 g of activated charcoal filtered through a Millipore 0.22 um filter unit). The cell plus DABA mixture was incubated for 30 min at 60° and stopped by addition of 1.5 ml 1 N HCl. The mixture was read in a spectrofluorometer at an excitation wavelength of 410 nm and an emission wavelength of 502 nm. The DNA was determined by constructing a standard curve with known amounts of DNA (Calf thymus, type I) in SDS.

RESULTS

IN VITRO ASSAYS

In vitro inhibition of pyroglutamyl peptide hydrolase by 5-oxoprolinal

5-oxoprolinal was synthesized according to the method of Saito et al. (1980) (Figure 4). It was found to be a potent inhibitor of pyroglutamyl peptide hydrolase in vitro. Analysis by the method of Dixon (1953), revealed a Ki of 20 nM (mean of 3 experiments) (see Figure 5) with the intersection of the lines of different substrate concentrations occurring above the x-axis, a condition indicative of competitive inhibition. The analysis of the kinetics of this tight binding inhibitor was also carried out by the method of Henderson (1972). In this method, the inhibitor concentration divided by the degree of inhibition is plotted on the ordinate and the velocity without inhibitor divided by the velocity with inhibitor is plotted on the abscissa. A linear plot was obtained for three different substrate concentrations and the slope was found to increase with increasing substrate concentrations, a condition indicative of competitive inhibition. When the slope of each of the three lines in this plot was plotted against the substrate concentration, a linear plot was obtained with the y-intercept of 26 nM equal to the Ki.

Other compounds related to 5-oxoprolinal were tested for

Figure 5

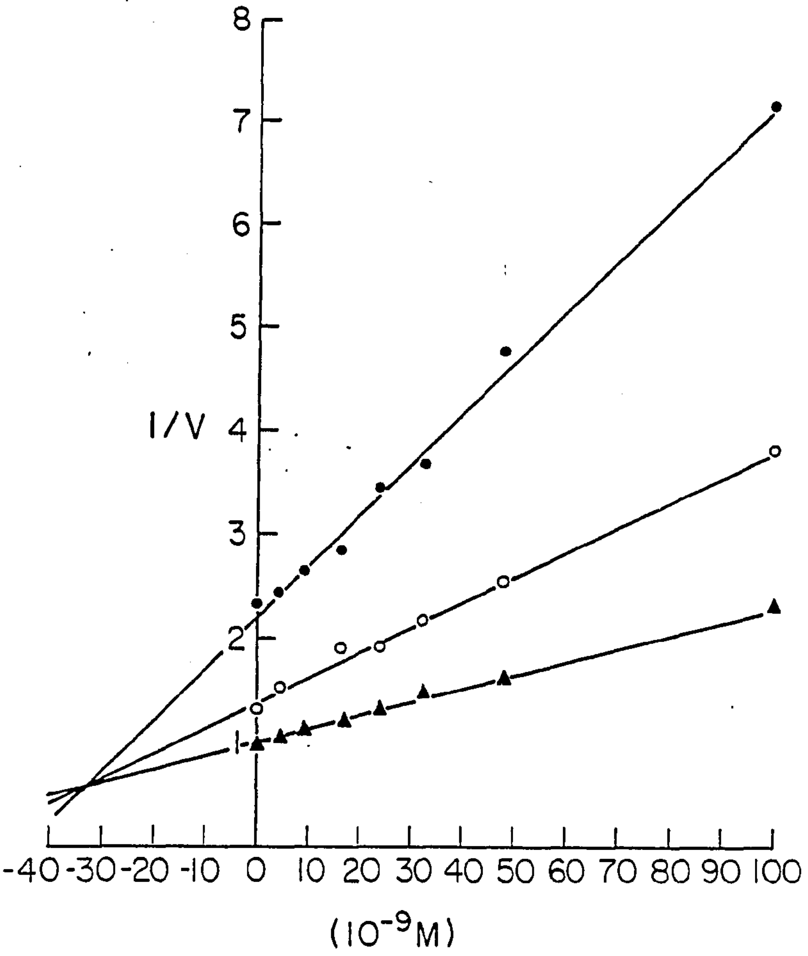


Figure 5

A Dixon plot of the inhibition of pyroglutamyl peptide hydrolase by 5-oxoprolinal. The inhibitor was preincubated for 10 min at 37° C with the enzyme and the reaction was initiated by addition of pGlu-2NA as described in the Methods section.

▲ conc. of substrate = 0.4 mM

○ conc. of substrate = 0.2 mM

● conc. of substrate = 0.1 mM

their ability to inhibit pyroglutamyl peptide hydrolase activity. As shown in Table 1, pyroglutamic acid (5-oxoproline) and the alcohol derivative of pyroglutamic acid (5-oxoprolinol) inhibited enzymatic activity with a Ki of 0.85 mM and 0.65 mM respectively. These Ki's are over 10,000 fold higher than that of the aldehyde. The Ki of Z-Pro-Prolinal, the specific aldehyde inhibitor of prolyl endopeptidase (Wilk and Orlowski. 1983a). on pyroglutamyl peptide hydrolase was found to be 7.5 mM. This is over 5 orders of magnitude higher than its Ki for prolyl endopeptidase. Two other 5-member heterocyclic compounds with an aldehyde carbonyl in the 2-position. were also tested for their effect on pyroglutamyl peptide hydrolase activity. As shown in Table 1, 2-thiophenecarboxaldehyde and N-methylpyrrole-2-carboxaldehyde at concentrations of 0.4 mM did not inhibit pyroglutamyl peptide hydrolase activity.

5-oxoprolinal was tested on other peptidases which might be considered as susceptible to inhibition by this aldehyde. The Ki of 5-oxoprolinal on prolyl endopeptidase was found to be 0.36 mM. which is over 4 orders of magnitude greater than its Ki for pyroglutamyl peptide hydrolase. At a concentration of 10-5 M. 5-oxoprolinal did not inhibit aminopeptidase M or diaminopeptidase IV (post-proline diaminopeptidase) activities.

Since 5-oxoprolinal was found to inhibit the cleavage of pGlu-2NA by pyroglutamyl peptide hydrolase, we chose to study

Table 1 Effect of inhibitors on calf liver pyroglutamyl peptide hydrolase activity

Inhibitor	Ki (uM)
Z-Pro-Prolinal	7500
5-oxoproline	850
5-oxoprolinol	650
5-oxoprolinal	0.026
2-thiophene carboxaldehyde	N. I.[1]
N-methyl pyrrole-2-carboxaldehyde	N. I.[1]

The Ki of pyroglutamyl peptide hydrolase was determined as described in the Methods section. The enzyme was preincubated with inhibitor for 10 min prior to addition of substrate. Ki values were determined by the method of Dixon (1953).

[1]N. I. - Did not inhibit at a final concentration of 0.4 mM.

the effect of the inhibitor on the cleavage of a biological peptide by this enzyme. LHRH was chosen since it is cleaved by pyroglutamyl peptide hydrolase between the pyroglutamyl[1] and histidine[2] residues (Bauer et al., 1981a). HPLC analysis of an incubation mixture containing LHRH and pyroglutamyl peptide hydrolase revealed a peak corresponding to intact LHRH (retention time=12 min). This peak decreased with time concomitantly with the appearance of a new peak with a retention time of 9 min which progressively increased with time. As shown in Figure 6A, after 3 hours there was only about 25% of intact LHRH remaining. The peak which appeared during the incubation was collected, hydrolyzed and analyzed on an amino acid analyzer. Analysis of the amino acid composition of the product was consistent with the structure of des-pGlu[1]-LHRH, confirming that cleavage between the 1 and 2 position occurred. As shown in Figure 6B, 5-oxoprolinal at a concentration of 10^{-5} M almost totally blocked the degradation of LHRH by pyroglutamyl peptide hydrolase. After 3 hours, mainly intact LHRH was present.

Pyroglutamyl-peptide hydrolyzing enzyme assay

An assay using a chromogenic substrate was developed to study the brain particulate fraction and serum pyroglutamyl-peptide hydrolyzing enzyme. Since the pyroglutamyl peptide hydrolyzing enzymes in brain particulate fractions and in serum were reported to prefer TRH-like peptides (Taylor and Dixon, 1978; Bauer and Nowak, 1979; Bauer et al., 1981b;

Fiqure 6

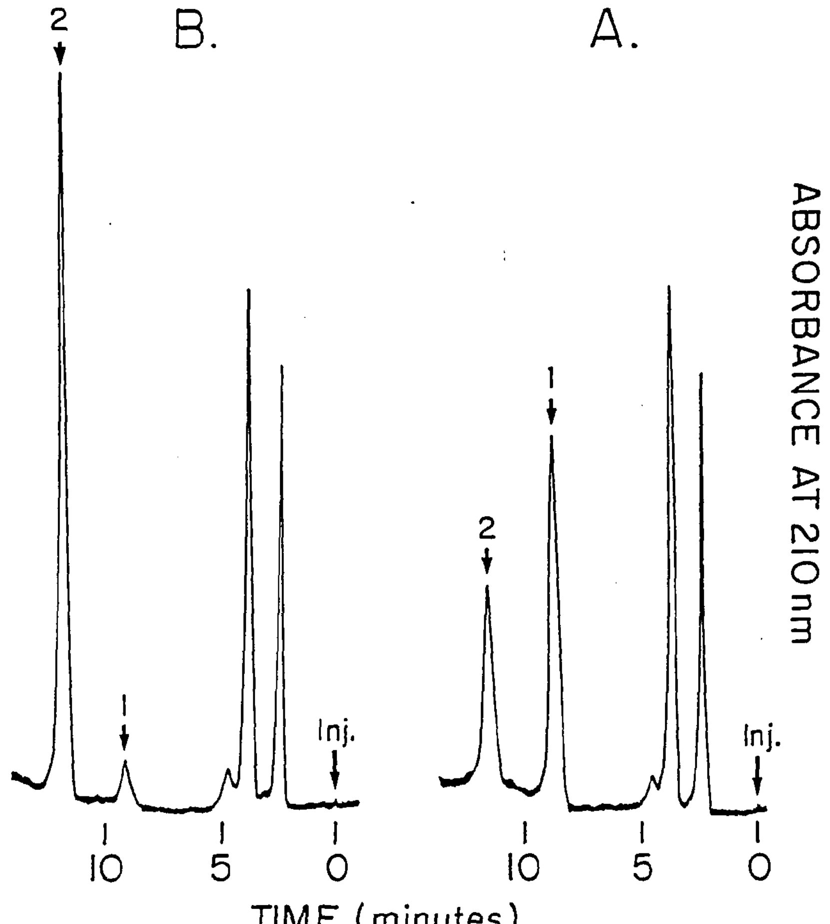


Figure 6

HPLC chromatogram of LHRH incubated with pyroglutamyl peptide hydrolase for 3 h at 37° C as described in the Methods section. A. Incubation without 5-oxoprolinal. B. Incubation in the presence of 5-oxoprolinal (10^{-6} M). A 20 ul aliquot of each incubation mixture was chromatographed on a 250 X 4 mm Bio-Sil OD-5S reverse phase column equilibrated with a mixture of acetonitrile and potassium phosphate buffer (0.05 M, pH 2.0). The starting concentration of acetonitrile was 20% and was linearly increased at a rate of 1%/ min. The flow rate was 1 ml/ min. Absorbance was monitored at 210 nm. Peak 1 is des-pGlu[1]-LHRH and peak 2 is LHRH.

O'Connor and O'Cuinn, 1984), a coupled assay using pGlu-His-Pro-2NA as the substrate and excess DAP IV was developed. We reasoned that the "TRH-specific" enzyme would also recognize pGlu-His-Pro-2NA.

The release of 2NA from pGlu-His-Pro-2NA in the absence of prolyl endopeptidase is dependent upon the initial cleavage of the pGlu-His bond (reaction 1) by an enzyme capable of removing the N-terminal pyroglutamyl residue (either PPH or presumably the membrane-bound pyroglutamyl hydrolyzing enzyme) followed by the removal of 2NA from His-Pro-2NA by DAP IV (reaction 2). Diaminopeptidase IV cleaves an N-terminal dipeptide residue from a peptide with a free N-terminus and a proline residue in the penultimate position (McDonald et al., 1971). The use of Z-Pro-Prolinal at a concentration of 10-5 M. will totally inhibit the action of prolyl endopeptidase which could otherwise cleave the Pro-2NA bond directly. Incubation of pGlu-His-Pro-2NA with DAP IV only did not release 2NA. Similarly, partially purified PPH did not release 2NA from this substrate when DAP IV was omitted from the incubation mixture. A combination of PPH and excess DAP IV readily released free 2NA.

The cleavage products of pGlu-His-Pro-2NA incubated with rat serum and rat washed brain pellets were studied by thin-layer chromatography. Unreacted pGlu-His-Pro-2NA has an Rf of 0.62 in solvent system I and 0.72 in solvent system II. Incubation of pGlu-His-Pro-2NA with rat serum and rat washed pel-

lets gave rise to a spot with an Rf of 0.09 in solvent system I and 0.17 in solvent system II which co-migrated with authentic His-Pro-2NA and with the product of pGlu-His-Pro-2NA incubated with partially purified PPH.

Degradation of TRH, pGlu-His-Pro-2NA and pGlu-2NA in rat tissues.

Radioimmunoassay of TRH allowed us to study its degradation since the degradation products did not cross react with the antiserum (Jeffcoate et al., 1973). Recovery of added TRH from hypothalamic samples was approximately 100% and the inhibitors, Z-Pro-Prolinal and PDMK, at a concentration much higher than in any degradation experiment, did not affect recovery.

Since in the literature, it was reported that in tissues only two enzymes, prolyl endopeptidase and PPH catalyze the initial degradation of TRH, it was of interest to examine whether addition of Z-Pro-Prolinal and PDMK, the inhibitors of these two enzymes would prevent TRH degradation. We therefore studied the degradation of TRH using rat brain fractions and rat serum in the presence and absence of these inhibitors. We were also interested in studying the affects of DTT and EDTA on TRH degradation since various others add these cofactors to their buffers and get different TRH degradation (see discussion). As shown in Table 2, in rat brain homogenates there was greater degradation of TRH in the absence of DTT and EDTA than in their presence. In the presence of DTT and EDTA,

Table 2 Degradation of TRH in rat brain homogenates in the presence of various inhibitors and activators

Addition	% TRH remaining[1] ± SEM
None	29.7 ± 6.5
Z-Pro-Prolinal	49.2 ± 6.8 [2]
PDMK	37.2 ± 11.6 [2]
Z-Pro-Prolinal + PDMK	60.0 ± 12.4[2] *
DTT/EDTA	48.4 ± 9.1
DTT/EDTA/Z-Pro-Prolinal	81.0 ± 8.3[3] *
DTT/EDTA/PDMK	72.4 ± 13.1[3]
DTT/EDTA/Z-Pro-Prolinal + PDMK	73.2 ± 14.2[3]

Degradation of TRH was determined by RIA as described in Materials and Methods. The final concentration in the assay was 10-5 M for PDMK and Z-Pro-Prolinal and 1.6 mM for EDTA and DTT in all experiments. Inhibitors and activators were preincubated 10 min before addition of substrate. Data are mean values of 4 to 7 determinations ± SEM.

[1]% TRH remaining was expressed as percentage of controls in which TRH was added at the conclusion of the incubation.

[2]The level of significance was found by comparing the percentage of degradation in the presence of inhibitor to the percentage of degradation in the absence of inhibitor by the two-tailed Student's T-test.

[3]The level of significance was found by comparing the percentage of degradation in the presence of inhibitor plus DTT

and EDTA to the percentage of degradation in the presence of DTT and EDTA alone by the two-tailed Student's T-test.

* $p < 0.05$

Z-Pro-Prolinal protected most of the TRH from degradation and PDMK also offered good protection. In the absence of DTT and EDTA. TRH was effectively degraded despite the presence of Z-Pro-Prolinal and PDMK, either separately or together. The use of specific inhibitors, therefore, indicates that in the absence of DTT and EDTA, an enzyme(s) other than prolyl endopeptidase and PPH is responsible for substantial TRH-degrading activity in brain homogenates.

In rat serum, TRH is degraded readily. As indicated in Table 3, after 2 h of incubation with 50 ul serum, TRH degradation was not affected by addition of Z-Pro-Prolinal (10-5 M). PDMK (10-5 M) or both inhibitors. However, o-phenanthroline (1 mM), a metal chelator, protected most of the TRH from degradation by rat serum. This data indicates that in serum, prolyl endopeptidase and PPH do not significantly degrade TRH. The degradation of TRH appears to be catalyzed by an enzyme which is inhibited by o-phenanthroline. These findings are consistent with the action of the previously described TRH-specific degrading enzyme thought to be a metalloenzyme (Taylor and Dixon, 1978; Bauer and Nowak, 1979).

Chromogenic substrates were used to further study the role of enzymes degrading TRH at the pGlu-His bond. As shown in Table 4. cleavage of pGlu-2NA in rat brain homogenates is almost totally dependent upon the presence of DTT and EDTA in the incubation mixture. This activity (upon activation with DTT and EDTA) was almost totally inhibited by PDMK. Thus

Table 3 Degradation of TRH in rat serum in the presence of various inhibitors

Inhibitor	% of controls ± SEM
None	13.7 ± 4.9
Z-Pro-Prolinal	10.8 ± 4.6
PDMK	10.4 ± 4.0
Z-Pro-Prolinal + PDMK	8.8 ± 3.2
o-phenanthroline	78.5 ± 11.5 *

Degradation of TRH was determined by RIA as described in Materials and Methods and was compared to controls in which TRH was added at the conclusion of the incubation. The final concentration in the assay was 10-5 M for PDMK and Z-Pro-Prolinal and 1 mM for o-phenanthroline in all experiments. Inhibitors were preincubated 10 min before addition of substrate. Data are mean values of 4 to 6 determinations ± SEM. The level of significance was found by comparing the percentage of degradation in the presence of inhibitor to the percentage of degradation in the absence of inhibitor by the two-tailed Student's T-test.

* $p < 0.005$

Table 4 The effect of inhibitors and activators on the pyroglutamyl-peptide hydrolyzing activities of rat brain fractions

	Specific activity (nmol/g tissue/h ±SEM)	
	[1]pGlu-His-Pro-2NA	[2]pGlu-2NA
Addition	Brain Homogenate	
None	290.0 ± 50.6	13.0 ± 9.7
-DAP IV	106.8 ± 48.8	
PDMK	241.2 ± 51.7	
o-phenanthroline	48.8 ± 20.0	
DTT/EDTA	471.4 ± 123.1	371.2 ± 72.9
DTT/EDTA/o-phenanthroline	15.0 ± 9.6	125.0 ± 60.3
DTT/EDTA/PDMK		6.2 ± 4.7
EDTA	235.0 ± 65.0	
DTT	363.8 ± 89.5	
	Brain Supernatant	
None	4.0 ± 2.5	0 ± 0
DTT/EDTA	61.0 ± 39.0	179.0 ± 74.7
DTT/EDTA/o-phenanthroline		13.0 ± 13.0
DTT/EDTA/PDMK		2.5 ± 2.2
	Brain Particulate Fraction	
None	298.7 ± 51.6	22.4 ± 6.8
-DAP IV	63.2 ± 12.5	
DTT	306.8 ± 58.0	55.0 ± 5.0
EDTA	191.2 ± 54.7	17.5 ± 4.3
DTT/EDTA	105.0 ± 16.6	87.5 ± 30.6
DTT/EDTA/PDMK	95.2 ± 15.9	21.4 ± 11.8

PDMK	275.0 ± 76.4	12.5 ± 6.3
o-phenanthroline	45.0 ± 26.7	
DTT/EDTA/o-phenanthroline	0 ± 0	

[1]Activity with pGlu-His-Pro-2NA was measured in the coupled assay as described in Materials and Methods.

[2]Activity with pGlu-2NA was measured as described in Materials and Methods.

The final concentration in the assay was 1 mM for o-phenanthroline. 10-5 M for PDMK and Z-Pro-Prolinal and 1.6 mM for EDTA and DTT in all experiments. Inhibitors and activators were preincubated 10 min before addition of substrate. Data are mean values of at least 4 determinations ± SEM.

pGlu-2NA in the presence of DTT and EDTA was degraded by the thiol-dependent PPH (EC 3.4.11.8). O-phenanthroline at a concentration of 1 mM also inhibited about 75% of the stimulated activity.

Cleavage of the pGlu-His bond of pGlu-His Pro-2NA was studied in rat brain homogenates in the presence of Z-Pro-Prolinal as described. Release of 2NA was greatly stimulated by exogenous DAP IV (Table 4). Basal activity in the absence of exogenous DAP IV is likely due to endogenous DAP IV (McDonald et al., 1971). The release of 2NA from pGlu-His-Pro-2NA was only slightly inhibited by PDMK when assayed in the absence of DTT and EDTA indicating that PPH contributed little to the cleavage under these conditions and that a distinct pyroglutamyl-peptide hydrolyzing enzyme degraded pGlu-His-Pro-2NA. Addition of DTT and EDTA increased the total activity in homogenates probably due to an increased contribution of PPH. O-phenanthroline markedly inhibited the activity both in the presence and absence of DTT and EDTA.

Cleavage of pGlu-2NA in the rat brain supernatant fraction is dependent upon DTT and EDTA and is virtually totally inhibited by PDMK (Table 4) indicating the presence of the thiol-dependent PPH. O-phenanthroline also strongly inhibited the cleavage of pGlu-2NA in the brain supernatant. In the supernatant pGlu-His-Pro-2NA is cleaved poorly; an activity probably due to PPH. This data indicates that pGlu-2NA is cleaved more readily than pGlu-His-Pro-2NA in rat brain supernatant.

In the washed rat brain particulate fraction the release of 2NA from pGlu-His-Pro-2NA greatly exceeded the release of 2NA from pGlu-2NA. The latter activity is stimulated by DTT and EDTA together and the stimulated activity is about 75% inhibited by PDMK indicating the presence of particulate PPH. PDMK did not inhibit the basal activity. The activity toward pGlu-His-Pro-2NA was markedly dependent upon exogenous DAP IV indicating initial cleavage of the pGlu-His bond. This activity is totally inhibited by a combination of DTT, EDTA and o-phenanthroline. PDMK did not inhibit this activity in either the presence or absence of DTT and EDTA indicating that in the washed particulate fraction, cleavage of pGlu-His-Pro-2NA was not due to PPH. The fact that in the particulate fraction pGlu-His-Pro-2NA is cleaved much more readily than pGlu-2NA is consistent with the report of a membrane-bound narrow-specificity pyroglutamyl-peptide hydrolyzing enzyme (O'Connor and O'Cuinn, 1984).

In rat serum, release of 2NA from pGlu-His-Pro-2NA exceeded the release of 2NA from pGlu-2NA (Table 5). The former activity was virtually totally inhibited by a combination of o-phenanthroline, EDTA and DTT. Omission of exogenous DAP IV from the assay only slightly reduced enzymatic activity. Serum contains relatively large amounts of DAP IV (McDonald et al., 1971). PDMK only slightly reduced the activity indicating that PPH was not responsible for the cleavage of pGlu-His-Pro-2NA in serum. Moreover, when pGlu-2NA was used as substrate, enzymatic activity was inhibited by o-phenanthroline

Table 5 The effect of inhibitors and activators on the pyroglutamyl-peptide hydrolyzing activities of rat serum

	Specific activity (nmol/ml serum/h ±SEM)	
	Substrate	
Addition	[1]pGlu-His-Pro-2NA	[2]pGlu-2NA
None	75.2 ± 5.0	27.1 ± 2.9
-DAP IV	55.8 ± 6.1	
o-phenanthroline	50.0 ± 12.9	6.0 ± 3.6
PDMK	67.5 ± 4.7	30.6 ± 3.8
DTT/EDTA	41.0 ± 11.8	26.2 ± 4.7
DTT/EDTA/o-phenanthroline	1.2 ± 0.7	2.5 ± 1.5

[1]Activity with pGlu-His-Pro-2NA was measured in the coupled assay as described in Materials and Methods.

[2]Activity with pGlu-2NA was measured as described in Materials and Methods.

The final concentration in the assay was 1 mM for o-phenanthroline. 10^{-5} M for PDMK and Z-Pro-Prolinal and 1.6 mM for EDTA and DTT in all experiments. Inhibitors and activators were preincubated 10 min before addition of substrate. Data are mean values of at least 4 determinations ± SEM.

but not by PDMK.

Determination of Ki for TRH

The affinity of the enzymes toward TRH was studied by determining the inhibition of synthetic substrates after addition of several concentrations of TRH The Ki of TRH competing with pGlu-His-Pro-2NA for the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme was found to be 0.25 mM. For comparison, the Ki of TRH for homogeneous rabbit brain prolyl endopeptidase competing with Z-Gly-Pro-SM and the Ki of TRH for highly purified bovine brain PPH competing with pGlu-2NA was determined. Values of 5.8 mM and 0.47 mM respectively were found. TRH showed competitive inhibition with all three enzymes.

ANIMAL STUDIES

Distribution of enzymatic activities in animal organs

The distribution of the membrane-bound pyroglutamyl-peptide hydrolyzing activity in rat organs is shown in Table 6. The highest activity was found in the brain with some activity also present in the lung and serum. In the liver, spleen, heart, skeletal muscle and kidney there was only trace activity. This distribution can be compared to the distribution of soluble PPH in mouse tissues (Table 7, control column). The activity of soluble PPH was highest in the kidney and liver with lower levels in other organs. The brain had the lowest activity of the organs studied. The distribution of prolyl

Table 6 Distribution of the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme in rat organs

Organ	Specific activity (nmol/mg protein/h ±SEM)
brain	4.38 ± 0.56
serum	1.18 ± 0.07
lung	1.18 ± 0.38
liver	0.11 ± 0.08
spleen	0.12 ± 0.12
heart	<0.10
skeletal muscle	<0.10
kidney	<0.10

Enzyme activity was determined in the coupled assay as described in Materials and Methods. Data are mean values of 4 samples ± SEM.

Table 7 Pyroglutamyl peptide hydrolase activity in mouse tissues after intraperitoneal administration of 5-oxoprolinal

Tissue	Enzymatic Activity (nmol 2NA/g tissue/h)			
	Control	Time after inhibitor administration		
		10 min	30 min	4 days (8 injections)
brain	540±100 (4)	210±24[2] (7)	300±70[1] (4)	290 (1)
heart	870±70 (4)	220±55[3] (7)	720±160 (4)	750 (1)
muscle	560±110 (4)	75±19[3] (7)	220±50 (4)	400 (1)
lung	980±130 (4)	120±27[3] (7)	450±50[1] (4)	810 (1)
spleen	1100±230 (4)	64±11[3] (7)	540±120[1] (4)	970 (1)
liver	4600±980 (4)	1600±340[1] (4)	2200±85[1] (4)	5300 (1)
kidney	8800±2500 (4)	250±69[2] (4)	565±70[1] (3)	6200 (1)

5-oxoprolinal (50 mg/kg) was administered intraperitoneally as a solution in 50% ethanol. Control animals received the vehicle. 10 and 30 min after injection, the animal was sacrificed and pyroglutamyl peptide hydrolase activity was determined as described in the Methods section. One animal received 8 injections over a period of 4 days and was sacrificed 16 h after the last injection. Data are mean values ± S.E. Values in parentheses represent the number of animals used. The level of significance was found by comparing the

activities of inhibitor-treated homogenates with that of control-treated homogenates by the one-tailed Student's T-test.

[1] $p < 0.05$. significantly different from controls.

[2] $p < 0.01$.

[3] $p < 0.001$.

endopeptidase in mouse tissues is listed in Table 8 (control column). The lung and liver had the highest activity with lower activity in skeletal muscle and duodenum and pancreas.

A comparison of the distribution in brain regions of the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme and soluble PPH is shown in Table 9. The distribution of soluble PPH is much more uniform with highest activity in the pituitary while for the membrane-bound enzyme, a more pronounced regional distribution is seen. For the latter activity, the highest levels are in the cortex and hippocampus and lowest levels in the pituitary. It is worth noting that in the pituitary, the level of soluble PPH is 100 times higher than the membrane-bound activity.

In vivo inhibition of enzymes in animals

The results of determination of prolyl endopeptidase activity in various tissues 30 min after an intraperitoneal injection of Z-Pro-Prolinal into mice are presented in Table 8. The data expressed in terms of umoles of SM released per g tissue per h show a greater than 90% inhibition of enzyme activity in most tissues. The lowest inhibition (85%) was found in liver. Similar results were obtained when the activity was related to the concentration of tissue protein, although data collected in this manner showed a somewhat greater variability.

The possibility was considered that the inhibition of the

Table 8. Prolyl Endopeptidase Activity in Mouse Tissues after Intraperitoneal Administration of Z-Pro-Prolinal

Tissue	Enzyme Activity (umol SM/g/h) Control	30 min. after Z-Pro-Prolinal	
Brain	35.2 ± 0.95	3.0 ± 0.3	(92)
Heart	33.0 ± 1.9	2.2 ± 0.3	(93)
Skeletal muscle	18.0 ± 1.6	0.7 ± 0.35	(96)
Lung	83.2 ± 4.7	6.7 ± 0.45	(92)
Spleen	39.7 ± 6.8	4.7 ± 0.7	(88)
Duodenum & Pancreas	17.2 ± 0.8	1.5 ± 0.2	(91)
Liver	75.7 ± 4.5	11.2 ± 0.8	(85)
Kidney	34.5 ± 1.8	3.0 ± 0.4	(91)

Z-Pro-Prolinal (1.25 mg/kg) was administered as a solution in 10% methanol. Enzyme activity was determined as described under "Materials and Methods". Data are mean values from four experiments ± S.E. Values in parenthesis represent percent inhibition.

Table 9 Distribution of PPH and the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme in rat brain regions

Brain region	Specific activity (nmol/mg protein/h ±SEM) membrane-bound activity	soluble PPH activity
pituitary	0.15 ± 0.15	15.0 ± 2.9
hypothalamus	2.8 ± 0.30	9.9 ± 2.0
cortex	6.8 ± 0.90	5.3 ± 1.4
hippocampus	6.5 ± 1.3	6.9 ± 2.4
striatum	1.4 ± 0.10	5.1 ± 2.0
brainstem	0.58 ± 0.23	8.3 ± 2.9
thalamus	2.9 ± 0.53	5.8 ± 2.0
cerebellum	2.0 ± 0.51	7.1 ± 2.5

The membrane-bound activity was measured in the coupled assay with pGlu-His-Pro-2NA as substrate in the presence of excess DAP IV as described in Materials and Methods. PPH was measured in the supernatant fraction of homogenates using pGlu-2NA as substrate in the presence of DTT and EDTA as described in Materials and Methods. Data are mean values from four to five rats ± SEM.

enzyme in homogenates after intraperitoneal administration of the inhibitor does not reflect a true in vivo inhibition, but rather results from exposure of the enzyme to the inhibitor after disruption of tissues by homogenization. This possibility deserved consideration because the dose used could have produced a tissue concentration in the uM range, while the Ki of the inhibitor is in the nM range. Furthermore, it was not known whether after intraperitoneal administration the inhibitor penetrates all the tissue compartments where the enzyme is localized. A true in vivo inhibition of enzyme activity could therefore. be claimed only by demonstrating that the inhibitor blocks the in vivo degradation of a prolyl endopeptidase substrate. It was elected accordingly, to follow the in vivo degradation of Z-Gly-Pro-SM. This prolyl endopeptidase substrate is hydrolyzed by the enzyme at the Pro-SM bond with the release of free SM which can be conveniently determined by a diazotization procedure. After administration of the inhibitor (5 mg/kg). Z-Gly-Pro-SM was injected intraperitoneally after various time intervals and the concentration of free SM was determined in tissues 30 min later. The results summarized in Table 10 show that the degree of inhibition, as expressed by a decrease in tissue SM concentration was highest after 45 min. This decrease was highest in the brain (85%) and lowest in the liver (64%). The degree of inhibition progressively decreased during the following 45 min but was still distinct after more than 6 hours. Thus after 6.5 hours the brain concentration of SM was lower than in controls by more

Table 10. In Vivo Inhibition of Sulfamethoxazole Release from Z-Gly-Pro-Sulfamethoxazole after Administration of Z-Pro-Prolinal

Tissue	Control (60 min)	Concentration of Sulfamethoxazole (ug/g tissue) 45 min.	60 min.	90 min.	390 min.
Brain	9.0±1.2	1.31±0.38*(85)	2.44±0.5*(73)	2.58±0.4*(71)	5.63±0.8*(37)
Heart	38.2±3.9	8.42±1.6 *(78)	10.7 ±1.8*(72)	16.2 ±2.5*(58)	25.7 ±1.3*(33)
Skeletal muscle	31.0±3.5	8.43±2.1 *(73)	9.47±2.4*(69)	14.2 ±2.6*(54)	21.9 ±1.8*(29)
Lung	46.3±4.3	11.8 ±1.2 *(75)	12.5 ±2.0*(73)	19.7 ±1.3*(57)	34.6 ±3.3*(25)
Spleen	34.7±4.3	9.0 ±2.3 *(74)	10.4 ±2.3*(70)	14.5 ±1.0*(58)	21.5 ±1.3*(38)
Duodenum & Pancreas	37.3±3.3	12.2 ±2.8 *(67)	15.2 ±2.0*(59)	20.9 ±4.1*(44)	28.5 ±1.9*(24)
Liver	62.4±5.4	22.5 ±2.3 (64)	29.1 ±4.6*(53)	41.8 ±5.8*(33)	53.2 ±4.5(15)
Kidney	57.0±5.7	14.5 ±2.6 *(75)	16.3 ±1.6*(71)	28.5 ±4.6*(50)	39.4 ±3.6*(31)

Z-Pro-Prolinal (5 mg/kg) was given by intraperitoneal injection followed at various time intervals (15, 30, 60, and 360 min) by Z-Gly-Pro-SM. Thirty minutes later the animals were killed and free SM was determined as described under "Materials and Methods". Control animals received the vehicle, followed by Z-Gly-Pro-SM 30 min later. Data are mean values ± S.E. from 4 to 6 experiments. Values in parenthesis represent prercent inhibition. *p<0.05 to 0.001.

than 35%. and significant decreases were observed in all of the other tissues except for the liver.

The relationship between the dose of Z-Pro-Prolinal and activity of prolyl endopeptidase in homogenates of various organs one hour after inhibitor injection is summarized in Table 11. An IC50 of about 1.25 mg/kg was found for most organs. The inhibition in brain for this dose was almost 80%: a dose of 0.5 mg/kg was sufficient to cause an almost 50% inhibition in brain. The highest inhibition at virtually all doses was found in the brain and the lowest inhibition in the liver. It is notable that 30 min after administration of a dose as low as 0.005 mg/kg, there was still a 39% inhibition of the enzyme in the brain, while a comparable inhibition of the enzyme in liver was seen only after a dose 100 times greater (0.5 mg/kg).

These results demonstrate that Z-Pro-Prolinal is an effective inhibitor of prolyl endopeptidase in vivo. In the brain, the inhibitor is both potent (significant inhibition at a dose of 0.005 mg/kg) and long-lasting (significant inhibition after 6.5 hours). This indicates that the inhibitor traverses the blood-brain barrier readily and could be used to study the role of prolyl endopeptidase in neuropeptide metabolism.

5-oxoprolinal was injected into mice to determine if it was an effective inhibitor of PPH in vivo. The results shown in Table 7 demonstrate greater than 60% inhibition of enzymatic activity in all organs tested 10 min after 5-oxoprolinal

Table 11. In Vivo Inhibition of Sulfamethoxazole release from Z-Gly-Pro-Sulfamethoxazole after administration of various doses of Z-Pro-Prolinal

Tissue	Concentration of Sulfamethoxazole (ug/g tissue)					
	Control	5mg/kg	1.25 mg/kg	0.5 mg/kg	0.02 mg/kg	0.005 mg/kg
Brain	9.0 ± 1.2	2.4 ± 0.48*(73)	2.0 ± 0.52*(78)	4.7 ± 0.61*(48)	4.9 ± 0.55*(45)	5.5 ± 1.1*(39)
Heart	38.2 ± 3.9	10.7 ± 1.8*(72)	14.9 ± 3.5*(61)	23.8 ± 2.3*(38)	30.2 ± 1.8 *(21)	31.2 ± 5.3 (18)
Skeletal muscle	31.0 ± 3.5	9.5 ± 2.4*(70)	11.8 ± 2.1*(62)	20.6 ± 2.4*(34)	25.1 ± 1.3 (19)	27.0 ± 3.1 (13)
Lung	46.3 ± 4.3	12.6 ± 2.0*(73)	18.8 ± 3.0*(60)	26.8 ± 2.9*(42)	36.9 ± 3.6 (20)	35.7 ± 6.3 (23)
Spleen	34.7 ± 4.3	10.4 ± 2.3*(70)	14.8 ± 2.9*(58)	23.7 ± 5.7 (32)	35.8 ± 4.6 (0)	29.0 ± 4.4 (16)
Duodenum & Pancreas	37.3 ± 3.3	15.2 ± 2.0*(59)	15.2 ± 3.1*(59)	27.0 ± 3.5*(28)	29.7 ± 2.3 (20)	31.3 ± 4.3 (16)
Liver	62.4 ± 5.4	29.1 ± 4.6*(53)	35.1 ± 3.3*(44)	37.2 ± 5.6*(40)	60.5 ± 2.2 (3)	59.5 ± 7.8 (5)
Kidney	57.0 ± 5.7	16.3 ± 1.6*(71)	23.7 ± 4.0*(58)	29.2 ± 4.0*(49)	42.7 ± 0.35*(25)	42.4 ± 3.6*(26)

Z-Pro-Prolinal at various doses (5 mg/kg, 1.25 mg/kg, 0.5 mg/kg, 0.02 mg/kg, and 0.005 mg/kg) was given by intraperitoneal injection followed after 30 min by Z-Gly-Pro-SM. Thirty minutes later the animals were killed and free SM was determined as described under "Materials and Methods". Control animals received the vehicle followed by Z-Gly-Pro-SM 30 minutes later. Data are mean values ± S.E. from 4 to 8 experiments. Values in parenthesis represent percent inhibition. *$p<0.05$ to 0.001.

injection (50 mg/kg) compared with control activity. This inhibition was statistically significant in all organs tested. After 30 min. the degree of inhibition decreased but significant inhibition occurred in all organs except the heart. The highest degree of inhibition at both times was in the kidney.

Since 5-oxoprolinal increased pyroglutamyl peptide hydrolase activity in GH3 cells (see below), the possibility of a similar increase in pyroglutamyl peptide hydrolase activity in vivo was considered. A mouse received 8 injections of 5-oxprolinal (50 mg/kg) over a period of 4 days. As shown in Table 7. PPH activity was not affected by chronic exposure to 5-oxoprolinal.

Pyroglutamyl diazomethyl ketone was found to be a very effective inhibitor of PPH in vivo (Table 12). A dose of 0.1 mg/kg almost totally inactivated PPH one hour after PDMK injection in all mouse tissues studied. A dose of 0.01 mg/kg also inhibited PPH in all organs, however the level of inibition was less in the brain than in other organs. Since the inhibition is irreversible, recovery of activity will be dependent upon synthesis of new enzyme protein. Approximately 50% inactivation is still observed 24 hours after administration of 0.1 mg/kg of inhibitor. 5 days after inhibitor administration. PPH returned to control levels (Table 12). These results indicate that pyroglutamyl diazomethyl ketone should be of value in studies on the turnover of the enzyme in various tissues.

Table 12 Inhibition of pyroglutamyl peptide hydrolase in mouse tissues in vivo by pyroglutamyl diazomethyl ketone

Organ	Control	0.1 mg/kg 1 h	0.1 mg/kg 24 h	0.1 mg/kg 5 d	0.01 mg/kg 1 h
	Enzymatic activity (nmol/g tissue/h)				
brain	540 ± 100	0	230 ± 46	480	380 ± 26
heart	870 ± 70	0	350 ± 120	1200	27 ± 4
muscle	460 ± 110	16 ± 16	130 ± 41	560	17 ± 17
lung	980 ± 130	10 ± 10	490 ± 100	1200	14 ± 14
spleen	1100 ± 230	0	630 ± 200	910	10 ± 10
liver	4600 ± 980	0	2200 ± 810	5800	500 ± 250
kidney	8800 ± 2500	66 ± 66	4200 ± 1400	5400	750 ± 130

Enzymatic activities were determined as described in Materials and Methods. Pyroglutamyl diazomethyl ketone was administered by the intraperitoneal route at either 0.01 mg/kg or 0.1 mg/kg. Animals were killed after either 1 h, 24 h or 5 days. Values are expressed as mean ± SEM. 4 animals were used as controls. 3 animals received 0.1 mg/kg for 1 h, 5 animals received 0.1 mg/kg for 24 h, 1 animal received 0.1 mg/kg for 5 days and 3 animals received 0.01 mg/kg for 1 h.

The possibility was considered that inhibition of prolyl endopeptidase and PPH would increase the levels of the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme since that would be the only known enzyme capable of degrading TRH. Z-Pro-Prolinal (5 mg/kg) and PDMK (1 mg/kg) were injected into rats. 10 hours later, a second injection of these two inhibitors was made. After an additional hour, the brain particulate pyroglutamyl-peptide hydrolyzing enzyme was assayed. The activity was not affected by inhibitor injection (controls 435 ± 46 nmol/g tissue/h, N=4; inhibitor-treated 428 ± 46 nmol/g tissue/h, N=4).

Effect of inhibitors on TRH, TSH and prolactin levels in rats

Radioimmunoassay was used to determine TRH, TSH and prolactin levels in rats. TRH-OH did not cross react with the TRH antiserum. Z-Pro-Prolinal or PDMK at a concentration many times higher than present in the RIA did not affect hormone measurement.

Z-Pro-Prolinal significantly raised TRH levels in the pituitary 15 minutes after administration (Table 13). A smaller nonsignificant increase was observed 30 minutes after Z-Pro-Prolinal administration. Z-Pro-Prolinal plus PDMK, however, did not alter TRH levels 15 minutes after injection. Only 4 animals received this inhibitor combination. In the frontal cortex, the combination of Z-Pro-Prolinal and PDMK raised TRH levels significantly 60 minutes after administration. Since, however, control levels of cortical TRH varied

Table 13 Effect of inhibitors on TRH levels in rat brain regions

Time	control	Z-Pro-Prolinal	Z-Pro-Prolinal±PDMK
	TRH (pg/mg tissue ± SEM)		
	Pituitary		
15 min	44.3 ± 7.1 (16)	101.4 ± 25.2 (11)*	51.3 ± 11.9 (4)
30 min	33.5 ± 8.9 (7)	56.7 ± 11.7 (7)	
60 min	47.9 ± 9.2 (11)	55.6 ± 7.2 (4)	40.9 ± 9.0 (8)
18 h	60.3 ± 4.2 (4)		54.6 ± 15.7 (4)
	Hypothalamus		
15 min	229.6 ± 22.1 (16)	219.6 ± 30.7 (12)	403.2 ± 147 (4)
30 min	213.4 ± 29.0 (12)	244.3 ± 31.8 (11)	
60 min	329.0 ± 57.2 (14)	173.2 ± 23.9 (6)	360.1 ± 73.2 (8)
2 h	116.7 ± 17.6 (4)	171.3 ± 37.7 (6)	
18 h	284.5 ± 24.7 (2)		162.2 ± 22.6 (4)
	Frontal cortex		
15 min	7.89 ± 1.9 (16)	5.46 ± 0.9 (11)	6.4 ± 4.2 (4)
30 min	1.58 ± 0.7 (7)	2.14 ± 0.7 (8)	
60 min	4.47 ± 1.5 (11)	3.60 ± 2.11 (4)	11.8 ± 3.4 (8)*
18 h	10.50 ± 5.5 (4)		9.6 ± 5.1 (4)

Rats received Z-Pro-Prolinal (5 mg/kg in 30% ethanol) or a solution of Z-Pro-Prolinal (5 mg/kg) plus PDMK (1 mg/kg) in 30% ethanol by intraperitoneal injection. Control animals received the vehicle. The animals were sacrificed 15, 30, 60 min and 18 h after injection and the pituitary, hypothalamus and frontal cortex were removed. TRH was extracted into 90% methanol and assayed by radioimmunoassay as described in the Methods section. Data are mean values ± SEM. Values in

parentheses represent the number of animals used. The level of significance was found by comparing TRH levels in inhibitor-treated animals to control animals.

* $p < 0.05$

widely in different experiments, this might be an artifact. There was no significant alteration of TRH levels after other times in the pituitary, hypothalamus or frontal cortex (Table 13). A wide variation in hormone levels between animals was observed.

Prolactin and TSH levels were measured by RIA in rat serum after injection of Z-Pro-Prolinal. As shown in Table 14, there was no significant changes in prolactin and TSH levels after 15, 30 and 60 minutes after inhibitor administration compared to controls. Wide variations in hormonal levels between animals was also observed.

EFFECT OF INHIBITORS ON LHRH HALF-LIFE IN RATS

Radioimmunoassay was used to measure LHRH levels in rat plasma. The 1-9 LHRH fragment did not cross react with the antiserum. Z-Pro-Prolinal at a concentration many times higher than present in the RIA did not affect LHRH measurements. The distribution of LHRH from rat plasma followed a biphasic elimination characteristic of a two-compartment model. A rapid first phase (α) corresponds to the distribution of hormone from blood to tissues while a second phase (β) corresponds to metabolism and elimination of the hormone from the animal. The DRUGFUN program was used to fit the pharmacokinetics of LHRH disappearance and calculate the initial and terminal half-lifes and the clearance and volume of distribution. The clearance and volume of distribution are expressed per body weight.

Table 14 Effect of Z-Pro-Prolinal on prolactin and TSH levels in rat serum

Time	ng hormone/ml serum	
	prolactin	
	control	Z-Pro-Prolinal
15 min	14.2 ± 6.2 (4)	16.7 ± 0.5 (4)
30 min	15.7 ± 5.3 (8)	32.0 ± 10.8 (8)
60 min	6.0 ± 1.0 (4)	19.3 ± 13.0 (4)
	TSH	
15 min	0.62 ± 0.14 (4)	0.60 ± 0.23 (4)
30 min	4.3 ± 1.7 (8)	4.9 ± 2.3 (8)
60 min	1.2 ± 0.36 (4)	5.6 ± 4.4 (4)

Rats received Z-Pro-Prolinal (5 mg/kg in 30% ethanol) by intraperitoneal injection. Control animals received the vehicle. 15, 30 and 60 min after the injection, the animals were sacrificed and serum was prepared as described in the Methods section. Data are mean values ± SEM. Values in parentheses represent the number of animals used. There were no statistically significant differences between control and Z-Pro-Prolinal-treated rats as determined by the Student's T-test.

As shown in Table 15 the initial half-life in controls was 1.47 ± 0.31 min while the terminal half-life was 16.1 ± 1.6 min. The mean volume of distribution was 173 ± 45 ml/kg while the clearance was 40 ± 10 ml/kg/min. Injection of Z-Pro-Prolinal (1.25 mg/kg) 2 min before injection of LHRH prolonged the terminal half-life in rats # 7-10, however in the next 6 rats studied (# 11-16) the half-life was not altered (18.3 ± 2.5 min: N=6) compared to controls The initial half-life, clearance and volume of distribution were also not significantly different from controls. The clearance was also not significantly altered. It appears that Z-Pro-Prolinal cannot be used to affect the pharmacokinetic parameters of LHRH elimination.

GH3 CELL STUDIES

Comparison of enzymatic activities in GH3 cells and rat anterior pituitaries

Initial studies indicated that many of the enzymatic activities declined with storage at 4°, especially cathepsin B, aminopeptidase and prolyl endopeptidase. Accordingly GH3 cell homogenates were used within 2 days of homogenization and anterior pituitary homogenates were used on the same day as homogenization, to obtain consistent enzyme activities.

Table 16 summarizes the results of activity measurements of the peptide-hydrolizing enzymes in GH3 cells as well as in anterior pituitary homogenates. There are statistically sig-

Table 15 Effect of Z-Pro-Prolinal on the pharmacokinetic parameters of LHRH disappearance in rats

Rat #	Half-life (min)		Volume of	Clearance
	1st comp	2nd comp	Distribution (ml/kg)	(ml/min/kg)
		Controls		
1	1.71	19.1	381	67
2	2.04	13.2	102	20
3	1.72	11.9	207	41
4	2.16	17.3	122	18
5	0.15	21.7	97	30
6	1.08	13.3	129	49
mean	1.47±0.31	16.1±1.6	173.0±45	40±10
		Z-Pro-Prolinal		
7	2.02	32.2	275	43
8	1.46	89.0	44	7
9	2.16	inf.	241	-
10	0.32	68.6	10	11
11	1.84	12.7	90	22
12	0.87	15.6	103	27
13	1.60	29.8	234	53
14	1.11	15.7	178	41
15	2.09	16.3	91	26
16	0.74	19.7	121	93
mean	1.42±0.20	33.3±9.0	138.7±28	35.9±8.7

Z-Pro-Prolinal (1.25 mg/kg) was injected into the femoral vein of an anesthetized rat 2 min before injection of LHRH (210 ug/kg). Blood was collected at various times and the

concentration of LHRH in the plasma was determined by RIA as described in the Methods section. The pharmacokinetic parameters of LHRH disappearance were analyzed by the DRUGFUN program of the PROPHET Computer System. Individual experiments and the mean ± SEM for control and Z-Pro-Prolinal-treated rats are given. The mean values for the 2nd half-life component and the clearance do not include rat #9 which had an infinite 2nd component of disappearance.

nificant differences between the two homogenates for all enzyme activities studied except for pyroglutamyl peptide hydrolase. The highest activity found in both preparations was for the lysosomal enzyme, cathepsin B. The activity of cathepsin B in GH3 cells was almost five times greater than its activity in anterior pituitary homogenates whereas the other lysosomal enzyme, cathepsin D, which had much less activity in both preparations, had higher activity in the anterior pituitary homogenates. Of the cytoplasmic enzymes, soluble metalloendopeptidase and prolyl endopeptidase had high activities in both preparations. Their activities were about twice as high in the GH3 cell homogenates than in anterior pituitary homogenates. The multi-catalytic protease complex had moderate activity in both preparations while pyroglutamyl peptide hydrolase activity was low in both preparations. The activity of aminopeptidase was moderate in both preparations, while the activity of membrane-bound neutral metalloendopeptidase was about four times less in the GH3 cell homogenates than in the anterior pituitary homogenates.

Table 16 Specific activities of peptide-degrading enzymes in GH3 cell homogenates and anterior pituitary homogenates

Enzyme	Substrate	Specific activity (nmoles/mg protein/h ±SEM) GH3 cells	anterior pituitary homogenates
soluble metallo-endopeptidase	Bz-Gly↓Ala-Ala-Phe-pAB	1269±172[1]	654.2±74.4
prolyl endopeptidase	Z-Gly-Pro↓SM	482.2±26.4[2]	269.5±38.5
pyroglutamyl peptide hydrolase	pGlu↓2NA	18.2±1.7	11.9±2.5
multi-catalytic protease complex (chymotrypsin component)	Z-Gly-Gly-Leu↓pNA	32.6±3.5[1]	80.+11.2
multi-catalytic protease complex (SDS-activated component)	Z-Leu-Leu-Glu↓2NA (with SDS)	229.1±24.3[2]	59.0±3.8
cathepsin B	Z-Leu-Leu-Arg↓2NA	1727±199[2]	363.2±50.5
cathepsin D	TFA-Phe-(o-Benzyl)Ser-↓Phe-Phe-Ala-pAB	52.+7.5[3]	94.5±15.8

Aminopeptidase	Leu↓-pNA	156.2±8.8[3]	203.2±13.3
Membrane-bound neutral metallo-endopeptidase	Glutaryl-Ala-Ala↓-Phe-2NA	21.+1.48[4]	77.+11.2

Activities were determined as described in the Methods section. Arrows indicate the initial site of the cleavage of each substrate. Between 4 and 6 determinations were made for each enzyme. The level of significance was found by comparing the activities of the enzyme preparations by using Student's t-test. Significantly different from anterior pituitary homogenate: [1]$p < 0.05$; [2]$p < 0.01$; [3]$p < 0.005$; [4]$p < 0.001$;

Since a given peptide substrate may be degraded by more than one proteolytic enzyme, specific, active-site directed inhibitors were used to verify the identities of the activities measured. These inhibitors fulfill the binding requirements of the active-site and interact with the enzyme to form analogs of the transition state for peptide hydrolysis. As shown in Table 17, N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, a specific inhibitor of soluble metalloendopeptidase (Chu and Orlowski, 1984), eliminated most of the activity cleaving Hip-Ala-Ala-Phe-pAB in both GH3 cell homogenates and anterior pituitary homogenates. Consistent with the requirement of this enzyme for a sulfhydryl group, elimination of DTT from the reaction mixture substantially reduced its activity. The metal chelator, o-phenanthroline, at a final concentration of 2 X 10-4 M, also eliminated most of the activity. The data are consistent with the activity due to that of the soluble metalloendopeptidase.

Z-Pro-Prolinal is a specific inhibitor of prolyl endopeptidase in vitro (Wilk and Orlowski, 1983a) and in vivo (Friedman et al., 1984b). At a final concentration of 10-6 M, Z-Pro-Prolinal inhibited the Z-Gly-Pro-SM-cleaving activity in both the GH3 cell homogenates and the anterior pituitary homogenates by more than 95%. Similarly leupeptin, a potent inhibitor of cathepsin B, at a dose of 10-6 M, inhibited by more than 92% the Z-Leu-Leu-Arg-2NA-cleaving activity activity in both preparations. These observations indicate that the two substrates measure prolyl endopeptidase and cathepsin B activ-

Table 17 Effect of inhibitors on peptide-degrading enzymes of GH3 cell homogenates and anterior pituitary homogenates

Enzyme	Inhibitor	% of control GH3 cell homogenates	% of control anterior pituitary homogenates
soluble metallo-endopeptidase	N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (10-4 M)	7.0%	15.3%
	o-phenanthroline (2X10-4 M)	18.7%	16.6%
	-DTT *	11.3%	21.6%
prolyl endo-peptidase	Z-Pro-Prolinal (10-6 M)	5.3%	3.2%
cathepsin B	leupeptin (10-6 M)	4.9%	7.5%
membrane-bound neutral metallo-endopeptidase	N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB (4X10-5 M)	79%	31.9%

Enzyme activities were measured as described in the Methods section. Numbers in parentheses indicate the concentration of inhibitor in the incubation mixture. No preincubation was performed with the inhibitors. * Full expression of this activity requires a thiol reducing agent such as DTT in the incubation mixture.

ity respectively.

N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB is a specific inhibitor of membrane-bound neutral metalloendopeptidase (Almenoff and Orlowski, 1983). This inhibitor at a final dose of 4 X 10-5 M inhibited about 70% of the activity in anterior pituitary homogenate preparations, while inhibiting only about 20% of the activity in the GH3 cell homogenates. At this dose of inhibitor the activity of membrane-bound neutral metalloendopeptidase should be totally inhibited. The fact that the activity in the GH3 cell homogenates was only slightly inhibited indicates that cleavage of Glutaryl-Ala-Ala- Phe-2NA in GH3 cell homogenates is in part catalyzed by other enzyme(s).

Effect of inhibitors on enzymatic activity in GH3 cells

Z-Pro-Prolinal significantly inhibited prolyl endopeptidase activity in GH3 cells after exposure of the inhibitor for both 1 h (control cells 31.7 ± 4.1 nmol/mg/h, N=3; 10-6 M Z-Pro-Prolinal 11.6 ± 3.0 nmol/mg/h, N=3, $p < 0.05$) and 3 days (control cells 11.0 ± 1.9 nmol/mg/h, N=3; 10-5 M Z-Pro-Prolinal 3.9 ± 1.2 nmol/mg/h, N=3, $p < 0.001$). Similar results were obtained when the media containing serum (described above) was replaced with Neuman-Tytell serumless media. While enzymatic activities were always compared to controls, it was observed that prolyl endopeptidase activity was lower in cells grown in wells (results presented here) than in flasks (Table 16). It is possible that the different growing conditions between

flasks and wells or the different degree of confluency are important for expression of enzymatic activity. As a control, Z-Pro-Prolinal (10-5 M) did not affect the soluble metalloendopeptidase activity in cells exposed for 3 days (control cells 141 ± 3 nmol/mg/h, N=3; Z-Pro-Prolinal 135 ± 32 nmol/mg/h, N=3, N.S.). These results indicate that Z-Pro-Prolinal can be used to effectively inhibit prolyl endopeptidase in GH3 cells.

5-Oxoprolinal (10-5 M) caused an unexpected 3-4 fold increase in PPH activity in GH3 cells after 3 days of exposure (Table 18). Figure 7 shows the concentration-related effect of 3 days exposure to 5-oxoprolinal on PPH activity in GH3 cells. The EC50 was 10-7 M with a maximum increase produced by 10-5 M 5-oxoprolinal. This increase in PPH activity upon exposure of cells to 5-oxoprolinal (10-5 M) was time dependent, showing a rapid increase in the first 24 hours with a continuous rise until 6 days (Figure 8)

Specificity of the increase in pyroglutamyl peptide hydrolase activity

The specificity of the increase in PPH activity by 5-oxoprolinal was studied. The activity of prolyl endopeptidase in cells exposed to 5-oxoprolinal for 3 days was unaltered (untreated cells 341.7 ± 55.5 nmol/mg/h, N=30 vs 10-5 M 5-oxoprolinal 343.0 ± 36.8 nmol/mg/h, N=18). In addition, Z-Pro-Prolinal, a specific, aldehyde inhibitor of prolyl endopeptidase, when incubated with GH3 cells for 3 days did not

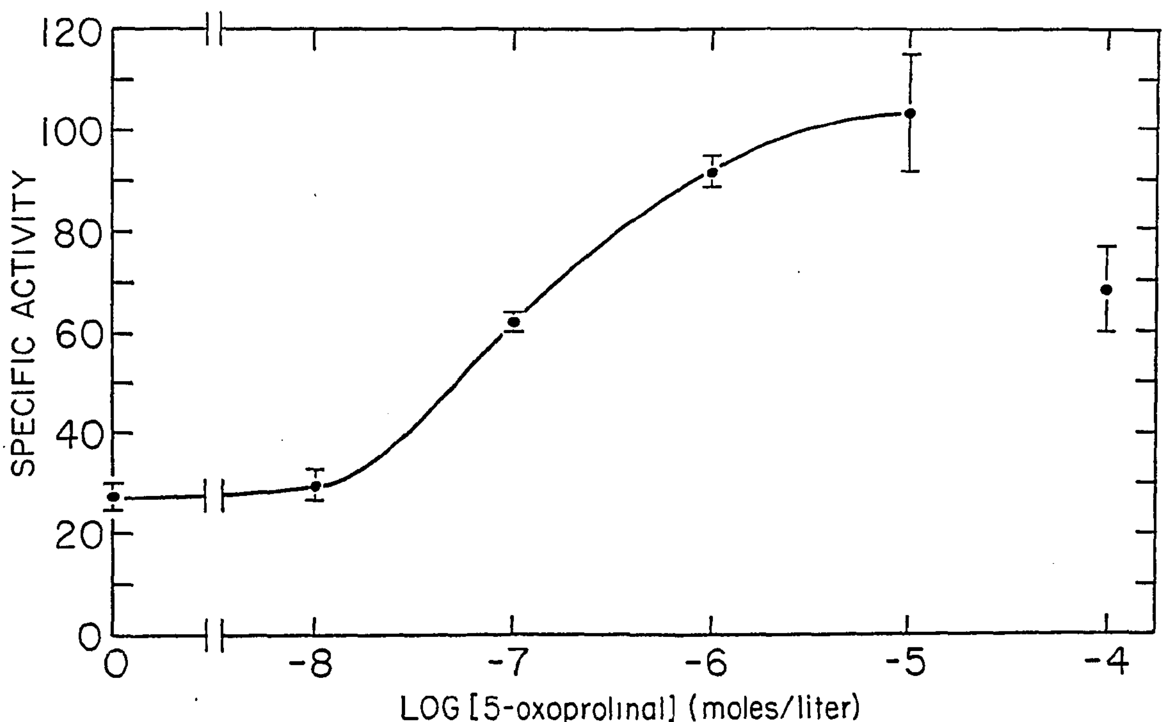
Table 18 The effect of TRH-degrading enzyme inhibitors, TRH and TRH metabolites on PPH activity in GH3 cells

Experiment	Specific activity (nmol/mg protein/h ± SEM)
5-oxoprolinal (10-5 M)	53.9 ± 3.9 (41) *
Control	18.6 ± 1.4 (45)
Z-Pro-Prolinal (10-5 M)	13.9 ± 3.5 (3)
Control	13.6 ± 0.3 (3)
TRH (10-6 M)	11.5 ± 2.3 (3)
Control	13.6 ± 0.3 (3)
cyclo-His-Pro (10-4 M)	35.5 ± 3.1 (6)
Control	26.9 ± 2.1 (6)
pyroglutamate (10-4 M)	22.7 ± 2.7 (3)
Control	21.4 ± 3.3 (3)

GH3 cells were grown as described in the Methods section. Two weeks after plating, 5-oxoprolinal (10-5 M) or inhibitor was added to the flasks. After 3 days of incubation at 37° C, cells were harvested, washed, homogenized and the homogenate was assayed for pyroglutamyl peptide hydrolase activity as described in the Methods section. The numbers in parentheses represent number of flasks used. The significance of changes in the specific activity between control and inhibitor-treated cells was determined using the Student's T-test.

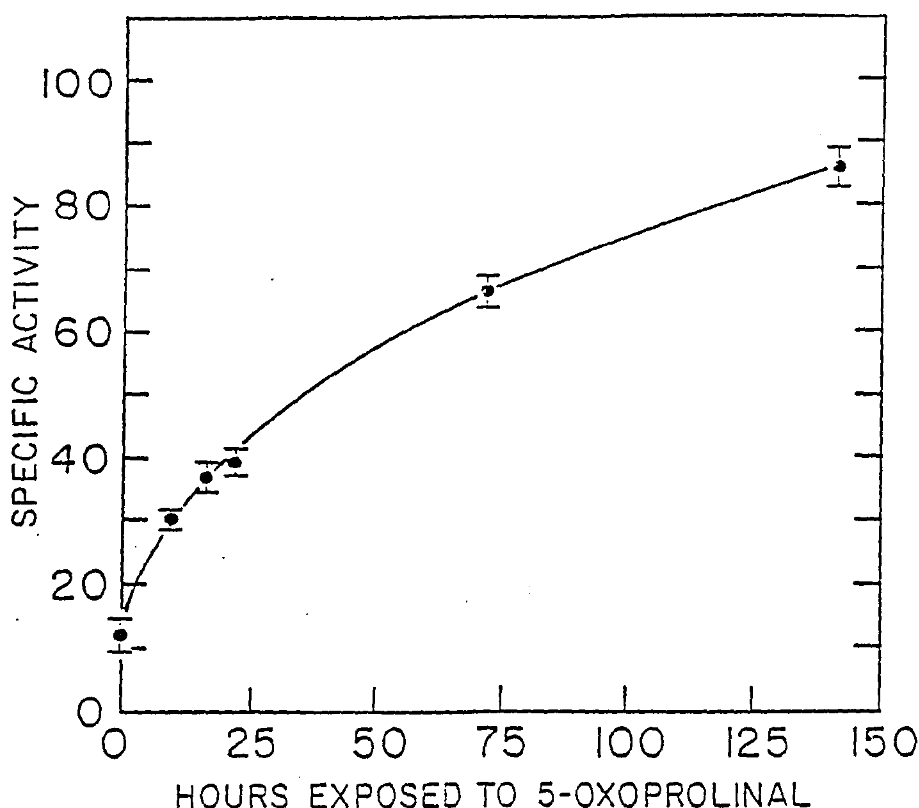
* $p < 0.001$

Fiqure 7



Concentration-response curve of 5-oxoprolinal on the specific activity of pyroglutamyl peptide hydrolase in GH3 cells. GH3 cells were grown as described in the Methods section. The cells received various concentrations of 5-oxoprolinal for 3 days. The cells were harvested, washed, homogenized and assayed for PPH activity as described in the Methods section. Specific activity is expressed as nmol/mg protein/h. Each point represents the mean ± SEM of 3 flasks of cells.

Figure 8



Time course of 5-oxoprolinal on the specific activity of pyroglutamyl peptide hydrolase in GH3 cells. GH3 cells were grown as described in the Methods section. Cells received 10-5 M 5-oxoprolinal for various times. The cells were harvested, washed, homogenized and assayed for PPH activity as described in the Methods section. Specific activity is expressed as nmol/mg protein/h. Each point represents the mean ± SEM of 3 flasks of cells.

affect PPH activity (Table 18) demonstrating that the effect of 5-oxoprolinal was not reproduced by other peptide aldehydes.

Since PPH catalyzes the degradation of TRH and TRH receptors are present on GH3 cells (Martin and Tashjian, 1977), we hypothesized that an inhibitor of PPH activity may alter PPH levels through TRH. To test this possibility TRH (10-6 M) was added to GH3 cell cultures for 3 days without effect on this enzyme. Similarly, the TRH metabolites, pyroglutamate and cyclo(His-Pro) (Bauer et al., 1978) failed to significantly alter PPH activity (Table 18).

To determine whether the stimulated pGlu-2NA-cleaving activity was indeed PPH and not a new enzyme which could also cleave pGlu-2NA, 5-oxoprolinal (10-5 M final concentration) was added to incubation tubes containing the stimulated enzyme. Under these conditions 5-oxoprolinal inhibited more than 90% of the pGlu-2NA-cleaving activity (stimulated activity 34.0 ± 4.6 nmol/mg/h, N=3; stimulated activity plus 5-oxoprolinal 3.0 ± 0.9 nmol/mg/h, N=3). Since 5-oxoprolinal is a specific PPH inhibitor, this experiment indicates that the stimulated activity is indeed PPH (EC 3.4.11.8). The membrane-bound pyroglutamyl-peptide hydrolyzing enzyme described above was not detectable in either control cells or cells treated with 5-oxoprolinal (10-5) for 3 days.

Experiments were conducted to determine whether the increase in activity of PPH requires the continuous presence

of 5-oxoprolinal. After exposure of GH3 cells to 5-oxoprolinal (10-5 M) for 1 hour, the media was replaced and the cells incubated for another 3 days. The activity of PPH in these cells was compared to the activity of PPH in cells exposed to 5-oxoprolinal continuously for 3 days. In this experiment, the activity of PPH in cells not exposed to inhibitor served as control. The activity of PPH in untreated cells was 25.4 ± 4.1 nmol/mg/h, N=6. PPH activity in cells exposed to 5-oxoprolinal for 1 h (76.4 ± 6.2 nmol/mg/h, N=6) was similar to the activity seen in cells exposed continuously to inhibitor for 3 days (69.1 ± 3.7 nmol/mg/h N=6). This indicates that a short exposure to 5-oxoprolinal is sufficient to cause an increase in PPH activity.

Since 5-oxoprolinal may be metabolized by GH3 cells, it was of interest to determine whether addition of fresh 5-oxoprolinal to the culture flasks each day for 3 days would increase PPH activity more than continuous exposure of cells to a single dose of 5-oxoprolinal added only at the start of the experiment. The increase in activity produced by addition of 5-oxoprolinal (10-5 M) each day for 3 days (untreated cells 25.4 ± 3.7 nmol/mg/h N=6; 5-oxoprolinal 60.9 ± 8.7 nmol/mg/h N=6) did not exceed the increase produced by continuous exposure to a single dose of 5-oxoprolinal (10-5 M) for 3 days (untreated cells 25.4 ± 4.1 nmol/mg/h N=6; 5-oxoprolinal 69.1 ± 3.7 nmol/mg/h N=6).

Experiments were performed to determine the stability of

5-oxoprolinal in the cell culture. In these experiments, pGlu-2NA was added to the flasks and the 2NA released was determined after 1 h and 3 days in the presence and absence of 5-oxoprolinal. After 1 h, 5-oxoprolinal (10-5 M) inhibited about 2/3 of the formation of 2NA from pGlu-2NA (0.4 mM) (controls 2.9 ± 0.1 nmol/ml media, N=3; 5-oxoprolinal-treated 0.97 ± 0.07 nmol/ml media, N=3). Three days after adding 5-oxoprolinal, there was no significant enzyme inhibition (controls 530 ± 13 nmol/ml media, N=3; 5-oxoprolinal-treated 490 ± 27 nmol/ml media, N=3). These results indicate that 5-oxoprolinal has been metabolized during the 3 day incubation.

The possibility that the increase in PPH activity is due to an increase in the affinity of the enzyme for its substrate, pGlu-2NA, was explored. The Km of PPH in cells exposed to 10-5 M 5-oxoprolinal was compared to the Km of PPH in control cells. Lineweaver-Burk plots, obtained by a linear regression analysis, were used to calculate the Km's. The Km of PPH from inhibitor-treated cells (Km = 0.16 mM) and control cells (Km = 0.18 mM) were virtually identical, indicating that the increase in enzymatic activity on exposure to 5-oxoprolinal was not due to increased affinity of the enzyme for the substrate.

The localization of the increased PPH activity in GH3 cells exposed to 5-oxoprolinal (10-5 M) for 3 days was compared to the localization of PPH in control cells. In control cells,

the activity of PPH (measuring the release of 2NA from pGlu-2NA by fluorometry) was 8.9 nmol/mg/h in the washed particulate fraction and 14.9 nmol/mg/h in the supernatant fraction. In inhibitor-treated cells, the activity was 10.0 nmol/mg/h in the washed particulate fraction and 76.3 nmol/mg/h in the supernatant fraction indicatng that the activated PPH is present in the cytosolic fraction of GH3 cells.

Effect of cyclcheximide on enzyme levels in GH3 cells

Cycloheximide was used to determine whether the increased enzymatic activity was due to induction of protein synthesis. As shown in Table 19, basal PPH activity decreased with cycloheximide alone, due to decreased protein synthesis. However even in the presence of cycloheximide, 5-oxoprolinal still caused an approximately 3 fold increase in PPH activity. These results suggest that new protein synthesis is not required for the increase in PPH activity. 5-oxoprolinal did not affect prolyl endopeptidase activity either in the presence or absence of cycloheximide, although the basal activity of the enzyme was lowered by cycloheximide.

Effect of inhibitors on prolactin response in GH3 cells

The addition of TRH (10 nM) to GH3 cells caused a significant increase in prolactin release (1 h) and synthesis (3 days) (Table 20) in agreement with earlier reports (Dannies and Tashjian, 1976). As shown in Table 20, 5-oxoprolinal (10-5 M) significantly decreased both basal and TRH-stimulated

Table 19 The effect of cycloheximide on PPH and prolyl endopeptidase activity in GH3 cells

	Specific activity (nmol/mg protein/h ± SEM)			
	control	5-oxoprolinal	cycloheximide	cycloheximide± 5-oxoprolinal
		PPH		
expt. 1	28.0 ± 0.7	39.7 ± 2.4 (140)	14.1 ± 0.4 (50)	24.7 ± 3.7 (175)
expt. 2	21.4 ± 2.2	56.3 ± 3.6 (263)	10.8 ± 2.9 (50)	30.7 ± 5.7 (284)
expt. 3	24.6 ± 0.3	42.4 ± 5.3 (173)	6.4 ± 3.9 (26)	28.0 ± 14.0 (438)
		Prolyl endopeptidase		
expt .1	216 ± 23	196 ± 23 (91)	236 ± 16 (109)	164 ± 12 (69)
expt. 2	220 ± 29	260 ± 31 (118)	144 ± 17 (65)	141 ± 23 (98)
expt. 3	299 ± 39	189 ± 12 (61)	163 ± 33 (54)	133 ± 18 (82)

GH3 cells were grown as described in the Methods section. In experiment 1, GH3 cells received cycloheximide (2ug/ml) for 10 h while in experiments 2 and 3, GH3 cells received cycloheximide (0.5 ug/ml) for 24 h. For each experiment, 3 flasks were used as controls, 3 flasks received 5-oxoprolinal (10-5 M). 3 flasks received cycloheximide and 3 flasks received cycloheximide ± 5-oxoprolinal (10-5 M). Cells were harvested, washed and homogenized and the homogenate was assayed for PPH and prolyl endopeptidase activity as described in the Methods section. The numbers in parentheses represent % of control except for "cycloheximide ± 5-oxoprolinal" column in which they represent % of cycloheximide.

prolactin release and synthesis. 5-oxoprolinal (10-5 M) plus TRH (10 nM) resulted in a 50% reduction in prolactin release and synthesis compared to TRH alone. Z-Pro-Prolinal (10-5 M) did not significantly alter prolactin release or synthesis either in the presence or absence of TRH. The combination of Z-Pro-Prolinal (10-5 M) and 5-oxoprolinal (10-5 M) in the presence of TRH (10 nM) also significantly decreased prolactin synthesis compared to TRH alone. Similar results were obtained when the prolactin response was related to the amount of DNA in the flask although data collected in this manner showed a somewhat greater variability.

In the above experiment, the inhibitor was present while prolactin was accumulating in the media. It was also of interest to examine the effects of preincubating the cells with 5-oxoprolinal,replacing the media and then adding TRH. As shown in Table 21, preincubation of GH3 cells with 5-oxoprolinal (10-5 M) for 3 days did not alter basal or TRH-stimulated prolactin release or synthesis. The results were similar whether the data was related to amount of DNA or amount of media.

Table 20 Prolactin response in GH3 cells

	Prolactin response (ng/ml media ± SEM)	
	1 h	3 days
control	44.8 ± 11.8 (9)	4250 ± 1280 (9)
TRH	72.3 ± 31.6[1] (9)	6950 ± 1130[2] (9)
5-oxoprolinal	30.3 ± 3.3[1] (6)	2070 ± 421[2] (6)
5-oxoprolinal/TRH	33.1 ± 3.8[3] (6)	3100 ± 425[4] (6)
Z-Pro-Prolinal	46.2 ± 12.8 (9)	4660 ± 1680 (9)
Z-Pro-Prolinal/TRH	74.0 ± 19.9 (9)	7810 ± 1430 (9)
Z-Pro-Prolinal/5-oxoprolinal	35.3 ± 1.6 (6)	3070 ± 443 (6)
Z-Pro-Prolinal/5-oxoprolinal/ TRH	45.0 ± 7.7 (6)	4730 ± 573[4] (6)

GH3 cells were grown as described in the Methods section. The media was replaced and Z-Pro-Prolinal (10-5 M), 5-oxoprolinal (10-5 M) and TRH (10 nM) were added to the flasks. The media was removed after 1 h and 3 days and prolactin was measured by radioimmunoassay as described in the Methods section. Data are mean values ± SEM. Numbers in parentheses represent the number of flasks used. The level of significance was found by comparing the prolactin response in inhibitor-treated cells to control cells.

[1] $p < 0.05$ from controls

[2] $p < 0.005$ from controls

[3] $p < 0.05$ from TRH

[4] $p < 0.005$ from TRH

Table 21 Prolactin response in GH3 cells preincubated with 5-oxoprolinal for 3 days

	Prolactin response (ng/ml media ± SEM)		Prolactin response (ng/ug DNA ± SEM)	
	1 h	3 days	1 h	3 days
control	39.6 ± 5.3	2214 ± 399	5.6 ± 1.5	407 ± 132
TRH	46.0 ± 5.8	3735 ± 384	6.7 ± 2.1	724 ± 120
5-oxoprolinal	36.7 ± 3.7	2407 ± 265	5.9 ± 1.2	417 ± 111
5-oxoprolinal/ TRH	43.4 ± 6.2	3702 ± 217	7.6 ± 2.0	587 ± 91

GH3 cells were grown as described in the Methods section. Two weeks after plating, 5-oxoprolinal (10-5 M) was added to half of the flasks. After 3 days of incubation, the media was removed. the cells were washed and new media was added. TRH (10 nM) was then added to half of the flasks. Media was removed after 1 h and 3 days. Prolactin was measured by radioimmunoassay and DNA was determined as described in the Methods section. Data are mean values ± SEM. 9 flasks were used for the ng/ml media experiments and 6 flasks were used for ng/ug DNA experiments.

Effect of substrates and inhibitors on TRH binding in GH3 cells

Since 5-oxoprolinal decreased prolactin response in GH3 cells. it was of interest to examine the effect of inhibitors and substrates of TRH-degrading enzymes on TRH binding. 5-oxoprolinal. Z-Pro-Prolinal, Z-Gly-Pro-SM and pGlu-2NA at a final concentration of 100 nM did not significantly alter ^{3}H-TRH binding (82 ± 6% , 82 ± 3.5%, 81 ± 6% and 73 ± 5%; % of control binding respectively, N=4). Unlabelled TRH (2 uM), however, totally deplaced ^{3}H-TRH from GH3 cell binding sites.

DISCUSSION

My initial studies demonstrated that Z-Pro-Prolinal is an effective inhibitor of prolyl endopeptidase in vivo. Maximal inhibition after intraperitoneal injection occurs in all organs in the first 45 min. The inhibition decreases progressively thereafter, but at a dose of 5 mg/kg it is still noticeable even after 6.5 h (Table 10). It is possible that the inhibition seen after 6.5 h is at least partially the result of an irreversible inactivation of the enzyme. This is based on the finding that Z-Pro-Prolinal behaves as a noncompetitive inhibitor of prolyl endopeptidase (Wilk and Orlowski, 1983) and dialysis of an enzyme-inhibitor mixture does not completely restore activity. It can be assumed that the decline in inhibition with time is caused by two factors. One is elimination of the inhibitor, presumably through the kidney and the gastrointestinal tract; the other is metabolism to the corresponding acid, probably by the action of aldehyde dehydrogenase, or to the corresponding alcohol by the action of alcohol dehydrogenase. Since both the alcohol and the acid are about 3000 times less inhibitory than the aldehyde (Wilk and Orlowski, 1983a), this metabolism would be expected virtually to terminate the action of the inhibitor. The rate of elimination of the inhibitor, as well as the rate of its metabolism, can be assumed to be contributing to the sensitivity of the brain enzyme to inhibition (Table 11). The lipophilic nature of the inhibitor apparently favors its accumula-

tion in brain and also its slow elimination from this organ. That this is, indeed, the case is indicated by the finding that doses of the inhibitor (0.005 mg/kg) capable of producing maximal tissue concentrations only in the nanomolar range exert a significant inhibitory effect in the brain, but not in other tissues, except the kidney (Table 11).

Aldehyde dehydrogenase and alcohol dehydrogenase, two enzymes potentially capable of metabolizing Z-Pro-Prolinal, have an unequal distribution in tissues. Relatively large amounts of both enzymes are present in the liver, with lesser amounts present in other organs, including the brain. For example, the activity of aldehyde dehydrogenase is about 30 times higher in rat brain (Deitrich, 1966), and the activity of alcohol dehydrogenase was reported to be more than 3000 times higher in rat liver than in brain (Raskin and Sokoloff, 1968). The low activity of the two enzymes in brain can thus be an important factor, favoring inhibition of the enzyme in this tissue. Conversely, the high activity of the two enzymes in the liver probably accounts for the relatively lower inhibition of the enzyme in this organ and for the higher doses required for inhibition (Table 11).

The concentration of SM in various tissues given in the control columns in Tables 10 and 11 are determined both by the actual activity of prolyl endopeptidase in the tissue and by access of the substrate, Z-Gly-Pro-SM, to the tissue and the enzyme. The relatively low values of SM in brain compared

with the liver and kidney are probably the result of a limitation of entry into the brain, imposed by the blood-brain barrier.

In our laboratory, we have previously shown that Z-Pro-Prolinal is a specific prolyl endopeptidase inhibitor. A variety of cellular endo- and exopeptidases are resistant to inhibition even by large doses of this inhibitor (Wilk and Orlowski, 1983a). The specificity, duration of action, and potency of Z-Pro-Prolinal as a prolyl endopeptidase inhibitor in brain led us to use it as a tool in studies on the role of this enzyme in neuropeptide metabolism. TRH was chosen as a model neuropeptide for studies on the effects of enzyme inhibitors on neuropeptide degradation.

Since the literature stated that in tissues, two major enzymes appear to be responsible for the initial degradation of TRH: deamidation catalyzed by prolyl endopeptidase and removal of the N-terminal pyroglutamyl residue catalyzed by pyroglutamyl peptide hydrolase (Griffiths et al., 1979a; Kreider et al., 1981; Busby et al., 1982; Bauer and Kleinkauf, 1980), we elected to synthesize an inhibitor to PPH. Since PPH specifically hydrolyzes 5-oxoproline from the N-terminus of peptides by a mechanism in which a cysteine residue in the active site is believed to be necessary for activity, it was expected that the aldehyde analog of pyroglutamate should form a tetrahedral thiohemiacetal with the cysteine in the active site. 5-oxoprolinal was therefore synthesized as an active-

site directed inhibitor of PPH. The observation that the Ki of 5-oxoprolinal is about five orders of magnitude lower than that of the corresponding alcohol or acid (Table 1) is consistent with the interpretation that an analog of the transition state intermediate is being formed in the interaction of the inhibitor with the enzyme.

The synthesis of 5-oxoprolinal proved to be quite difficult. Although the DMSO-DCC method gave low yields and impurities which comigrated with 5-oxoprolinal on silica gel columns, it was more successful than other methods including reduction of pyroglutamate by the thexylborane reagent (Brown et al., 1972), reduction of the ester derivative by $LiAlH_4$ (Brown et al., 1982), and oxidation of the alcohol derivative by pyridinium dichromate (Stanfield et al., 1981) or by alcohol dehydrogenase (Andersson and Wolfenden, 1982). 5-oxoprolinal appears to be labile to acidic or basic conditions, possibly due to lactam ring-opening. The use of the neutral catalyst, PTFA, increased the yield of the DMSO-DCC oxidation. Attempts to catalyze the DMSO-DCC oxidation by oxalic acid (Omura and Swern, 1978), dichloroacetic acid (Thompson, 1973) and phosphoric acid (Thompson, 1977) met with little success.

The NMR and IR spectra are consistent with the structure of 5-oxoprolinal as depicted in Figure 2. To confirm this structure, the 2,4-dinitrophenylhydrazone derivative of this compound was prepared and isolated as a solid. The NMR and IR

spectra and the elemental analysis are correct for the derivative's structure depicted in Figure 2. The derivative was used to prepare a standard curve for the DNP assay and used to quantitate 5-oxoprolinal in subsequent preparations. The curve was found to be similar to that of butyraldehyde, which confirms that the DNP reaction using butyraldehyde accurately quantitated 5-oxoprolinal in the earlier experiments. Since α-aminoaldehydes are particularly prone to racemization and since this tendency is fostered by exposure to silica gel (Hamada and Shiori, 1982), it is likely that 5-oxoprolinal is a racemate.

5-oxoprolinal was found to act as a competitive inhibitor as demonstrated by the Dixon and Henderson plots (Figure 5). This is in contrast to Z-Pro-Prolinal which was found to be a noncompetitive inhibitor of prolyl endopeptidase (Wilk and Orlowski, 1983a). Although the Ki for 5-oxoprolinal (26 nM) is similar to that for Z-Pro-Prolinal (14 nM), 5-oxoprolinal appears to be a less potent and shorter acting inhibitor in vivo. The degree of inhibition was much lower at 30 min than at 10 min for 5-oxoprolinal (Table 7) while the degree of in vivo inhibition for Z-Pro-Prolinal remained high at 30 min (Table 8). At a dose of 5 mg/kg, Z-Pro-Prolinal inhibited greater than 90% of prolyl endopeptidase activity in most organs after 30 min, while a dose of 50 mg/kg of 5-oxoprolinal was required to give between 17% and 94% inhibition depending on the organ after 30 min. The lower potency and shorter duration of 5-oxoprolinal inhibition might be due to the fact

that its binding to the enzyme is readily reversible and also that its metabolic inactivation and elimination might proceed faster than that of Z-Pro-Prolinal. It may be expected that alcohol dehydrogenase and aldehyde dehydrogenase might contribute to the metabolism of the inhibitor.

It is possible that the inhibition of PPH in homogenates after intraperitoneal administration of the inhibitor does not reflect true in vivo inhibition, but rather results from exposure of the enzyme to the inhibitor only after disruption of the tissue by homogenization. This possibility is especially relevant since the dose used was relatively high. To exclude this possibility. future studies in which the inhibitor is used to block the degradation of a substrate of PPH in vivo need to be carried out.

Since 5-oxoprolinal was found to be a relatively short-lived and weakly potent inhibitor of PPH in vivo, PDMK, an irreversible inhibtor of PPH which alkylates the sulfhydryl residue in the active site of the enzyme, was used to inhibit PPH in vivo. This compound was found to be an extremely potent inhibitor in vivo with substantial inhibition one hour after a dose of 0.01 mg/kg in all tissues studied (Table 12). The inhibition was also found to be long-lasting as substantial inhibition was detected 24 hours after administration of a dose of 0.1 mg/kg of PDMK. PDMK was found to be a much more effective inhibitor than 5-oxoprolinal and was therefore used in further animal studies.

Several investigators have concluded that in tissues there are only two enzymes, prolyl endopeptidase ("TRH deamidase") and pyroglutamyl peptide hydrolase (also called pyroglutamyl aminopeptidase), capable of the initial degradation of TRH. There are discrepancies in the literature on the relative contributions of these two enzymes to TRH degradation in brain fractions in vitro. These discrepancies can be resolved by examining which subcellular fraction was used and whether thiols or metal chelators were included in the assay. Prolyl endopeptidase is activated by DTT and is largely a cytosolic enzyme. PPH is a predominantly cytosolic thiol protease which requires DTT and is activated by EDTA. In the particulate fraction there is an enzyme which on the basis of its cleavage of the pGlu-His bond of TRH has also been referred to as pyroglutamyl peptide hydrolase (Griffiths et al., 1979a; Browne et al., 1981; Prasad et al., 1983) but is inhibited by DTT and EDTA (Browne et al., 1981; O'Connor and O'Cuinn, 1984; Garat et al., 1985). Prasad and Peterkofsky (1976), Bauer and Kleinkauf (1980) and Busby et al. (1982) all used DTT and EDTA in their incubation mixtures and found most of the TRH-degrading activity in the supernatant with little or no activity in the particulate fraction. Under these conditions, TRH is deamidated to TRH-OH and cleaved at the pGlu-His bond to form either His-Pro-NH2 or cyclo-His-Pro, the nonenzymatic cyclization product of His-Pro-NH2 (Bauer et al., 1978). In contrast, Kreider et al. (1981), Schock (1977) and Griffiths and coworkers (Griffiths et al., 1979 a, b; 1980; 1982) omitted

DTT and EDTA and found more activity in the particulate fraction than in the supernatant. In the supernatant, only TRH-OH was formed (Griffiths et al., 1979 a, b; 1980) since PPH requires DTT. In the particulate fraction, TRH was cleaved at the pGlu-His bond (Griffiths et al., 1979 a, b; 1980: Kreider et al., 1981).

Since in both particulate and supernatant fractions, TRH was cleaved at the pGlu-His bond, until recently it was assumed that only one enzyme, PPH, was responsible. Browne et al. (1981) first proposed that the particulate and soluble enzymes were probably distinct. The particulate enzyme was found to have a high molecular weight (~180,000) and was inhibited by DTT and EDTA while the soluble enzyme, correctly characterized as PPH (EC 3.4.11.8), had a molecular weight of about 24.000 and was activated by DTT and EDTA (Browne and O'Cuinn. 1983). This group obtained a highly purified preparation of soluble PPH from guinea pig brain. More recently O'Connor and O'Cuinn (1984), Charli et al. (1985) and Garat et al. (1985) identified the particulate TRH-degrading enzyme in brain synaptosomal membranes. It cleaved the pGlu-His bond of TRH. was inhibited by metal chelators and had a molecular weight of 230.000 (O'Connor and O'Cuinn, 1984).

We were interested in verifying the contributions mentioned in the literature concerning the role of PPH and prolyl endopeptidase in the degradation of TRH in vitro in the presence and absence of metal chelators and thiols with the use of PDMK

and Z-Pro-Prolinal, specific inhibitors of the two enzymes. Additionally PDMK was used to determine whether the brain particulate fraction and serum contain a distinct TRH-degrading enzyme which cleaves the pGlu-His bond of this peptide. If the enzymes are distinct, they would not be inhibited by PDMK, a specific inhibitor of PPH (EC 3.4.11.8).

We found that Z-Pro-Prolinal and PDMK protected TRH from degradation only in the presence of DTT and EDTA. In the absence of DTT and EDTA, TRH was effectively degraded and the inhibitors did not block degradation. These results are therefore consistent with the presence of another TRH-degrading enzyme(s) whose activity is expressed in the absence of DTT and EDTA. This enzyme previously had to be measured with TRH as the substrate. We elected to develop a chromogenic assay to measure this enzyme.

Chromogenic substrates have previously been used in the study of TRH-degrading enzymes. We introduced the use of pGlu-His-Pro-2NA in a coupled assay with excess DAP IV to measure pyroglutamyl-peptide cleaving enzymes. This assay will specificically measure enzymes capable of cleaving the pGlu-His bond of the substrate provided that prolyl endopeptidase is inhibited. Moreover, if PPH is also inhibited, the assay will detect the "TRH-specific" enzyme which until now had to be measured with TRH. Identification of the products generated by incubation of pGlu-His-Pro-2NA with rat serum and rat brain washed pellets revealed that cleavage of the pGlu-

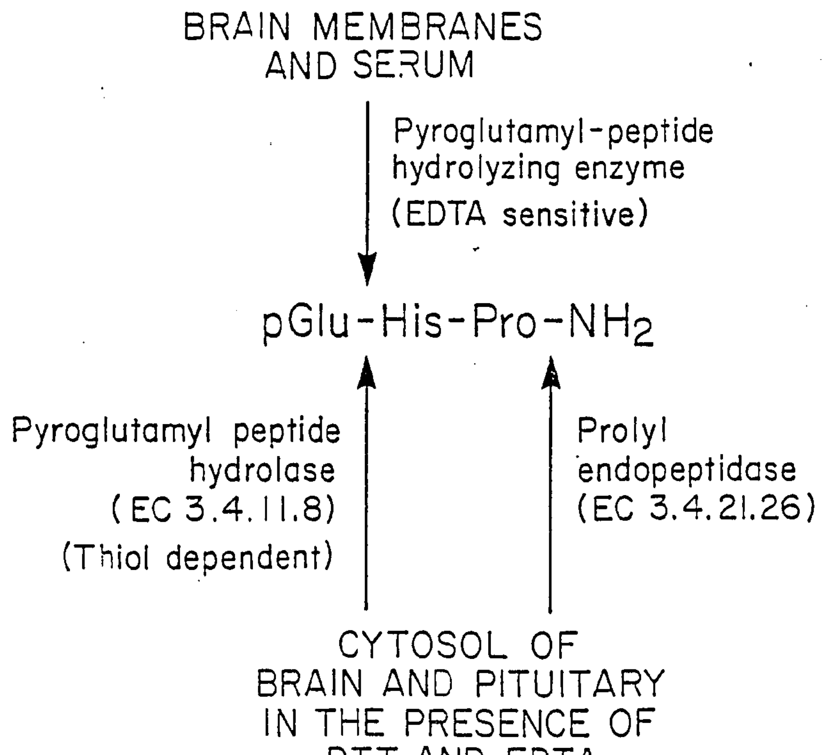
His bond had occurred.

Our studies using chromogenic substrates and specific inhibitors allow us to propose pathways of TRH degradation shown in Figure 9. In rat brain supernatant, in the presence of DTT and EDTA, the N-terminal pyroglutamyl residue and the C-terminal amide bond of TRH are cleaved by PPH and prolyl endopeptidase respectively. PPH is totally dependent on the presence of DTT and is specifically inhibited by PDMK. Prolyl endopeptidase is activated by DTT and is specifically inhibited by Z-Pro-Prolinal.

In the rat brain particulate fraction, there is a second pyroglutamyl-peptide hydrolyzing activity capable of cleaving the pGlu-His bond of TRH. pGlu-His-Pro-2NA is cleaved more readily than pGlu-2NA by this enzyme. This activity is not inhibited by PDMK indicating that it is not due to PPH. It has properties of a metalloenzyme being moderately inhibited by EDTA and strongly inhibited by o-phenanthroline.

Early reports on the degradation of TRH in serum found catabolism to TRH-OH and attributed this degradation to a TRH-deamidase now known to be prolyl endopeptidase (Nair et al., 1971; Redding and Schally, 1972). However several laboratories (Bauer et al., 1978; Visser et al., 1977; Knigge and Schock, 1975; Vale et al., 1971; Benuck and Marks, 1976) have not reproduced this finding but rather found cleavage of the pGlu-His bond (Bauer et al., 1978; Safran et al., 1982) and the His-Pro bond (Visser et al., 1977; Benuck and Marks, 1976)

Figure 9



Pathways of TRH Degradation

of TRH. The presence of a specific TRH-degrading enzyme in serum has been reported (Taylor and Dixon, 1978; Bauer and Nowak, 1979; Bauer et al., 1981b). This enzyme was partially purified from rat (Taylor and Dixon, 1978) and porcine (Bauer and Nowak, 1979) serum, found to have a molecular weight of 260,000 and to degrade TRH by cleavage of the pGlu-His bond. Since this enzyme was inhibited by DTT and EDTA and was not affected by 2-iodoacetamide, in contrast to the thiol enzyme PPH, which requires DTT and EDTA for full activity and is inactivated by 2-iodoacetamide, it was suggested that the serum enzyme differs from PPH (Bauer and Nowak, 1979).

Our findings demonstrate that in rat serum, Z-Pro-Prolinal and PDMK, specific inhibitors to prolyl endopeptidase and PPH respectively do not protect TRH from rapid inactivation. Although prolyl endopeptidase is present in serum (Yoshimoto et al., 1979), the inability of Z-Pro-Prolinal to protect TRH from degradation indicates that prolyl endopeptidase is not important in the degradation of TRH in serum and therefore TRH-OH would be an unlikely serum metabolite. The inability of PDMK to protect TRH from degradation indicates that PPH is also not important in serum TRH degradation and that serum contains a TRH-degrading enzyme which is neither prolyl endopeptidase nor PPH.

The assays using chromogenic substrates allowed us to screen tissues for pyroglutamyl-peptide degrading activities. The distribution of the membrane-bound pyroglutamyl-peptide

degrading enzyme was determined in various tissues using the coupled assay with the substrate pGlu-His-Pro-2NA in the presence of excess DAP IV. It is of considerable interest that this enzyme is highly localized to the brain (Table 6). Its selective localization in brain suggests that this membrane-bound enzyme may be important in inactivation of pyroglutamyl peptide putative neurotransmitters, such as TRH and LHRH. We have studied the distribution of prolyl endopeptidase in rabbit (Orlowski et al., 1979) and mouse (Table 8, control column) tissues and PPH in mouse tissues (Table 7, control column). These enzymes were found to have a much more uniform distribution.

The selective localization of the membrane-bound enzyme in brain prompted us to examine its distribution within brain and to compare its distribution to that of PPH. We found striking differences between the two enzymes in various regions of rat brain and in the pituitary (Table 9). The soluble PPH activity was highest in the pituitary. In the pituitary, PPH was 100 times higher than the membrane-bound activity. The membrane-bound activity was highest in the cortex and hippocampus. Since TRH is thought to have both endocrine and putative neurotransmitter actions, one could speculate that PPH could degrade TRH at the site of its endocrine actions (pituitary) while the membrane-bound enzyme could terminate the neurotransmitter actions of TRH in the CNS. In this respect it is of significance that O'Cuinn and coworkers (Greaney et al., 1980; O'Connor and O'Cuinn, 1984) and Charli and coworkers (Garat et

al.. 1985; Charli et al., 1985) have localized the membrane-bound activity to synaptosomes.

These results as well as those of O'Connor and O'Cuinn (1984) strongly suggest that the serum and membrane-bound brain pyroglutamyl hydrolyzing enzymes are similar or the same. As can be determined from comparing Table 4 (brain particulate fraction) and Table 5, both enzymes prefer pGlu-His-Pro-2NA to pGlu-2NA. Examination of their inhibition profile reveals that both enzymes are not affected by PDMK, are inhibited somewhat by DTT plus EDTA and are totally inhibited by the combination of DTT and EDTA and o-phenanthroline. Purified enzymes from both sources will be required to determine if they are indeed the same and whether brain tissue or possibly lung tissue is the source of the serum enzyme.

We have determined the Ki of TRH for the rat brain membrane-bound pyroglutamyl-peptide hydrolyzing enzyme to be 0.25 mM. For comparison, the Ki of TRH for homogeneous rabbit brain prolyl endopeptidase was 5.8 mM and the Ki of TRH for partially purified bovine brain PPH was 0.47 mM. TRH was found to inhibit all enzymes competitively. Since, for purely competitive inhibitors, the Ki can be assumed to equal the Km, these values can give an estimate of the relative affinity of TRH for the three enzymes. The membrane-bound pyroglutamyl hydrolyzing enzyme appears to have the highest affinity for TRH. TRH appears to be a relatively poor substrate for prolyl endopeptidase and PPH. However, since the membrane-bound

activity was not purified and since the other enzymes were obtained from different species, these results should be interpreted with caution. Full characterization of TRH as a substrate for these three enzymes awaits kcat/ Km ratios using homogeneous enzymes from similar sources.

The availability of the purified membrane-bound enzyme will allow its full characterization and classification. Although the particulate enzyme cleaves the pGlu-His bond of pGlu-His-Pro-2NA and presumably of TRH, its primary specificity may not be directed toward this bond. One cannot rule out hydrolysis of this bond by an endopeptidase with a specificity other than directed toward the pyroglutamyl-histidyl bond. Hydrolysis of the Phe-Gly bond of enkephalins by a crude membrane preparation was mistakenly attributed to a highly specific peptidyl dipeptidase termed "enkephalinase" (Malfroy et al., 1978). When the enzyme was purified it was shown to be neither a peptidyl dipeptidase nor enkephalin-specific (Almenoff et al., 1981). Substrate specificity studies of the purified membrane-bound enzyme will determine the amino acids required for recognition and whether this enzyme is indeed TRH-specific.

Finally it should be noted that this in vitro study does not reflect the contributions of these enzymes to the degradation of TRH in vivo. It would be of great value to have a specific inhibitor of the membrane-bound enzyme which could be used together with Z-Pro-Prolinal and PDMK for in vivo studies.

We demonstrated that Z-Pro-Prolinal and PDMK are potent and long-lasing inhibitors of brain prolyl endopeptidase and PPH respectively in vivo. If prolyl endopeptidase or PPH are the enzymes important in the physiological degradation of TRH, then it would be expected that the in vivo administration of Z-Pro-Prolinal plus PDMK would raise endogenous levels of TRH. As shown in Table 13, TRH levels were only increased significantly in the pituitary 15 minutes after administration of Z-Pro-Prolinal. A noticeable but not significant increase in TRH levels in the pituitary 30 minutes after administration of Z-Pro-Prolinal was observed. Given the fact that Z-Pro-Prolinal readily enters the brain and inhibits prolyl endopeptidase for up to 6.5 hours (Table 10), it is surprising that the effects of Z-Pro-Prolinal on TRH levels are limited to only short times in the pituitary with no effects in the hypothalamus or cortex.

Since TRH causes an increase in TSH (Schally et al., 1973) and prolactin (Mueller et al., 1974) levels in vivo, it was expected that if Z-Pro-Prolinal prevented TRH degradation in the pituitary, serum prolactin and TSH levels should increase. However, Mueller et al. (1974) reported that the effect of TRH on prolactin in rats was not dose-related and reported that TRH raised prolactin levels only in estrogen-treated rats. As shown in Table 14, no significant changes in either TSH or prolactin levels were noted in rats who received Z-Pro-Prolinal. Large variations in prolactin and TSH levels between animals were observed which may obscure any effects of the

inhibitor. In the literature, wide variations in the levels of these hormones both between animals and within an animal at different times have been reported. For example, Fink et al. (1983) reported TSH levels in rats which varied by over 30 fold. Prolactin and TSH exhibit nyctohemeral variations (Fukuda et al., 1975) and appear to increase with stress (Krulich and Illner, 1973; Blake, 1974). Although the time and stress variations were controlled by treating vehicle- and inhibitor-injected animals in a similar manner, large variations between animals were still observed.

The location of the physiologically important degradation-site of TRH is not known. The site of the endocrine action of TRH is the pituitary. TRH is synthesized in the hypothalamus and transported to the pituitary by the hypothalamic-hypophyseal portal blood system. The levels of TRH are approximately 5 times higher in the hypothalamus than in the pituitary (Table 13, control column). Although the physiological relevance of the large amount of TRH present in the hypothalamus is unknown, it is possible that excess TRH is degraded enzymatically in the hypothalamus. Alternatively, the physiological degradation of TRH might occur in the portal blood system or at its site of action, the pituitary. Additionally, TRH might be degraded in a different manner in the cortex where it functions as a neurotransmitter. As discussed above, PPH levels are highest in the pituitary and hypothalamus while the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme levels are highest in the cortex and hippocampus. Prolyl endopepti-

dase levels are highest in the entorhinal cortex, hippocampus and striatum with lower levels in brainstem regions (Orlowski et al., 1979). The elevation of TRH by Z-Pro-Prolinal only in the pituitary and the pronounced regional distribution of the pyroglutamyl-peptide hydrolyzing enzyme suggest that TRH degradation varies at different sites. It is possible that in the pituitary, a region with extremely low membrane-bound pyroglutamyl-peptide hydrolyzing activity, TRH is degraded primarily by prolyl endopeptidase. In other brain regions, prolyl endopeptidase may contribute little to the physiological degradation of TRH and hence it's inhibition does not result in elevated TRH levels. The membrane-bound pyroglutamyl-peptide hydrolyzing enzyme might be the important enzyme in the cortex and hippocampus, likely locations of TRH as a neurotransmitter. If an inhibitor of this enzyme could be designed and synthesized, the effects of this inhibitor on TRH levels would be worthy of investigation.

Surprisingly, there was less (although not significantly less) of an increase in TRH levels in the pituitary 15 minutes after administration of Z-Pro-Prolinal plus PDMK than after Z-Pro-Prolinal alone. The lack of effect of the two inhibitors together after 15 minutes might be due to the small sample size (4 animals). Alternatively, a drug interaction could have occurred between Z-Pro-Prolinal and PDMK. For example PDMK could have blocked the transport of Z-Pro-Prolinal to the enzyme and therefore decreased its inhibitory actions. 5-Oxoprolinal stimulated PPH activity in pituitary (GH3) cells

(discussed below). This regulation of an enzyme by an inhibitor suggests that PPH is the principal rate-limiting enzyme in the pituitary. The finding that PPH levels within GH3 cells are highly regulated is inconsistent with the lack of effect of PDMK on TRH in the pituitary of rats.

The limited effects of Z-Pro-Prolinal and PDMK on altering endogenous TRH levels is consistent with the in vitro experiments on TRH degradation. Substantial in vitro degradation of TRH was found in the presence of both Z-Pro-Prolinal and PDMK when the incubation was performed in the absence of DTT. If Z-Pro-Prolinal and PDMK do not protect TRH from in vitro degradation by rat brain homogenates, it seems unlikely that they would protect TRH from in vivo degradation. The co-localization of thiol-reducing agents such as glutathione and TRH-degrading enzymes is currently unknown. It appears that the presence of a thiol-reducing environment would favor degradation by prolyl endopeptidase and PPH while the absence would favor degradation by membrane-bound pyroglutamyl-peptide hydrolyzing enzyme.

In GH3 cells, when PPH is inhibited by 5-oxoprolinal, the cells, by some unknown mechanism, increases PPH activity. It appears that the cells are compensating for enzymatic blockade by increasing their enzymatic levels. It is possible that over a period of time the animal also responds to inhibition of an important enzyme by some sort of compensation; either an increase of that enzyme or another enzyme capable of neuropep-

tide degradation. In these cases, blockage of prolyl endopeptidase or PPH might not raise endogenous TRH levels due to some regulatory compensation. However, chronic administration of PDMK to rats did not alter PPH levels (Table 7, 4 day column) indicating that an in vivo increase in PPH activity did not occur in rats unlike the situition in GH3 cells. Additionally, administration of PDMK and Z-Pro-Prolinal did not alter the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme in rats. Although in rats, a compensation in enzymatic activity upon inhibition was not detected experimentally, it is still possible that some as yet unobserved compensation upon inhibition of prolyl endopeptidase or PPH did occur and prevented endogenous TRH levels from being elevated.

Since in vivo experiments are beset with problems such as variations between animals and stress and nyctohemeral variations as well as metabolism or elimination of the inhibitor, we elected to also study the effect of enzyme inhibitors in a cell culture system. GH3 cells, which respond to TRH by secreting prolactin, were chosen. We were initially interested in measuring neuropeptide-degrading enzymes in GH3 cells in order to determine whether these cells would be suitable to study these enzymes. The activities of neuropeptide-degrading enzymes in the GH3 cell homogenate preparations were compared to activities in rat anterior pituitary homogenate preparations (Table 16). The activities of the TRH-degrading enzymes are of particular interest. In the GH3 cell homogenates, the activity of prolyl endopeptidase is over 25 times that of PPH.

The levels of both of these enzymes are 1 1/2 to 2 times higher in GH3 cells homogenates than in rat anterior pituitary homogenates. No membrane-bound pyroglutamyl-peptide hydrolyzing enzyme was detected in GH3 cells.

The activity of cathepsin B is much higher in the GH3 cell homogenates than in anterior pituitary cell homogenates, while the activity of cathepsin D is lower. Both of these enzymes are lysosomal proteases optimally active at an acidic pH and are probably involved in protein metabolism (Orlowski, 1983). It is of interest that the activities of the two lysosomal proteases are divergent in the tumor cells. Poole et al. (1978) found cathepsin B but not cathepsin D higher in breast carcinoma cultures than in normal breast tissue. They suggested that cathepsin B might be important as a mediator of tumor dissemination. The fact that cathepsin B is higher in GH3 cell homogenates than in anterior pituitary homogenates suggests that the high enzyme activity might be an expression of neoplastic transformation of the GH3 cells.

The soluble metalloendopeptidase was found to have high levels in both GH3 cell homogenates and rat anterior pituitary homogenates. The multi-catalytic protease complex, the aminopeptidases and the membrane-bound metalloendopeptidase have lower activities in both of these preparations. These enzymes do not appear to degrade TRH and the physiological substrates for these enzymes are not known.

The profile of activities of peptide metabolizing enzymes differs in GH3 cell homogenates and anterior pituitary homogenates. The explanation for these changes, while not totally apparent from a study of this nature, is worthy of speculation. Two possible reasons for these differences in enzyme activities are that the GH3 cells are a single type of cell cloned to secrete growth hormone and prolactin and therefore have a different functional status than all of the other anterior pituitary cells or that they are cells which have undergone neoplastic transformation which affected their enzyme activities. The observation that cathepsin B, an enzyme known to be higher in tumor cells, has higher activity in the GH3 cell homogenate supports the hypothesis that the neoplastic transformation might be responsible for the change in the activity of this enzyme. It could be postulated that the high levels of cathepsin B, prolyl endopeptidase and the soluble metalloendopeptidase are due to the enzymes being present in high levels in a few specialized cells in the anterior pituitary which were then propogated in the GH3 cell population. The fact, however, that growth hormone-secreting and prolactin-secreting cells make up respectively about 45% and 25% of the total number of cells in the anterior pituitary support the idea that the cells have undergone some sort of change and do not represent a small amount of cells present in the normal pituitary with a specialized function. It is still possible, however, that part of these cell's altered enzyme levels might be related to their selectivity of secreting prolactin and

growth hormone. Possibly the high levels of cathepsin B, prolyl endopeptidase and soluble metalloendopeptidase imply either a functional change or a neoplastic transformation of the GH3 cell. A further study using cells with a different secretory function might clarify this question.

Since prolyl endopeptidase and PPH are present in GH3 cells (Table 16) (Friedman et al., 1984b), it was of interest to determine whether Z-Pro-Prolinal and 5-oxoprolinal could be used to inhibit these enzymes within the cells. Z-Pro-Prolinal inhibited prolyl endopeptidase as expected, however 5-oxoprolinal caused an unexpected 3 to 4 fold increase in PPH levels in the GH3 cells. This increase was characterized and found to be dependent on the concentration of 5-oxoprolinal and on the time of exposure to this inhibitor. The increase in PPH activity was specific to 5-oxoprolinal. Z-Pro-Prolinal. pyroglutamate and cyclo(His-Pro) did not significantly alter PPH activity (Table 18). Similarly, 5-oxoprolinal did not affect prolyl endopeptidase activity. The increased activity was indeed PPH and not another enzyme capable of cleaving the pGlu-2NA bond since the degradation of this substrate by a homogenate from 5-oxoprolinal-treated cells was totally inhibited by the addition of 5-oxoprolinal.

The mechanism of the 5-oxoprolinal-stimulated increase in PPH activity remains unknown. The results presented here indicate that the increase in PPH is due to 5-oxoprolinal directly and not to its effects on TRH. The increased activ-

ity is not due to a higher affinity of PPH for its substrate since the Km of PPH did not change upon exposure to 5-oxoprolinal. The increased PPH activity is not consistent with enzyme induction since cycloheximide at relatively high doses did not block the activation. Higher doses of cycloheximide then those used here may indeed block the increase in PPH activity. however under the conditions of these experiments. higher doses resulted in significant cellular toxicity. An experiment in which cycloheximide and actinomycin D, inhibitors of protein synthesis at two different sites, might be more effective at blocking the 5-oxoprolinal-stimulated increase in PPH activity. It is possible that 5-oxoprolinal increases PPH activity in GH3 cells by protecting the enzyme from inactivation. PPH, a cysteine protease is very susceptible to oxidation (Szewckuk and Kwiatkowska, 1970). 5-Oxoprolinal may prevent oxidation of the active-site cysteine by formation of the reversible thiol hemiacetal adduct (Friedman et al., 1965). Alternatively it is conceivable that 5-oxoprolinal may inhibit a proteolytic enzyme responsible for degrading PPH. However, the observation that only a brief exposure to 5-oxoprolinal is sufficient to increase PPH activity argues against these possibilities. The mechanism of stimulation of PPH remains to be elucidated.

GH3 cells are often used as a model for mammotroph function in the pituitary gland. Within the pituitary, the initial degradation of TRH appears to proceed both through deamidation catalyzed by prolyl endopeptidase and cleavage of the pGlu-His

bond catalyzed by PPH. Both enzymes are present in GH3 cells (Table 16) but their physiological significance remains unknown. Our data suggest that PPH activity appears to be highly regulated in GH3 cells. Since rate limiting enzymes are often regulated, these studies suggest that PPH may be the principal rate limiting enzyme in TRH degradation within the GH3 cells and by inference within the pituitary gland.

In control cells, about 2/3 of the PPH activity is located in the cytosol while 1/3 of the activity is membrane-bound. In 5-oxoprolinal-treated cells, the increase in PPH activity is totally cytosolic. Prolyl endopeptidase is also a cytosolic enzyme. The location of these enzymes is important because most researchers state that the binding of TRH to receptors on the plasma membrane initiates the stimulation of TSH and prolactin (Gershengorn, 1982). The presence of cytosolic enzymes might have little effect on the function of TRH on pituitary cells. One group has reported TRH entering the cell and binding to nuclear receptors to stimulate prolactin synthesis (Lavarriere et al., 1981). Only if internalized TRH affects intracellular function or somehow regulates TRH binding to the plasma membrane could cytosolic enzymes physiologically affect TRH function. The action of TRH to stimulate prolactin release appears to occur within seconds to minutes (Aizawa and Hinkle, 1985) while the degradation of TRH in GH3 cells occurs over a period of hours to days (Hinkle and Tashjian, 1975). This data argues against an effect of prolyl endopeptidase or PPH inhibiton on prolactin production in GH3

cells.

Z-Pro-Prolinal was found to not affect either basal or TRH-induced prolactin release or synthesis in GH3 cells (Table 20). This finding argues that either prolyl endopeptidase is not an important TRH-degrading enzyme in the GH3 cells or that internalized TRH does not affect prolactin response. 5-Oxoprolinal, however, did decrease both basal and TRH-stimulated prolactin release and synthesis when added at the same time as TRH (Table 20). This is a surprising result since if 5-oxoprolinal inhibited PPH, an enzyme capable of degrading TRH, then prolactin levels should increase. With the finding that 5-oxoprolinal stimulated rather than inhibited PPH activity in GH3 cells (Table 18), the possibility was considered that the stimulated PPH could degrade TRH more effectively and therefore the TRH-stimulated prolactin response would be decreased. This is unlikely since in this experiment excess 5-oxoprolinal was present in the GH3 cell media during prolactin accumulation and would be able to inhibit the newly formed PPH (the activation is seen only when the inhibitor is removed by washing). This possibility was tested by preincubating the GH3 cells for 3 days with 5-oxoprolinal, replacing the media to remove the inhibitor and then allowing prolactin to accumulate in the presence and absence of TRH. If the stimulated PPH did indeed degrade TRH more effectively and therefore decrease the prolactin response, then an even larger reduction in prolactin should be noted in this experiment. The prolactin levels, however, were not affected by preincubation with

5-oxoprolinal (Table 21), suggesting that the decrease in prolactin and the increase in PPH activity, although both due to 5-oxoprolinal, are probably independent findings.

5-Oxoprolinal could affect prolactin levels by mechanisms such as alteration of TRH binding, proteolysis of prolactin or processing and secretion of prolactin. It was demonstrated that 5-oxoprolinal did not affect TRH binding. To study if 5-oxoprolinal affect of prolactin levels is by alteration of secretion, an experiment in which intracellular prolactin would be measured would be of interest. If the inhibitor is blocking secretion of prolactin than intracellular prolactin would be expected to rise. Since 5-oxoprolinal appears to blunt the TRH-induced rise in prolactin secretion, it would be of interest to see if 5-oxoprolinal would affect the K^+-induced increase in prolactin. Rappay and coworkers (Rappay et al., 1984; 1985) found that the tripeptide aldehyde, t-butyloxycarbonyl-D-Phe-Pro-Arg-H, a serine protease inhibitor, as well as other tripeptide aldehydes inhibited (at relatively high doses) prolactin release in dispersed anterior pituitary cells. The authors postulated that this effect was due to altering of proteinases involved in hormone secretory processes. It is possible that 5-oxoprolinal decreases prolactin production by a similar mechanism.

With the synthesis of 5-oxoprolinal and Z-Pro-Prolinal, potent and specific inhibitors of two enzymes responsible for the degradation of TRH are available. These aldehydes inhibit

brain prolyl endopeptidase and PPH respectively in vivo. Z-Pro-Prolinal and 5-oxoprolinal might be useful in studies on TRH turnover or in studies on the biosynthesis of TRH in which it would be desirable to prevent TRH degradation. 5-oxoprolinal or PDMK could also be used to evaluate the role of PPH in the degradation of other pyroglutamyl-containing peptides such as neurotensin, LHRH and bombesin. The physiological and behavioral consequences of inhibition of PPH and prolyl endopeptidase are also worthy of investigation.

As discussed above, TRH appears to be a fairly poor substrate for prolyl endopeptidase and PPH. The suitability of TRH for the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme awaits kcat/km detrminations with the availability of the purified enzyme. My results showing the pronounced regional distribution of this enzyme and the literature findings of the localization of the enzyme on synaptosomal membranes as well as its apparent high specificity for TRH suggest that this enzyme may be the most important of the three enzymes in TRH metabolism. The physiological contributions of this enzyme are currently unknown since an inhibitor for this enzyme is not yet available. It is possible that limited proteolysis is not the correct mechanism for termination of action of TRH. If an inhibitor for the membrane-bound pyroglutamyl-peptide hydrolyzing enzyme, Z-Pro-Prolinal and PDMK together did not elevate endogenous TRH levels after injection, then it would appear that degradation of TRH is not an important mechanism for termination of action. Alternatively,

redistribution, elimination or uptake terminates the action of TRH. Recent studies on uptake of TRH by cerebellar slices (Pacheco et al., 1981) and hypothalamic slices (Charli et al., 1984) suggest that inactivation of TRH could occur by uptake. Other neuropeptides which are more efficiently degraded by endopeptidases could be examined for the effects of inhibitors on their endogenous levels. The use of enzyme inhibitors other neuropeptides capable of degradation by prolyl endopeptidase and PPH are worthy of investigation.

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